THE SOIL/PLANT/PATHOGEN ASPECTS OF POTATO LATE BLIGHT

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Oospores of *Phytophthora infestans* produced *in vitro* and *in planta,* were exposed to a variety of environments and their survival was assessed. 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 at 18°C, or in soil under natural field conditions. In comparison, viability as measured by germination was lower overall, but appeared to increase after storage in soil. Oospores produced *in planta* buried in the field were capable of infecting both tomato and potato leaflets when recovered after eighteen months, including two winters.

Soil samples collected from the central highlands of Mexico (center of diversity for *Phytophthora infestans*) were evaluated for suppressive activity to *P. infestans*. A New York soil was used for comparative analysis. Even though both mating types are known to cohabit these regions of Mexico no evidence of oospores was found in the soil samples. No significant differences were observed between the Mexican and New York soils in terms of their influence on survival and infectivity of *P. infestans.* Bacterial diversity in the different soil types was investigated using terminal restriction fragment length polymorphism analysis (T-RFLPs).

Foliar and tuber resistance to *Phytophthora infestans* were evaluated in a mapping population (n = 94) developed between two *Solanum tuberosum* breeding lines, NY121 x NY115. Foliar disease severity of the progeny clones was measured by the area under the disease progress curve (AUDPC) in field tests in 2004 and 2005. Correlation analysis of AUDPC values with amplified fragment length polymorphism

(AFLP) molecular markers revealed that 56 %, 53 %, and 52 % of the quantitative phenotypic variance for resistance were associated with markers PCTMATC 184, PATMATA 171, and PATMACT 236, respectively, located on a chromosome V homolog derived from NY121 ($p < 0.00001$). The genetic component of tuber blight variance was much smaller than the foliar blight component, but still these three markers were correlated ($p < 0.01 – 0.10$) with tuber blight resistance and explained 3-8 % of the variance.

BIOGRAPHICAL SKETCH

Hilary Mayton was born and raised in the central river valley in Connecticut, where she spent much of her time outdoors on farms and in fields and woods. Hilary has always been interested in biology and mathematics. She began her career at Cornell University, in 1990 and has worked for the Soil, Crop and Atmospheric Sciences, Horticulture, and Plant Pathology Departments. She obtained her BS degree in 1993, at Cornell University, with a major in Soil Science, and then went on to graduate school and received a Master's degree at Cornell University with a major in Plant Pathology and a minor in Soil Science in 1996. Upon completion of her MS degree she began working for the Department of Plant Pathology, at Cornell University, in Dr. William E. Fry's laboratory conducting research on the late blight pathogen *Phytophthora infestans*. Hilary's research interests are in host resistance, epidemiology, and biological control. She is interested in sustainable agriculture and the development of environmentally friendly management strategies that will protect our natural resources and produce the needed supply of agricultural commodities necessary to sustain the world's expanding population.

In addition to her interest in agriculture she is particularly interested in the biology of consciousness, quantum field theory, quantum biophysics, and undiscovered or not yet recognized energy fields that influence our daily lives.

To my parents and my daughters Mesa and Vicki.

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CHAPTER ONE

GENERAL INTRODUCTION

Late blight, caused by the oomycete *Phytophthora infestans* (Mont.) de Bary, is one of the most devastating diseases of potato. In North America and Europe the disease was first recognized and became infamous in the mid-19th century, as it was responsible for many devastating epidemics, including the epidemic in Ireland which led to the great potato famine in 1845-1846 (Goodwin et al., 1994a). Late blight is a polycyclic disease and both the belowground (tubers) and aboveground (foliar) parts of the plant can be affected. Although the pathogen, *P. infestans,* has a complex life cycle (Figure 1.1) which includes both aerial and soil phases, it is most often viewed as a typical, aerial pathogen with prolific production of short lived asexual infective sporangia (Erwin and Ribeiro, 1996). Under conducive environmental conditions, late blight can completely destroy a potato field within two weeks. Because late blight of tubers is often associated with soft rotting bacteria and other secondary pathogens, an almost insignificant amount of tuber blight (2-5%) can lead to complete loss of potatoes in storage (Dorrance and Inglis, 1998). Currently, when environmental conditions favor the pathogen there are no methods to completely control late blight once it has become established within a field. Similarly, when infected tubers are stored at high humidity and moderate temperature little can be done to prevent losses. The primary methods of control are the use of less susceptible cultivars and prophylactic fungicide applications to foliage in the field.

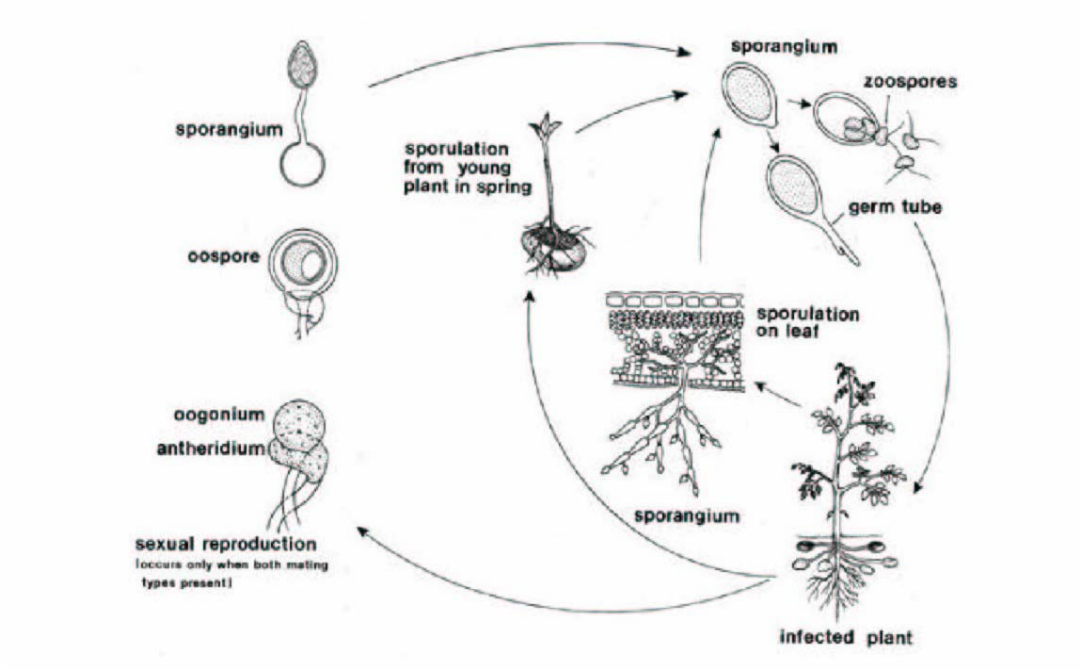

Figure 1.1. Life cycle of *P. infestans.*

The causal agent of late blight was first identified and characterized as the fungus, *Botrytis infestans*, by Charles Montagne in 1845 in Paris, France (Bourke, 1964). In 1876, Anton de Bary conducted extensive research with *B. infestans* and was the first to describe the asexual life cycle, which at the time was thought to be the full life cycle, and renamed the pathogen *Phytophthora infestans* (plant destroyer) (Bourke, 1964). Due to its morphological appearance, and filamentous fluffy hyphae, *P. infestans* was mistakenly classified as a fungus and has only recently been moved to the Kingdom Stramenopila, which also includes diatoms and brown algae. *P. infestans* belongs to the phylum Oomycota, class Oomycetes, order Peronosporales, family Pythiaceae (Alexopolus et al., 1996). Oomycetes is the largest class of Stramenopiles and includes many important plant pathogens. The majority of species in the genus *Phytophthora* are destructive plant pathogens. Most *Phytophthora* species cause root and stem rots, however, *P. infestans* causes both foliar and tuber blight (Erwin and Ribeiro, 1996).

Phytophthora infestans is an heterothallic oomycete and requires two mating types (A1 and A2) to reproduce sexually. The result of sexual recombination is a thick walled oospore which can survive for long periods in soil (Andrivon, 1995). Germination of oospores produces a germ sporangium releasing zoospores which can lead directly to infections of tubers, but which also can be splashed to leaf tissue to initiate foliar epidemics. Sexual reproduction results in new genotypic diversity. Several studies on the population biology of *P. infestans* have presented evidence that a single (A1 compatibility type) clonal lineage was distributed throughout the world and was, until very recently, the primary lineage responsible for late blight epidemics outside of Mexico (Goodwin et al., 1994b). As a result of the occurrence of only the A1 compatibility type worldwide, this organism was thought to be essentially asexual. However, in 1956, Gallegly and Galindo (Gallegly and Galindo, 1957) reported oospores on naturally infected potato leaves in the Toluca Valley, Mexico. They also observed that, in Mexico, the A1 and A2 mating types were present in equal proportions and sexual reproduction was probably common. Through genetic analysis it has been confirmed that the population of *P. infestans* in Mexico exists in Hardy-Weinburg equilibrium and it is now believed that central Mexico is the pathogens center of diversity (Goodwin et al., 1994a).

The population dynamics of *P. infestans* have changed dramatically over the past three decades. A world-wide migration of diverse strains (A1 and A2 mating types) of *P. infestans* from Mexico in the late 20th century created the opportunity for sexual reproduction and the potential for soil-borne inoculum (Fry et al., 1993) outside of Mexico. Prior to the discovery of oospores in nature and the elucidation of the complete life cycle of *P. infestans*, most research and control efforts had been focused on the asexual foliar phase of late blight.

Due to its destructive nature, foliar late blight has been extensively researched for over 150 years. A crucial component of the foliar late blight disease cycle, which enables an epidemic to progress at an exponential rate, is the rapid production of secondary inoculum. After the initial infection, the asexual production of sporangia on leaf tissue can be very fast, 3-4 days, under high relative humidity (> 95%) and moderately cool temperatures (60-80 º F). The sporangia are then wind or splash dispersed to infect healthy plant tissue. A typical late blight lesion on a potato leaflet can have as many as 300,000 sporangia (Mayton et al., 2001). The caducous, semipapillate sporangia of *P. infestans* are extremely sensitive to sunlight and desiccation (Minogue and Fry, 1981; Mizubuti et al., 2000). Mizubuti et al. (Mizubuti et al., 2000) exposed sporangia to varying levels of solar irradiance (SI) and observed that viability decreased by almost 95% after one hour when subjected to SI measured at > 600 W/m^2. However, under cloudy conditions at high relative humidity, airborne sporangia can travel very long distances and infect fields 10 km away (Aylor et al., 2001). The majority of sporangia are produced in the lower half of the canopy where the cool moist conditions are most conducive for sporangial development (Mayton et al., 2001). Sporangia deposited on the soil surface can then be moved within the soil profile and infect potato tubers, stems, and leaflets near the soil surface. Infected tubers exhibiting no disease can harbor the pathogen and when planted can initiate primary infections.

In temperate climates such as the Northeast USA, infected tubers are the primary source of inoculum. After planting infected seed tubers, the mycelia can grow into the sprouting shoots and emerge through the stem and leaf tissue (Figure 1.1). Cull piles containing infected tubers can also be a source of primary inoculum. In Mexico, both infected tubers and oospores produced in infected tissue can initiate primary infection of potato stems (Fernandez-Pavia et al., 2003; Grunwald and Flier,

2005). Now that both A1 and A2 mating types occur in many locations worldwide, the role of oospores in regions other than central Mexico is currently under investigation. Outside of central Mexico, oospores are very probably an important new component in the epidemiology of late blight.

In contrast to the research on the aerial phase of late blight, we still know very little about the ecology of *P. infestans* in soil. This is unfortunate because, tuber infections initiated in the soil are potentially very important. There was some interest in the soil phase of *P. infestans* in the late 19th and early 20th centuries, specifically on its ability to survive saprophytically in soil and on plant debris (de Bruyn, 1922). It was determined that *P. infestans* was not a good soil saprophyte and little further research was initiated until after the discovery of oospores in Mexico.

In 1995, Andrivon (Andrivon, 1995) summarized the scarce amount of data that had been published primarily on the asexual soil phase of *P. infestans*. He reported on several research studies conducted on the saprophytic fitness of *P. infestans* in soil, which found that sporangia were able to survive for longer periods of time in sterile versus non-sterile soil. It has been hypothesized that microorganisms in soil may influence the survival and infectivity of *P. infestans.* Several studies have found microorganisms in soil to be antagonistic to other *Phytophthora* species and other plant pathogens. Conversely, isolates of *Trichoderma* have been shown to stimulate oospore production in isolates of *Phytophthora* (Erwin and Ribeiro, 1996). Moisture and soil type can also influence survival of *P. infestans*. In sandy soils survival of sporangia tends to be shorter than in clay or loamy soils. Survival of sporangia in soil is favored by moderate/cool temperatures and moist conditions as compared to very warm or very dry or saturated conditions (Andrivon, 1995). Other factors that have been demonstrated to play a role in the persistence and infectivity of

sporangia in soil are temperature, pH, and aluminum concentration (Andrivon, 1995; Lacey, 1965).

Recent work in the Toluca Valley of Mexico by Pavia-Fernandez et al. (Fernandez-Pavia et al., 2003) revealed that oospores could survive winter fallow periods in soil and could lead to infections in emerging potato plants. They also reported differences in the survivability of the oospores collected from different soils, which may indicate some type of suppressive activity of the soils examined. The highlands of central Mexico are the only areas where for many years, the soil component of the life cycle of *P. infestans* has had an important role in the natural development of late blight epidemics (Andrivon, 1995; Gallegly and Galindo, 1957). However, there is very little information about the structure, diversity or composition of populations of microorganisms relative to *P. infestans* in those soils. The potential occurrence of oospores in many regions of the world outside of Mexico now exacerbates the threat to potato production worldwide. Information on the soil phase of *P. infestans* would be of major benefit to global potato production, because this information could be used to mitigate tuber infections and also to suppress initial inoculum.

Tuber blight, caused by *P. infestans,* is one of the most important diseases of potato in storage. In the USA over 60% of the potatoes grown are stored for at least 2 months (Dorrance and Inglis, 1998). Economic losses due to late blight and associated soft rot infections in storage can be devastating. A few of the primary factors thought to be involved in the incidence of tuber blight are the occurrence and severity of foliar blight, soil temperature and soil moisture (Sato, 1979). However, the relationships are complicated, and the amount of late blight in the foliage is often not correlated with the amount of tuber blight. Despite this inconsistency, the suppression of foliar blight remains the major strategy to preventing tuber blight.

Host resistance is recognized as potentially the most effective and environmentally acceptable method to control late blight. Unfortunately, most commercially cultivated potato varieties, have moderate to little resistance to both foliar and tuber blight. Resistance to late blight is conferred by dominant, single (vertical, R-gene, monogenic, qualitative) and multiple, minor genes (horizontal, polygenic, quantitative). Single dominant alleles (genes) from wild *Solanum* species have been introgressed into the cultivated *Solanum tuberosum* for resistance. However, monogenic resistance for control of late blight has not been a successful disease management approach. This is because R gene resistance in potato is quickly defeated by the appearance of new races of the pathogen *P. infestans* (Niederhauser and Mills, 1953). In addition, tubers with R-genes sometimes responded differently to *P. infestans* than did the foliage with R-genes. Because of the lack of success in controlling late blight with R-genes, a more intensive effort has been initiated to produce potato cultivars with horizontal or polygenic resistance.

Selection of resistant germplasm is unfortunately focused almost exclusively on foliar blight. As with R-gene resistance, quantitative resistance in the foliage does not always correlate well with tuber resistance (Rasmussen et al., 1998; Stewart et al., 1992). In one study, Stewart et al. (Stewart et al., 1992), found a negative correlation between foliar and tuber resistance and concluded that different genes may be involved in control of both phases of resistance. However, in a later study Stewart (Stewart et al., 1994) observed that quantitative resistance in foliage and tubers were correlated (Stewart et al., 1994). A systematic summary of the literature on the correlation between foliar and tuber blight resistance would suggest that the genes and defense pathways involved do sometimes, but not always, overlap. The relationship between foliar and tuber blight resistance is not clear-cut.

Since its first appearance in Europe, late blight has been a challenging disease to control. Significant progress has been made on the host pathogen interactions of the asexual foliar phase, yet many questions remain unanswered concerning the epidemiology of *P. infestans* in soil. In this thesis I investigate several aspects of the soil-phase of the biology of *P. infestans* and its soil-borne pathogenicity. These include: 1) epidemiological implications of sexual reproduction; 2) survival of sporangia in soils from the central highlands of Mexico and 3) host resistance to *P. infestans* in foliage and tubers.

LITERATURE CITED

Alexopolus, C.J., C.W. Mims, and M. Blackwell. 1996. Introduction to Mycology John Wiley and Sons, Inc., New York.

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

Aylor, D.E., W.E. Fry, H. Mayton, and J.L. Andrade-Piedra. 2001. Quantifying the rate of release and escape of *Phytophthora infestans* sporangia from a potato canopy. Phytopathology 91:1189-1196.

Bourke, P.M.A. 1964. Emergence of potato blight, 1843-1846. Nature 203:805-808.

de Bruyn, H.G.L. 1922. The overwintering of *Phytophthora infestans* (Mont.) de Bary. Phytopathology 16:121-140.

Dorrance, A.E., and D.A. Inglis. 1998. Assessment of laboratory methods for evaluating potato tubers for resistance to late blight. Plant disease 82:442-446.

Erwin, D.C., and O.K. Ribeiro. 1996. Phytophthora diseases worldwide The American Phytopathological Society, APS Press, St. Paul, MN.

Fernandez-Pavia, S.P., N.J. Grunwald, M. Diaz-Valasis, M. Cadena-Hinojosa, and W.E. Fry. 2003. Soilborne oospores of *Phytophthora infestans* in central Mexico survive winter fallow and infect potato plants in the field. Plant Disease 88:29-33.

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

Gallegly, M.E., and J. Galindo. 1957. Mating types and oospores of Phytophthora infestans in nature in Mexico. Phytopathology 48:274-277.

Goodwin, S.B., B. Cohen, A.,, and W.E. Fry. 1994a. Panglobal distribution of a single clonal lineage of the Irish potato famine fungus. National Academy of Sciences 91:11591-11595.

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

Grunwald, N.J., and W.G. Flier. 2005. The biology of Phytophthora infestans at its center of origin. Annual Review of Phytopathology 43:171-190.

Lacey, J. 1965. The infectivity of soils containing *Phytophthora infestans*. Annuals of Applied Biology 56:363-380.

(ed.) 2001. 8th International workshop on plant disease epidemiology, Ouro Preto, Brazil.

Minogue, K.P., and W.E. Fry. 1981. Effect of temperature, relative humidity, and rehydration rate on germination of dried sporangia of *Phytophthora infestans*. Phytopathology 71:1181-1184.

Mizubuti, E.S.G., D.E. Aylor, and W.E. Fry. 2000. Survival of *Phytophthora infestans* sporangia exposed to solar radiation. Phytopathology 90:78-84.

Niederhauser, J.S., and W.R. Mills. 1953. Resistance of Solanum species to *Phytophthora infestans* in Mexico. Phytopathology 43:456-457.

Rasmussen, J.O., J.P. Nepper, H.G. Kirk, K. Tolstrup, and O.S. Rasmussen. 1998. Combination or resistance to potato late blight in foliage and tubers by intraspecific dihaploid protoplast fusion. Euphytica 102:363-370.

Sato, N. 1979. Effect of soil temperature on the field infection of potato tubers by *Phytophthora infestans*. Phytopathology 69:989-993.

Stewart, H.E., J.E. Bradshaw, and R.L. Wastie. 1994. Correlation between resistance to late blight in foliage and tubers in potato clones from parents of contrasting resistance. Potato Research 37:429-434.

Stewart, H.E., J.E. Wastie, J.E. Bradshaw, and J. Brown. 1992. In heritance of resistance to late blight in foliage and tubers of progenies from parents differing in resistance. Potato Research 35:313-319.

CHAPTER TWO

OOSPORE SURVIVAL AND PATHOGENICITY OF SINGLE OOSPORE RECOMBINANT PROGENY FROM A CROSS INVOLVING US-17 AND US-8 GENOTYPES OF *Phytophthora infestans*[*]

ABSTRACT

Oospores of *Phytophthora infestans* produced *in vitro* and *in planta*, from a cross between US-17 and US-8 genotypes, were exposed to a variety of environments and their survival was assessed. Additionally the pathogenic characteristics of some resultant progeny isolates were assessed. 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 at 18°C, or in soil under natural field conditions. In comparison, viability as measured by germination was lower overall, but appeared to increase after storage in soil. Oospores produced *in planta* were buried in the field in the fall of 1998, and were capable of infecting both tomato and potato leaflets when recovered in May 1999. Single oospore progeny (n=53) from the *in vitro* cross were analyzed individually for genetic and pathogenicity characteristics. All 53 progeny tested for with probe RG57 were hybrids. All but one progeny produced sporulating lesions on detached potato and /or tomato leaflets in growth chamber tests, but most lesions were smaller and developed more slowly than those produced by either parental isolate. In a further test of pathogenicity, under field conditions, none of a subset of 10 A2 progeny was capable of initiating a detectable epidemic in small plots of either potatoes or tomatoes.

[*] H. Mayton, C. D. Smart, B. C. Moravec, E. S. G. Mizubuti , A. E. Muldoon, and W. E. Fry 2000. Oospore Survival and Pathogenicity of Single Oospore Recombinant Progeny from a Cross Involving US - 17 and US - 8 Genotypes of *Phytophthora infestans.* Plant Dis. 84:1190-1196

INTRODUCTION

Recent introductions of *Phytophthora infestans* strains into the United States and Canada have created the opportunity for sexual reproduction to become an important component of the life cycle. Prior to 1992, only A1 strains were common, but by the end of the 1990s, strains of both A1 and A2 mating types had become widely distributed (Fry and Goodwin, 1997a). Most isolates have been individuals representing a small number of clonal lineages, as determined by neutral markers (Goodwin et al., 1998). A clonal lineage contains the asexual descendants of a single genotype, and individuals differ from each other primarily as a result of genetic changes originating by mutation (Anderson and Kohn, 1995). Thus, individuals within a lineage are more similar to each other than to individuals in other lineages in terms of many ecologically important characteristics. For *P. infestans* in the United States and Canada, epidemiological characteristics predictable from knowledge of clonal lineages have included metalaxyl sensitivity, probable pathogenic specialization, and germination response to temperature (Goodwin et al., 1995a; Goodwin et al., 1996; Kato et al., 1997; Legard et al., 1995; Mizubuti and Fry, 1998; Sujkowski et al., 1996).

Recently introduced lineages replaced the predominant US-1 lineage (A1 mating type) in the 1990s in the United States and Canada (Fry and Goodwin, 1997a). The US-8 lineage (A2 mating type) achieved a continental distribution within only a few years of its initial detection in 1992 (Fry and Goodwin, 1997b). A few lineages of A1 mating type also became widely distributed and problematic in the United States during the 1990s, including US-6, US-11, and US-17 genotypes (Fry and Goodwin, 1997a). In newly dominant strains, the absence of genetic markers associated with the US-1 clonal lineage (*86* allele for the *Glucose-6-phosphate isomerase* (*Gpi*) locus, *92* allele for the *Peptidase* locus, and mitochondrial haplotype Ib) indicate that the new strains have probably not arisen via sexual reproduction involving the US-1 lineage

(Fry and Goodwin, 1997a). However, newly dominant lineages appear to be fertile with each other (Lee et al., 1999), so the opportunity for sexual reproduction appears to be very much greater at the end of the 1990s than previously.

Sexual reproduction involves oospore production which could create change in the ecology/epidemiology of *P. infestans* in the United States and Canada. Oospores are thick-walled spores produced by many *Phytophthora* species. They are an important source of inoculum for diseases caused by soil borne Phytophthoras and have the longest survival period for all infective propagules of that genus (Erwin and Ribeiro, 1996). Oospores of *P. infestans* survived a range of temperatures, from freezing temperatures to 40°C in short term laboratory experiments (Fay and Fry, 1997). In the United Kingdom (Pittis and Shattock, 1994), oospores remained viable but did not germinate when recovered from field soil after 10 months incubation. In other studies, oospores survived and could germinate after a winter in the field in both the Netherlands and Canada (Drenth et al., 1995; Medina and Platt, 1999). Thus, production of oospores probably creates additional opportunities for between-season survival of this pathogen. As an asexual organism, *P. infestans* is essentially an obligate parasite in natural or agricultural systems. If oospores of *P. infestans* survive in soil, they may serve as a source of initial inoculum - perhaps even some years after a susceptible host had been grown in that soil. There has been a suggestion that for at least one field in Sweden, the soil served as the source of *P. infestans* (Andersson et al., 1998).

Sexual recombination will also produce new genetic combinations creating the possibility of unusually fit or pathogenic individuals or individuals with novel ecological/epidemiological characteristics. This possibility has been termed the “Red Queen” hypothesis (Barton and Charlesworth, 1998; Wuethrich, 1998) and is particularly important for organisms which reproduce both asexually and sexually.

The US-11 clonal lineage may have been produced by sexual recombination in the United States or Canada, and it has been problematic in many potato and tomato production systems in the United States (Gavino, 1999; Gavino et al., 2000; Goodwin et al., 1998). While it seems probable that some epidemiologically important genotypes (such as the US-11 genotype) are produced via sexual reproduction, it also seems likely that some progeny will not be aggressive plant pathogens. However, we do not now know what proportion of progeny are likely to be important, aggressive pathogens. Finally, in some agro-ecosystems where both mating types are known to occur, sexual reproduction is apparently not a factor in the life history of *P. infestans* (Mosa et al., 1991; Mosa et al., 1993).

The potential importance of sexual reproduction in *P. infestans* populations in the United States stimulated our investigation of the ecological characteristics of this aspect of the life history. We tested three individual hypotheses: that two isolates of *P. infestans* currently widespread in New York State could produce viable sexual progeny; that their oospores could survive under field conditions; and that the oospore progeny were generally as pathogenic as the parental isolates.

MATERIALS AND METHODS

Oospore production *in vitro* and *in planta*. Oospores of *P. infestans* were produced *in vitro* by pairing two isolates of different mating types on Rye B agar (Caten and Jinks, 1968). All *P. infestans* isolates were obtained from the culture collection at Cornell University and the isolate numbers relate to that collection. The A1 isolate was US970001, and is a representative of the US-17 clonal lineage. This lineage is characteristically highly resistant to metalaxyl and highly pathogenic to tomatoes (Goodwin et al., 1998). The A2 isolate was US940480, a representative of the US-8 clonal lineage. This lineage is characteristically highly pathogenic to

potatoes and resistant to metalaxyl (Goodwin et al., 1995c). These isolates were selected as parents because they have both been detected in New York State, and may have had opportunity for sexual reproduction in our region. Progeny from the *in vitro* mating, designated as cross 82, were obtained into pure culture and subsequently stored in the Cornell University culture collection. After four weeks incubation in the dark at 18°C, oospores were extracted from agar medium by blending the mycelium and agar in water using a Polytron Homogenizer (Brinkmann Instruments, Westbury, NY). The oospore suspension was then treated with Novozyme 234 (Interspex Products Inc., Foster City, CA) at a concentration of 5g/liter to remove sporangia and mycelial debris. Oospore suspensions were then washed with sterile distilled water to remove the Novozyme, and distributed aseptically on water agar (0.7%, w/v) in 100 x 15 mm Petri plates. Oospores were incubated at 18°C in diffuse light and observed for germination almost daily during the subsequent 6-8 weeks. Germinating oospores were transferred aseptically to Petri plates containing Rye A agar (Caten and Jinks, 1968) and maintained on Rye B agar.

Oospores were also produced in both tomato (cv Sunrise) and potato tissue (cv Superior) to assess survival of *in planta*-produced oospores under field conditions. The A1 and A2 strains were US970001 (US-17) and US940480 (US-8), respectively, as described above. Plant tissues (stems, fruits and tubers) were inoculated with sporangia of US-17 alone, sporangia of US-8 alone, or with a 1:1 mixture of US-17 and US-8 sporangia. The inoculated tissue was then incubated in a plastic box containing moistened paper towels. After three weeks, the co-inoculated tissues were examined for oospores by visual inspection under a compound microscope.

Genetic analysis of progeny. Single oospore progeny that were obtained from the *in vitro* cross were analyzed for mating type, allozyme genotype at the *Gpi* locus, and for genomic fingerprint as detected by probe RG57. Mating type was assessed as

described by Lee et al (Lee et al., 1999). Techniques for allozyme analysis utilized cellulose acetate electrophoresis as described by Goodwin et al (Goodwin et al., 1995b) and techniques for DNA fingerprinting were those described by Goodwin et al (Goodwin et al., 1992) . Because both parental strains had the same (I-a) mitochondrial haplotype (Gavino, 1999), the progeny were not assayed for this characteristic.

Metalaxyl sensitivity. The metalaxyl sensitivity of the single-oospore progeny and parental isolates was assessed as described by Matuszak et al (Matuszak et al., 1994). Each isolate was grown in Rye B agar without metalaxyl (control) or media containing 5 or 100 μg/ml. Cultures were started with a 7mm disc of agar containing mycelia taken from the edge of a culture growing on Rye B medium. Horizontal and vertical measurements of the colony on control and test media in two replicate plates were obtained over time. The growth of each individual culture at 5 and 100 μg/ml metalaxyl was compared to growth in the absence of metalaxyl. The trial was done twice and the data were averaged. An isolate of the US-1 clonal lineage (US940507) was included as a metalaxyl sensitive control.

Oospore survival. Survival of oospores produced *in vitro* was assessed in an experiment beginning in the fall of 1997. Oospores in an agar-water slurry were placed in three different environments: 1) in Falcon tubes (VWR, Rochester, NY) in an incubator at 4°C; 2) in soil (Honeoye silt-loam soil obtained from the experimental field site) in the greenhouse at 18°C; and 3) buried at a depth of 32 cm in the field. For burial in the field and in field soil in the greenhouse, subsamples were placed in nylon bags constructed from 10 μm mesh nylon (Tetko Inc., Kansas City, MO). The bags were used to prevent escape of any of the oospores. In the field, soil moisture and temperature were monitored at a depth of 32 cm. Soil moisture was measured with a model CS615 water content reflectometer, and soil temperature was recorded using a

model 107B soil probe (Campbell Scientific, Logan, UT). Data were collected and stored twice a day (noon and midnight) with a CR10 Datalogger (Campbell Scientific, Logan, UT) from September 1997 through May 1999. Individual bags containing oospores were harvested from the field soil and from soil in the greenhouse at 3-5 month intervals from January 1998 to May 1999. The oospores were then analyzed for viability as described in the section below.

Survival of oospores produced *in planta* was also assessed after a winter season. Tomato stems and fruits and potato stems and tubers containing oospores were buried at a depth of 30 cm in nylon mesh bags (as described above) in November 1998 at three different sites in central New York State. Additionally, tissues that had been inoculated with only the A1 or A2 parent were also buried in nylon mesh bags at those sites. The bags were retrieved from the field in May 1999.

Viability, germination and infectivity of oospores. Viability of oospores was evaluated by the plasmolysis method reported by Jiang and Erwin (Jiang and Erwin, 1990), and also by germination. Oospores were recovered and suspended in water. An aliquot (50-150 µL) was placed in a 35 x 10 mm Petri plate; 2x the volume of 4M NaCl (Sigma) was added and oospores (300 in total) were assessed after 30 min, using a compound microscope at 20 or 40X, for whether or not they had plasmolyzed. To assess germination, a second aliquot of each oospore suspension was spread on 1.5 % water agar plates containing ampicillin (100 mg/liter, Sigma, St. Louis, MO). The plates were incubated under blue fluorescent light at 22°C (Fay and Fry, 1997). After 20 days of incubation, 300 oospores were observed microscopically for evidence of germination.

At the conclusion of the storage period, both *in vitro* and *in planta*-produced oospores were assayed for their ability to initiate infection on both tomato and potato leaflets. Oospore samples were placed individually in 150 x 15 mm Petri plates with

10-25 ml distilled water and were maintained at 18°C. After two days at 18°C, both tomato and potato leaflets were added to each Petri plate. Leaflets were examined for infection after 5-10 days. Lesions which appeared on the detached leaflets were excised and the *Gpi* genotype, mating type and RG57 genomic fingerprint of each isolate were determined.

Pathogenicity of single oospore progeny. All oospore progeny recovered from the *in vitro* mating were tested for pathogenicity on detached leaflets of both potato and tomato in moist chambers. Whatman #1 filter paper was placed in the bottom of a Petri plate (150 x 15 mm), and 1-2 ml distilled water was added to the filter paper. Four potato leaflets (cv Superior) or four tomato leaflets (cv Sunrise) were placed in each moist chamber . Leaflets were obtained from the middle third of plants grown in the greenhouse. Sporangia from 2-3 week old cultures grown on Rye B medium were used as inoculum. Sporangia were washed from the medium and adjusted to a concentration of 3000 – 7000/ml. Sporangia were stimulated to release zoospores by incubating the suspension at 4°C for two hours prior to inoculation. A droplet (40 µl) of the suspension was placed on the abaxial surface of the tomato or potato leaflet. Inoculated leaflets were maintained in the moist chambers for 7-10 days at 18°C in diffuse light. Pathogenicity was scored on the basis of lesion size and the degree of sporulation as a result of each inoculation. A pathogenicity score of (+++) was given if inoculation with a progeny isolate consistently resulted in a sporulating lesion >3 cm in diameter. An isolate was rated as (++) for pathogenicity, if a sporulating lesion was consistently 1-3 cm in diameter. Lastly, a score of (+) was given if inoculation with a progeny isolate resulted in a lesion of 0.1-1 cm in diameter. Assessments were made at least twice for each progeny isolate. Isolates were considered to be pathogenic if a visible sporulating lesion was observed. The progeny

isolates were assayed in several groups. Parental isolates were included as controls with each group. Each progeny isolate was assayed independently at least twice.

Because detached leaflet assays indicated that most of the progeny were less aggressive than the parents (see Results), a subset of progeny isolates was analyzed for pathogenicity in a field test. The assay plants were tomatoes (cv Sunrise) and potatoes (cv Superior). Neither cultivar is known to have R-genes. A randomized complete block design with three replicates was used. Each replicate contained experimental units of both potato and tomatoes. The trial was conducted at the Homer C. Thompson Experimental Farm, located in Freeville, NY. Potato tubers were planted 19 June 1998. Each experimental unit consisted of three rows with five plants per row, spaced 23 cm apart within the row. Tomatoes, started from seed in May, were transplanted 19 June 1998. Each experimental unit consisted of three rows with three plants per row, spaced 23 cm apart.

The field trial included 26 treatments which consisted of 10 single-oospore progeny, a water control, the US-8 A2 parent, and a US-7 (US940486) A2 tomato aggressive isolate (Legard et al., 1995) on potato plots and tomato plots. Because our field experiments involved field isolates of A2 mating types, we restricted our analysis to only A2 progeny – to avoid the possibility of sexual reproduction among isolates and resultant oospore contamination of soil at our field test site. The replicated field test was conducted only in 1998 and not repeated because results confirmed those from *in vitro* pathogenicity tests.

Inoculation was done on the evening of 14 August 1998. Plants were irrigated via overhead sprinklers prior to inoculation so that the soil and foliage were wet. Inoculum for each individual unit consisted of a sporangial suspension (1700 sporangia/ml) of the appropriate isolate. Sporangial suspensions were cooled at 4°C for 2 h prior to inoculation to induce zoospore formation. The center of each

experimental potato/tomato unit was inoculated by atomizing 5 ml of the appropriate sporangial suspension onto leaves in the middle of the canopy. The plants were monitored daily so that any aggressive strains could be detected and destroyed before significant sporulation and spread to adjacent field plots occurred. The incidence of primary and secondary lesions was monitored almost daily and the *Gpi* genotype of the isolates sporulating from a subset of individual lesions was determined.

Statistical analysis. Statistical analyses were performed on some of the data. Analysis of variance (General Linear Model Procedure) was used to analyze sensitivity data of isolates to metalaxyl. Regression analysis was used to examine the effect of environment and time on oospore germination and viability. These analyses were computed using Statistical Analysis System 6.12 (SAS Institute, Cary, NC). The genotypic data using neutral markers and the pathogenicity data were not subjected to statistical analysis.

RESULTS

Production and genetic characterization of *in vitro* produced oospore progeny. A total of 53 single-oospore progeny was obtained from the *in vitro* cross between US-17 and US-8 (Table 2.1). Thirty-five of the 53 were A1 mating type and 18 were A2 mating type. All of the progeny examined were products of recombination as determined by segregation of allozyme alleles and mating type (Table 2.1). Eight of the ten theoretically possible *Gpi* genotypes were observed in the progeny (Table 2.1).

All of the 53 progeny isolates were characterized for DNA fingerprint with probe RG57. The parental strains were polymorphic at loci for bands 3, 5, 7, 10, 18 and 23. Therefore, these bands were instructive in determining if the progeny were hybrids as opposed to selfs. All 53 progeny isolates had genotypes that could only be indicative of hybrids (Table 2.1).

Table 2.1. Genetic and pathogenic characteristics of progeny and parental isolates of *P. infestans*.

Isolate [1]	Mating Type	*Gpi* genotype	Genotype at RG57 bands [2]						Pathogenicity [3]	
			3	5	7	10	18	23	Potato	Tomato
US940480	A2	100/111/122	0	1	0	1	0	1	+++	+++
US970001	A1	100/122	1	0	1	0	1	0	+++	+++
CS82001	A1	100/100/111	1	1	1	1	0	0	+	+
CS82002	A1	100/100	1	1	1	1	0	0	+	+
CS82003	A2	100/111	1	1	1	1	1	1	+++	+++
CS82004	A1	100/122	1	1	1	1	1	0	++	++
CS82005	A1	111/122	1	1	1	1	0	0	++	++
CS82006	A1	100/111/122	1	1	1	1	0	0	+++	+++
CS82007	A1	100/122	1	0	1	1	1	1	+	+
CS82008	A1	100/111	1	1	1	1	0	1	+	+
CS82009	A2	111/122	1	1	1	1	0	0	+	+
CS82010	A1	122/122	1	1	1	1	0	0	++	++
CS82011	A2	100/122	1	1	1	1	1	1	+	+
CS82012	A1	100/122	1	1	1	1	0	0	++	++
CS82013	A1	100/122	1	1	1	1	0	1	++	++
CS82014	A1	122/122	1	0	1	1	1	1	++	++
CS82015	A1	100/122	1	1	1	1	1	1	+	+
CS82016	A1	100/100	1	1	1	0	1	0	+	+
CS82017	A2	100/100	0	1	0	1	1	1	+	-
CS82018	A1	100/111	1	1	1	0	0	0	++	+

[1] US970001 + US940480 are parental isolates of cross 82. Other isolates are the progeny from this cross.

[2] Bands 3,5,7,10,18 and 23 as described by Goodwin et al 1992 were scored as present (1) or absent (0) for the parental and progeny isolates.

[3] Pathogenicity was scored as follows: (+++) = large (>3 cm in diameter) lesions with sporulation consistently resulting from each inoculation; (++) = intermediate (1-3 cm in diameter) lesions produced at most inoculations; (+) = small (0.1-1 cm in diameter) lesions resulting from only some inoculations; (-) = no sporulating lesions.

Table 2.1 (Continued).

Isolate [1]	Mating Type	*Gpi* genotype	Genotype at RG57 bands [2]						Pathogenicity [3]	
			3	5	7	10	18	23	Potato	Tomato
CS82019	A1		1	0	1	0	0	1	+	+
CS82021	A1	100/122	1	1	1	1	0	0	+	+
CS82022	A1	100/100	1	1	1	1	0	1	++	++
CS82023	A1	111/122	1	1	1	1	1	1	++	++
CS82024	A1	100/122	1	1	1	1	1	1	++	++
CS82025	A1	100/122	1	1	1	0	1	0	+	+
CS82026	A2	100/122	0	1	1	1	0	1	+++	+++
CS82027	A1	100/122	1	1	1	1	1	0	++	++
CS82028	A1	100/122	1	1	1	1	0	0	++	++
CS82029	A1	122/122	1	1	1	0	1	1	++	+
CS82030	A2	100/122	1	1	1	1	1	0	++	++
CS82031	A1	111/122	1	1	1	1	1	1	++	++
CS82032	A1	100/122	1	1	1	0	0	1	+	+
CS82033	A2	100/122	0	1	0	1	1	1	+	+
CS82034	A2	100/122	1	1	1	1	0	1	+	+
CS82035	A1	100/122	1	0	1	1	1	0	+	+
CS82036	A2	100/100	0	1	0	1	1	0	+	+
CS82037	A1	111/122	1	1	1	1	1	1	++	+
CS82038	A2	100/111	1	1	1	1	0	0	++	++
CS82039	A2	100/122	1	1	1	1	0	1	++	++
CS82040	A1	100/100/122	1	0	1	1	1	1	+	+
CS82041	A2	100/100	0	1	0	1	1	1	+	++
CS82042	A2	122/122	1	1	1	0	1	1	+	+
CS82043	A1	100/111	1	1	1	0	0	0	++	++
CS82044	A1	100/122	1	1	1	1	0	0	+	+
CS82045	A2	100/100	1	1	1	1	1	1	+	-

Table 2.1 (Continued).

Isolate [1]	Mating Type	*Gpi* genotype	Genotype at RG57 bands [2]						Pathogenicity [3]	
			3	5	7	10	18	23	Potato	Tomato
CS82046	A1	100/122	1	1	1	1	0	0	+	+
CS82047	A1	100/111	1	1	1	1	1	1	+	+
CS82048	A2	100/111	1	1	1	1	0	1	+	+
CS82049	A2	100/122	1	1	1	1	0	1	+	+
CS82050	A2	100/111/122	1	0	1	0	1	1	+	+
CS82051	A1	100/122	0	1	0	0	0	1	+	+
CS82052	A1	100/122	1	1	1	1	0	0	-	+
CS82053	A1	111/122	1	1	1	1	0	1	+	+
CS82054	A2	100/122	1	1	1	1	0	0	-	-

All of the progeny tested were insensitive to metalaxyl (growth at 5 μg/ml metalaxyl > 40 % of the growth in the absence of metalaxyl) (Figure 2.1). However, the relative growth rates differed significantly among the progeny; at 5 μg/ml growth ranged from 40% to well above 100% of the controls. Growth of some progeny was apparently stimulated by the presence of metalaxyl. Some progeny were more sensitive than either parental isolate, while others were more resistant than either parental isolate.

Pathogenicity of progeny. All but one of the progeny were capable of infecting tomato and/or potato in the *in vitro* detached leaf assays. Most isolates produced smaller lesions than the parental strains, but several produced lesions that were similar to those produced by the parental isolates (Table 2.1).

In the field test, the progeny isolates had much lower pathogenicity than either of the control field isolates. Only half of the progeny tested produced primary lesions as a result of the initial inoculation; the other half produced no lesions (Table 2.2).

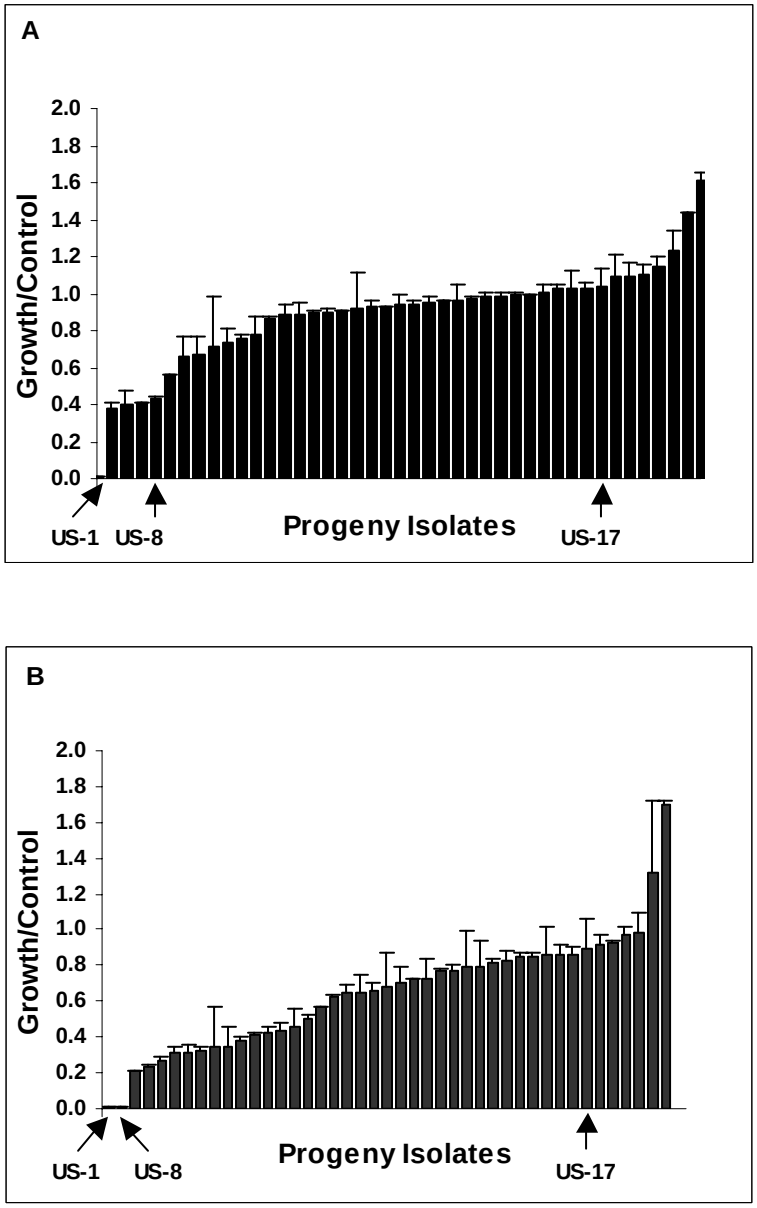

Figure 2.1. Sensitivity of US-17 X US-8 progeny isolates to metalaxyl. (**A**) 5 μg/ml; (**B**) 100 μg/ml. Error bars represent the standard deviation of the mean growth of two replicates of each isolate.

Table 2.2. Incidence of primary and secondary late blight lesions in small field plots of potatoes and tomatoes inoculated with progeny isolates, US-8 parent and a US-7 field isolate.

Isolate	Potato		Tomato	
	Primary lesions	**Secondary lesions**	**Primary lesions**	**Secondary lesions**
US940480 (US-8)	+	+	+	+
US940486 (US-7)	+	+	+	+
CS82003	+	-	+	-
CS82009	+	-	+	-
CS82017	-	-	-	-
CS82026	+	-	+	-
CS82030	+	-	+	-
CS82038	+	-	+	-
CS82039	-	-	-	-
CS82041	-	-	-	-
CS82048	-	-	-	-
CS82050	-	-	-	-
WATER	-	-	-	-

While some primary lesions appeared, they did not expand rapidly and infected leaflets did not die within two weeks of the original inoculation. In those plots containing primary lesions caused by progeny isolates, there were no secondary lesions near the primary lesions (in the center of the plot) before the plots were destroyed. In contrast, leaflets with primary lesions died within five to six days after

lesions appeared and there were many secondary lesions surrounding the primary lesions in plots inoculated with either of the control field isolates. In some of the plots inoculated with progeny, there were a few lesions not near the initial inoculation site, and these were confirmed by *Gpi* analysis to be caused by either of the control field isolates and thus were contamination. Therefore, there was no evidence of secondary lesions caused by any of the progeny isolates. We destroyed all plots within two weeks after inoculation to preclude the population increase of any progeny.

Oospore survival. Oospores produced *in vitro* on agar plates survived in all environments during the study period (Figure 2.2). The plasmolysis technique provided a higher estimate of viability (maximum of 50%) compared to the germination assay (maximum of 9%) (Figure 2.2). The plasmolysis technique also provided results that were less variable over time (Figure 2.2). Of the oospores recovered, there was no significant change in germination for those stored at 4°C (P = 0.74) in the laboratory. However, there was a trend (P = 0.07) toward an increased rate of germination for oospores held in soil, in the growth chamber, at 18°C over the 18 months, and a highly significant increase in germination rate (P = 0.001) for oospores recovered after field burial. In contrast, there was a significant decline in viability, as determined by plasmolysis, over time for oospores stored at 4°C (P = 0.001) and in soil at 18°C (P = 0.001), but not in soil under field conditions (P = 0.115). The decline in viability was most pronounced at 4°C (Figure 2.2).

The soil environment in the field was generally moderate during the assay period. Soil temperatures remained fairly constant during the winter, ranging from 1-5°C. (Figure 2.3). Soil temperatures increased steadily in the spring and early summer months and peaked at 21°C in Jul and Aug 1998 (Figure 2.3). Soil moisture was high during the winter and early spring, and was lower in May and the soil remained dry during the summer months (Figure 2.3).

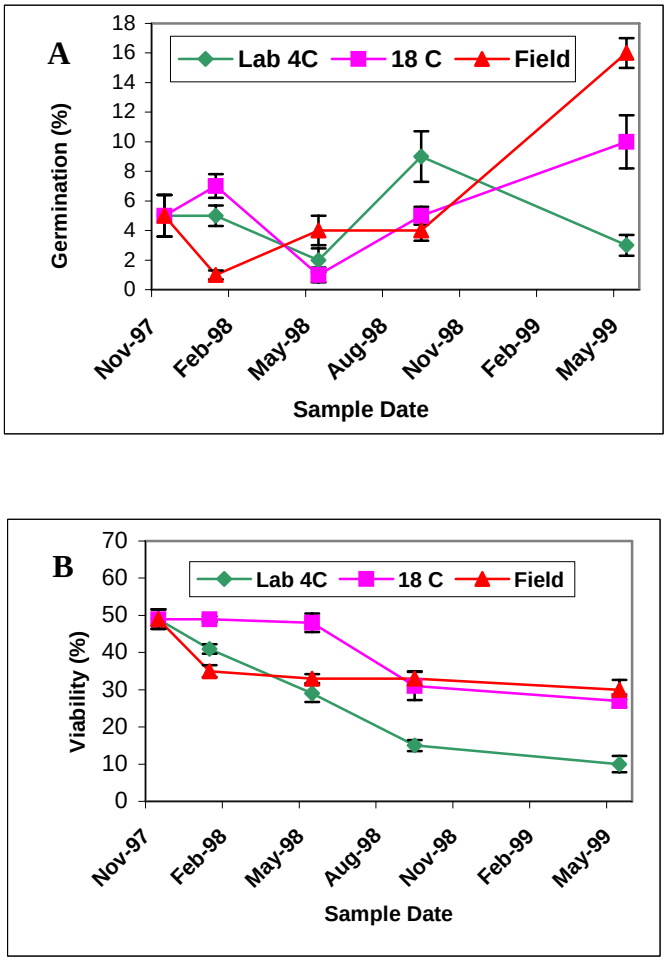

Figure 2.2. Assessment of viability of *in vitro* produced oospores from a US-17 X US-8 mating as determined by **(A)** germination on water agar and **(B)** ability to plasmolyze in NaCl. Oospores were produced *in vitro* and subsequently stored in the lab at 4°C, in soil at 18°C, or buried in the field under natural weather conditions. Error bars represent the standard error of the mean of three replicates obtained from each environment at each sample date.

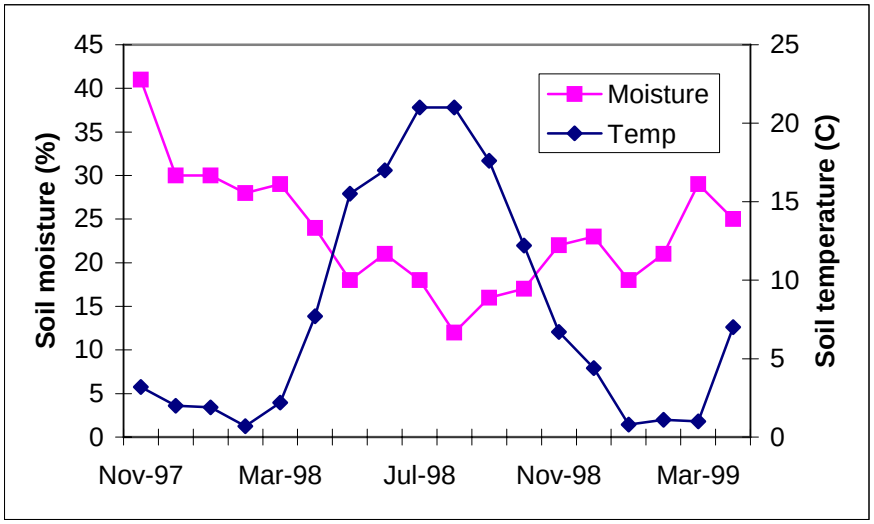

Figure 2.3. Soil moisture and temperature recorded in the field where oospores were buried.

Oospore infectivity. Some of the oospores produced *in vitro* and *in planta* were infectious after several months in the field or after several months in soil at 18°C. In contrast, no oospores produced from the *in vitro* cross stored at 4°C in the lab over the 18 month interval produced lesions. From two of the three field sites, some *in planta* produced oospores recovered after over-wintering were capable of infecting potato and tomato leaflets. For example oospores from tomato fruits and from stems of both tomatoes and potatoes were infectious, whereas oospores from potato tubers did not cause infection. Allozyme and RFLP analyses revealed that all 22 isolates recovered from infected potato and tomato leaflets were recombinant hybrid progeny. Oospores were required for long term survival, as no viable *P. infestans* was recovered from control tissue (tomato stems and fruits and potato stems and tubers infected with US-8 or US-17 alone) after long term storage in any of the three environments.

DISCUSSION

The results of this study provided data concerning the potential significance of sexual reproduction in *P. infestans* in northeastern USA. We determined that the US-17 and US-8 lineages of *P. infestans*, occurring commonly in New York State during the late 1990s, were fertile and could produce sexual hybrid progeny; thus our first hypothesis was not rejected. We further learned that oospores survived for at least 18 months in a variety of environments including in soil in the field, thus preventing us from rejecting our second hypothesis: that oospores can survive in the field. However, while progeny were generally capable of infecting both tomato and potato in detached leaflet tests, most progeny appeared to have lower pathogenicity than parental strains, and of a subset of 10 progeny, none produced secondary lesions in the field. Under the same conditions a parental strain and another field isolate were able to initiate rapid epidemics in the field. Thus we reject our third hypothesis that progeny are generally as pathogenic as field-adapted parental strains.

The ability of common strains of US lineages to produce oospores capable of germinating after exposure to field conditions in NY, differs from the data reported from Wales and Japan. Viable oospores were produced (as determined by ability to plasmolyze) by Welsh strains of *P. infestans,* but did not germinate after storage in the field for 10 months (Pittis and Shattock, 1994). In Japan, oospores produced in matings between the common strains there did not germinate (Mosa et al., 1991; Mosa et al., 1993). However, our study is consistent with reports from the Netherlands and Canada, where sexually compatible strains occurred and where survival in nature of some progeny has been inferred (Drenth et al., 1995; Medina and Platt, 1999).

An important result from our study is that oospores can survive in a variety of environments including in the field, greenhouse, and cold storage. Field survival is potentially very important, therefore we compared long-term monthly averages of soil

temperature data obtained from a monitoring station in the northeastern USA with those encountered in the 18 months of our study. Soil temperature in our field experiment, measured at 32 cm, never went below 0° C. Data from the soil monitoring station revealed that soil temperatures at a depth of 30 cm rarely went below 0°C (USDA Forest Service, Durham, NH; http://www.hbrook.sr.unh.edu/data/soil/stemp/cstdoc.htm). Soil moisture levels were typical of soils in the region. The soil moisture level in our field plot was close to saturation during the winter and early spring and became drier during the summer.

Oospore viability as determined by plasmolysis declined with time in several environments, while storage in soil appeared to promote germination of oospores. A decline in oospore viability as determined by plasmolysis is consistent with the study of Hanson and Shattock (Hanson and Shattock, 1998). Although the large variance in oospore germination limited conclusions, we observed an apparent increase in germination of oospores after storage in soil. This is potentially important. If something in the soil stimulates oospores to germinate, their role in initiating epidemics would be increased. Soil-stimulated germination of oospores is also important if this phenomenon can be utilized to improve oospore germination rates in genetics studies. The low and inconsistent germination percentages that we observed are not unusual for oospores of *P. infestans* (Medina and Platt, 1999; Pittis and Shattock, 1994).

We hypothesize that the viability of oospores of *P. infestans* declines slowly over time, but that they could remain viable for extended periods (>18 months). Presumably, the actual germination data are an underestimate of the oospore viability, because dormancy may prevent some oospores from germinating. However, ability to plasmolyse might be an overestimate of viability. It seems appropriate to regard the

plasmolysis data as maxima for viability and the germination data as minima for viability.

The segregation for metalaxyl sensitivity is consistent with the model of a single or linked gene(s) of major effect with additional genes of minor effect (Fabritius et al., 1997; Judelson and Roberts, 1999; Lee et al., 1999). The occurrence in the progeny of individuals that were more insensitive than either parent supports this model and indicates that sexual recombination can provide combinations of genes that might produce individuals with adaptability and virulence factors that are different than those of either parent.

Interestingly, all oospore progeny analyzed were found to be recombinant hybrids and were not the product of selfing or apomixis. This was true of progeny produced *in vitro* as well as *in vivo*. This was somewhat unexpected, as one of the strains used in this study (US940480) has been shown to be self-fertile in agar culture (Smart et al., 1998).

Despite the potential for new combinations of genes, the generally reduced pathogenic potential of most progeny relative to either parent is of considerable interest and of some potential importance. While several progeny appeared to produce lesions as large as those of either parent in growth chamber assays on detached leaflets, the majority appeared less pathogenic than either parent. Similarly, the entire subset of 10 progeny seemed dramatically less pathogenic than the A2 parental strain or another field isolate in a field test. This seemed counter-intuitive to us for an organism that has essentially no active saprophytic stage in its life history. We had expected genes for pathogenicity to be fixed in an organism such as *P. infestans*. However, this result is consistent with the data of Miller et al (Miller et al., 1998) and Gavino et al (Gavino et al., 2000) who reported the ephemeral appearance of a group of isolates (probable siblings) in the Pacific northwest of the United States, from

which only one isolate, giving rise to the US - 11 clonal lineage, survived for several seasons.

One conclusion concerning the epidemiology of *P. infestans* is that some progeny (and indeed if our results are generally applicable, then most progeny) are not as pathogenic as the parental strains. Thus, most progeny will not be selected and will not survive as epidemiologically important individuals. However, the rapid asexual reproduction of epidemiologically fit individuals enables even a small number of fit individuals to become important very rapidly.

LITERATURE CITED

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

Andersson, B., M. Sandstrom, and A. Stromberg. 1998. Indications of soil borne inoculum of *Phytophthora infestans*. Potato Research 41:305-310.

Barton, N.H., and B. Charlesworth. 1998. Why sex and recombination. Science 281:1986-1989.

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

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

Erwin, D.C., and O.K. Ribeiro. 1996. Phytophthora diseases worldwide The American Phytopathological Society, APS Press, St. Paul, MN.

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

Fay, J.C., and W.E. Fry. 1997. Effects of hot and cold temperatures on the survival of oospores produced by United States strains of *Phytophthora infestans*. American Potato Journal:315-323.

Fry, W.E., and S.B. Goodwin. 1997a. Re-emergence of potato and tomato late blight in the United States. Plant Disease 81:1349-1357.

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

Gavino, P.D. 1999. Diversity in the mitochondrial genome of *Phytophthora infestans*. PH. D., Cornell University, Ithaca.

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

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

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

Goodwin, S.B., R.E. Schneider, and W.E. Fry. 1995b. Use of cellulose-acetate electrophoresis for rapid detection of allozyme genotypes of *Phytophthora infestans*. Plant Disease 79:1181-1185.

Goodwin, S.B., L.S. Sujkowski, and W.E. Fry. 1996. Widespread distribution and probable origin of resistance to metalaxyl in clonal genotypes of *Phytophthora infestans* in the United States and Western Canada. Phytopathology 86:793-800.

Goodwin, S.B., L.S. Sujkowski, A.T. Dyer, B.A. Fry, and W.E. Fry. 1995c. Direct detection of gene flow and probable sexual reproduction of *Phytophthora infestans* in northern North America. Phytopathology 85:473-479.

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

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

Jiang, J., and D.C. Erwin. 1990. Morphology, plasmolysis, and tetrazolium bromide stain as criteria for determining viability of *Phytophthora* oospores. Mycologia 82:107-113.

Judelson, H.S., and S. Roberts. 1999. Multiple Loci Determining Insensitivity to Phenylamide Fungicides in *Phytophthora infestans*. Phytopathology 89:754-760.

Kato, M., E.S. Mizubuti, S.B. Goodwin, and W.E. Fry. 1997. Sensitivity to protectant fungicides and pathogenic fitness of clonal lineages of *Phytophthora infestans* in the United States. Phytopathology 87:973-978.

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

Legard, D.E., T.Y. Lee, and W.E. Fry. 1995. Pathogenic specialization in *Phytophthora infestans*: Aggressiveness on tomato. Phytopathology 85:1356-1361.

Matuszak, J.M., J. Fernandez-Elquezabal, W.K. Gu, M. Villarreal-Gonzalez, and W.E. Fry. 1994. Sensitivity of *Phytophthora infestans* populations to metalaxyl in Mexico:distribution and dynamics. Plant Disease 78:911-916.

Medina, M.V., and H.W. Platt. 1999. Viability of oospores of *Phytophthora infestans* under field conditions in Northeastern North America. Canadian Journal of Plant Pathology 21:137-143.

Miller, J.S., D.A. Johnson, and P.B. Hamm. 1998. Aggressiveness of isolates of *Phytophthora infestans* from the Columbia Basin of Washington and Oregon. Phytopathology 88:190-197.

Mizubuti, E.S.G., and W.E. Fry. 1998. Temperature effects on developmental stages of isolates from three clonal lineages of *Phytophthora infestans*. Phytopathology 88:837-843.

Mosa, A.A., K. Kobayashi, A. Ogashi, M. Kato, and N. Sato. 1991. Formation of oospores by *Phytophthora infestans* in inoculated potato tissues. Annals of the Phytopathological Society of Japan 57:334-338.

Mosa, A.A., K. Kobayashi, A. Ogashi, M. Kato, and N. Sato. 1993. Isozyme polymorphism and segregation in isolates of *Phytophthora infestans* from Japan. Plant Pathology 42:26-34.

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

Smart, C.D., M.R. Willman, H. Mayton, E.S.G. Mizubuti, R.W. Sandrock, A.E. Muldoon, and W.E. Fry. 1998. Self-Fertility in Two Clonal Lineages of *Phytophthora infestans*. Fungal Genetics and Biology 25:134-142.

Sujkowski, L.S., S.B. Goodwin, and W.E. Fry. 1996. Changes in specific virulence in Polish populations of *Phytophthora infestans*: 1985-1991. European Journal of Plant Pathology 102:555-561.

Wuethrich, B. 1998. Why sex? Putting theory to the test. Science 281:1980-1982.

CHAPTER THREE

ANALYSIS OF SOIL FROM CENTRAL MEXICO FOR SURVIVAL AND INFECTIVITY OF SPORANGIA OF *Phytophthora infestans*

ABSTRACT

Soil samples collected from the central highlands of Mexico, the center of diversity for *Phytophthora infestans,* were evaluated for suppressive activity to *P. infestans*. A New York soil was used for comparative analysis. Through detached leaflet infectivity bioassays it was determined that sporangia can survive and remain infective for several weeks in these soils. Even though both mating types are known to co-habit these regions of Mexico, no evidence of oospores was found in the soil samples. No significant differences were observed between the three mountain and two valley soils from Mexico and the New York soil in terms of their influence on survival and infectivity of *P. infestans.* Terminal restriction fragment length polymorphism analysis (T-RFLP) of the bacterial communities present in these soils was conducted in conjunction with detached leaflet bioassays with the goal of characterizing the composition of potentially suppressive soil bacterial communities and identifying possible antagonists to *P. infestans*. Bacterial community composition was similar in the two Mexican valley soils, but these differed substantially from that in the mountain and New York soils. The New York soil shared some population characteristics in common with both of the Mexican soils, but clustered separately in most analyses. In canonical correspondence analysis, the main environmental factors correlated with mountain soil bacterial community T-RFLP fingerprints were soil organic matter (%), Al and Ca concentrations and CEC. Soil P and NO_3 concentrations were more important variables related to community composition in the valley soils, which are

cropped regularly. Since none of the Mexican soils was suppressive for *P. infestans*, soil microbial communities were not characterized further.

INTRODUCTION

Biological control of plant pathogens in soil is an area of intense interest due to the need to minimize the use of synthetic pesticides to control plant diseases. Two aspects typically desired in a fungicide are a short residual life and low mobility in soil, so that it will have limited environmental impact. A logical result is that such fungicides are typically not very effective against soil-borne pathogens (Nelson, 1996). In contrast, soil fumigants have been effective against a wide range of soil-borne pathogens and pests but they are also toxic to non-target organisms. In addition to the undesirable effects on non-target organisms, methyl bromide, a particularly effective soil fumigant, also contributes to depletion of the ozone layer (Gilreath et al., 2005). Environmentally friendly and more sustainable methods for control of soil-borne pathogens are needed. Soil ecosystems are complex environments and like most natural systems have biological limits for growth and survival of organisms within a specific niche. Plant pathologists (and farmers) have long recognized the ability of some soils to suppress plant pathogens (Baker and Cook, 1982; Hornby, 1983). Analysis of the nature of pathogen suppressive soils is a rapidly growing area of research for control of soil-borne diseases (Mazzola, 2002).

Suppressive soils inhibit plant pathogen growth and disease development even under conditions that are favorable or conducive for disease to occur (Baker and Cook, 1982; Haas and Defago, 2005). In suppressive soils, the pathogen may not be able to flourish and reproduce in sufficient numbers to cause disease or cannot sustain itself once it has become established. Suppressive soils are most often characterized as having either general or specific suppression to plant pathogens (Baker and Cook,

1982; Garbeva et al., 2004). General suppression is defined as broad-based control of soil pathogens. Agricultural fields subject to frequent crop rotations often exhibit general suppression of diseases caused by nematode and fungal pathogens. This type of general suppression is often attributed to an increase in biodiversity of non-plant pathogenic soil organisms (Baker, 1991; Garbeva et al., 2004). Soil biodiversity, type and quality of organic matter, and the physical nature of soils are all components of general suppression (Mazzola, 2002). Specific suppression is the inhibition of a particular pathogen by antagonistic organism/s (Baker and Cook, 1982). Many studies on suppressive soils have focused on identifying factors involved with specific suppression due to the interest in exploiting antagonistic organisms for biocontrol. Examples of suppressive soils have been documented for several plant pathogen systems (Baker and Cook, 1982; Erwin and Ribeiro, 1996; Haas and Defago, 2005; Mazzola, 2002). The most well known examples of suppressive soil systems are those that control *Fusarium* wilts, caused by *Fusarium oxysporum*, and take-all of wheat caused by *Gaeumannomyces graminis* var. *tritici*; (Garbeva et al., 2004; Mazzola, 2002).

Potato late blight, caused by the oomycete *Phytophthora infestans*, is extremely destructive to foliage and tubers in soil. Tubers are infected by sporangia of *P. infestans* that are produced in the leaf canopy and then washed through the soil. These sporangia can also lay dormant in soil and initiate infections of tubers during or after harvest. Late blight is an extremely destructive disease and can destroy potato fields within two weeks after infection by *P. infestans* (Fry et al., 2002). Tuber blight is often accompanied by soft rot in tubers and other secondary pathogens. As a result, minimal amounts of tuber blight (2-4%) can lead to complete loss of potatoes in storage (Dorrance and Inglis, 1998). Infected asymptomatic tubers can be a mechanism for distributing the pathogen and can also initiate primary infections when

planted (Erwin and Ribeiro, 1996). Due to the potential for catastrophic loss, large amounts of protectant fungicides are applied to prevent foliar blight each year. At the present time there are no fungicides available for control of tuber blight.

The recent occurrence of both mating types of *P. infestans* in many locations worldwide (including the USA) makes the production of oospores possible in regions where none have existed previously (Goodwin et al., 1995). Oospores are the most resilient form of the pathogen and can survive for long periods of time in soil (Erwin and Ribeiro, 1996; Mayton et al., 2000). Oospores can initiate a foliar epidemic or they can directly infect tubers (Fernandez-Pavia et al., 2003; Grunwald and Flier, 2005) .

Discovery of antagonistic organisms that are able to reduce the number of viable sporangia/oospores of *P. infestans* in soil could lead to an ideal sustainable method of control of late blight. Many genera of several soil-dwelling microorganisms are able to parasitize and lyse *Phytophthora* propagules in soil (Erwin and Ribeiro, 1996). A likely location to find antagonists or parasites of an organism is in a location where that organism has existed for a long time (Baker and Cook, 1982). The highlands of central Mexico is the region with the greatest diversity of *P. infestans* and it is also believed that *P. infestans* co-evolved with wild *Solanum* species in that part of the world (Fry and Goodwin, 1997; Fry et al., 1993). Until the latter half of the 20th century, the highlands of central Mexico were the only regions in the world, where oospores were routinely produced and played an important role in the life cycle of *P. infestans* (Andrivon, 1995; Gallegly and Galindo, 1957). Recently it was shown that some soils from this region were more conducive for germination and infectivity of oospores than other soils (Fernandez-Pavia et al., 2003).

The importance of the population structure of microbial communities in soil to agriculture and the overall soil ecosystem has become increasingly more apparent over

the past several years. In addition to their influence on suppression of plant diseases, soil organisms are essential for critical biogeochemical cycling of both organic and inorganic compounds (Kirk et al., 2004). Unfortunately, research on the complexity and diversity of soil microbial communities has been challenging, due to the fact that less the 1% of soil microorganisms can be cultured and identified (Daniel, 2005). Fatty acid methyl ester analysis, diversity in carbon (C) utilization and other traditional biochemical based methods in combination with new molecular nucleic acid-based methods have enhanced our ability to differentiate organisms in soils (Kirk et al., 2004)

Terminal restriction fragment length polymorphism (T-RFLP) analysis is one of several new nucleic acid-based molecular methods now used to assess microbial diversity in natural systems (Blackwood et al., 2003; Clement et al., 1998; Liu et al., 1997). In T-RFLP, polymerase chain reaction (PCR) is used to amplify DNA extracted from environmental samples. The PCR product is tagged with a fluorescent label at the 5' terminal end. The amplicon is then digested with restriction enzymes and the terminal restriction fragment size polymorphisms are analyzed with an automated sequencer (Osborn et al., 2000). Ribosomal RNA genes are often used as the molecular markers of choice because they are involved in many vital cellular processes and are highly conserved. The most commonly amplified target for prokaryotic profiling is the 16S rRNA gene (Blackwood et al., 2003; Hill et al., 2000; Osborn and J., 2005). T-RFLP analysis can provide estimates of quantitative microbial diversity as well as be used to identify the predominant phylogenetic groups present in natural soils (Blackwood et al., 2003). With the use of T-RFLP analysis, we can potentially identify specific phylogenetic groups that may be involved in both general and specific pathogen suppression in soils.

The objectives of this project were to determine the effects of different soils from the highlands of central Mexico on the survival and infectivity of *P. infestans*, and to determine if soils from different locations in central Mexico had similar or distinct bacterial community structures. Our approach was to assess the survival of sporangia in both sterile and non-sterile soils to determine if biotic or abiotic mechanisms influenced survival time. If some soils were suppressive, we would attempt to identify possible antagonists for use in biocontrol of *P. infestans* in soil.

MATERIALS AND METHODS

Soils. Composite soil samples were collected from the A horizon (5-10 cm depth) in fields located in the central highlands of Mexico in November 2001. Three soils were sampled in the Sierra Mountains just north of Mexico City in Santa Catarina del Monte, Municipio de Texcoco (Table 3.1). Both cultivated potatoes and wild S*olanum* species are commonly infected with *P. infestans* in these mountain regions. Two of these samples (Mt1 and Mt2) were collected from fields planted to corn in rotation with potatoes. The other mountain sample (Mt3) was obtained from the forest adjacent to the cultivated fields. Two soils were sampled from the Toluca Valley (V1 and V2), near Metepec, State of Toluca, Mexico (Table 3.1). A New York soil (used as a control) was sampled in 2001 from a field that had not been cropped for more than five years and presumably had never been planted to potatoes. Soil samples collected from the fields were subdivided; some were stored in plastic containers at 4° C and others stored at -20° C (for subsequent microbial diversity analysis). Mexican soil samples were stored under quarantine at the USDA Golden Nematode facility on the Cornell University campus. All experiments were conducted with soils that had been sieved with a 2 mm mesh screen. Physical analysis of the soils was conducted by the Cornell Agricultural and Life Sciences Nutrient Analysis Laboratory (CNAL).

***P. infestans* isolates.** Two different isolates of *P. infestans* were used to assess survival of sporangia in the diverse soil samples. One isolate (US940480, ATCC # 208834) belonged to the US8 clonal lineage and had been obtained from potatoes in New York in 1994. The other isolate (MX990005 = MX5) had been collected from infected potatoes in the Toluca Valley, Mexico, in 1999. All manipulations were conducted under the provisions of a Cornell University quarantine permit, with all materials involving exotic strains being autoclaved when experiments were completed. Isolates were grown on Rye B (Erwin and Ribeiro, 1996) medium in a growth chamber at 15°C and transferred to new media every other week. Sporangia for experiments were obtained from sporulating lesions on leaves of the potato cultivar Katahdin or Atlantic which had been incubated in a moist chamber at 15°C for 7 d following inoculation.

Survival and infectivity of *P. infestans* in infested soil samples. Survival of sporangia over time in the soil samples was measured through infectivity bioassays. Infectivity of *P. infestans* sporangia was assessed in both non-sterile and sterile soils. Sterile soils were created by autoclaving a soil sample (500 g) three times for 60 minutes on three successive days. The infectivity bioassay was modified from that described by Drenth (1995). It involved floating leaf disks (1 cm diameter) over 5 g of saturated soil in a well of a sterile, six-well polystyrene cell culture plate (3 cm x 1.5 cm) (Corning Inc., Corning, NY). Three of the six wells were non-infested soil controls and three were infested with 2 ml of a sporangial suspension (2500 sporangia ml^{-1}). An additional 3 ml of sterile distilled water was added to each well to saturate the soil. Control (non-infested) wells received 5 ml of sterile distilled water. Additional water was added when necessary to maintain soil at near saturation during the experiment. If the leaf disk became infected after incubation for 5-10 d at 15°C, the sample was concluded to contain viable *P. infestans*. A total of seven, six-well

polystyrene plates were infested at the same time (day one). On day one and then once a week for seven weeks, potato leaf discs were added to a new six-well polystyrene plate that was infested on day one of the experiment. Each week three potato leaf discs (cork bore #10, 1 cm diameter) were placed on the surface of the soil of each well (total 18 leaf discs per plate) in a "floating leaf" bioassay to determine infectivity. Infectivity was scored as a percentage of leaf discs infected.

Survival and infectivity of *P. infestans* in soil extracts. The effects of soil extracts on survival of sporangia were measured through infectivity of detached potato leaflets. Soil extracts were obtained by adding 10 ml water to 10 g soil in 50 ml centrifuge tubes. The tubes were shaken for 2 h and then centrifuged at 3000 rpm for 10 min (Zan, 1962). The supernatant (soil extract) from each soil mixture was decanted and placed in a 15 ml sterile centrifuge tube. Four ml of a sporangial suspension (20,000 sporangia ml^{-1}) of *P. infestans* was added to each tube containing the soil extract. Soil extracts and sporangia were stored at 15°C in the dark for the duration of the experiments. Nine potato leaf discs (cork bore # 10) per soil extract were placed in mini-moist chambers (100 x 15 mm, 1% water agar Petri plates) and were inoculated with 100 µl extract suspension on day 0 and then once a week for 6-7 weeks. Infectivity was scored as a percentage of the leaf discs infected. Bioassays with both the US8 and MX5 isolates were conducted individually. All trials were repeated and the data were combined.

T-RFLP bacterial community composition analysis. DNA was extracted from soil according to manufacturer's instructions with the UltraClean™ Soil DNA kit (MoBio Laboratories, Inc., Solana Beach, CA). Two separate DNA extractions were conducted for all soil samples. Three, replicate 1 g samples of each soil were used for each of the two soil DNA extractions. The extracted DNA was purified with a PCR Kleen Spin Column (Bio-Rad Laboratories, Hercules, CA) and then quantified prior to

PCR amplification with a DyNA Quant 2000 Fluorometer (Hoefer Pharmacia Biotech, Inc., San Francisco, CA). The 16S rRNA genes of soil bacteria were amplified using the primers 27F-FAM and 1492R (Moeseneder et al., 1999). The 27F-FAM primer was labeled at the 5′ end with phosphoramidite fluorochrome 5-carboxyfluorescein (5' 6-FAM, Searing, Belgium) (Moeseneder et al., 1999) to facilitate downstream laser detection and sizing of the terminal fragment. PCR reaction (50 µl total volume) reagents and concentrations were: ultra pure water, 10X Taq buffer; 25 mM $MgCl_2$; 10 mM dNTPs, 10 µM of each primer, 10 mg ml^{-1} BSA, 5U μl^{-1} Amplitaq and 1 ng target DNA. A Hybaid PCR Express thermal cycler (Hybaid Limited, Middlesex, TW) with the following PCR program was used to amplify 16S rDNA from each sample: 1 cycle, 94°C for 3 min; 30 cycles, 94°C for 30 sec, 62°C 15 sec, 72°C (60 seconds) and a final extension at 72°C for 15 minutes. PCR products were confirmed by gel electrophoresis consisting of either 1% or 1.5% agarose gels with 2 µl (10 mg ml^{-1}) ethidium bromide added to detect bands using ultraviolet light. PCR products were purified with a QIAquick PCR purification kit (Qiagen, Inc. Valencia, CA). The cleaned PCR amplicons were digested (30 µl total volume) for 4.5 h at 37ºC with two different restriction enzymes, *Hha*I (Promega), and *Sau96*I (New England Biolabs, Beverly, MA), in separate reactions. Reactions were terminated by heating at 70ºC for 15 minutes. The digested products were purified using a Performa® DTR 96-well purification plate (Edge Biosystems, Gaithersburg, MD). Sizes of terminal restriction fragments from sample digests were determined with an ABI 3730 fragment analysis system at the Cornell University Biotechnology Resource Center (Ithaca, NY). Electropherograms of terminal restriction fragments (T-RFs) were generated using Genemapper software version 3.0 (Applied Biosystems).

Data analysis. Means of the percentage of infected leaflets in bioassays were computed for the soil infestation and soil extract bioassay trials and subjected to

analysis of variance using the Proc GLM procedure (General Linear Model Procedure) with SAS 9.1 (SAS institute, Cary, NC). Polynomial curves and linear regression lines were fitted to the infectivity data of *P. infestans* sporangia over time in diverse soils and soil extracts with Microsoft Excel Data Analysis software. Hierarchical cluster analysis of T-RFs of 16S rDNA amplified from soil samples (Ward's single linkage analysis), bacterial community diversity values and canonical correspondence analysis (CCA) of T-RFs with soil characteristics were accomplished using PC-ORD (Multivariate Analysis software MjM Software, Glendon Beach, OR).

RESULTS

Sites. The names, designations, soil texture and global positioning system (GPS) coordinates for all sites sampled are given in Table 3.1.

Table 3.1. Name of regions where samples were collected, physical texture, and GPS.*

Soil sample	Desig.	Texture	GPS
Huiloapan	Mt1	Fine sandy loam	3140 M[a] N 19° W 98°
La Cabana	Mt2	Fine sandy loam	3160 M N 19° W 98°
Mt Forest	Mt3	Sandy loam	3170 M N 19° W 98°
El Corral	V1	Fine sandy loam	2640 M N 19° S 99°
Torquesa	V2	Sandy loam	2640 M N 19° S 99°
New York	NY	Silt loam	307 M N 43° W 76°

* Global positioning satellite coordinates

Soil analyses. Soils collected from the five Mexican and one NY field site had similar soil texture (Table 3.1). The three mountain soils, Mt1, Mt2, and Mt3, had higher concentrations of aluminum (Al) and calcium (Ca), higher cation exchange capacity (CEC) and higher OM contents than did the valley soils (Table 3.2). Soils ranged in pH from 5.6 to 6.6. The valley soils were more characterized by (Mt3) soil had the highest organic matter (Table 3.2).

Table 3.2. Selected soil characteristics of diverse soil samples collected from Mexico and New York.

Soil	NO_3^-	P	K	Ca	Mg	Al	pH	OM	CEC
	----------(mg/kg)----------							(%)	(cmol kg^{-1})
Mt1	9.4	0	158	1476	97	208	6.5	6.9	31.2
Mt2	0	0	103	1517	90	157	6.6	6.3	27.7
Mt3	0	1	304	2257	96	195	5.9	14.6	43.2
V1	47.2	11.3	341	567	125	20	5.2	2.4	10.5
V2	9.0	2.8	165	1007	193	20	6.0	3.4	13.6
NY	11.7	0.7	73	918	137	23	5.9	4.4	11.3

Isolates. No significant differences attributable to isolate were observed in any experiment. Therefore, all subsequent results and figures presented here are based on experiments with the MX5 isolate.

Survival and infectivity of *P. infestans* in infested soil samples. Data obtained from the bioassays on survival and infectivity in the Mexican and New York soils infested with *P. infestans* sporangia revealed that sporangia survive longer in sterile than in non-sterile soil (Figures 3.1 and 3.2). The overall mean infectivity over time was slightly higher in the Mountain soils than in the two Valley or the single New York soils. Little variation was observed in the length of survival of sporangia in the different soils.

Survival and infectivity of *P. infestans* in soil extracts. Initial infectivity of sporangia was higher in non-sterile and sterile than in soils infested with *P. infestans* sporangia (Figures 3.3 and 3.4). However, *P. infestans* sporangia survived for a shorter time in soil extracts than in soils. There were no significant differences in survival of sporangia in the non-sterile versus the sterile soil extracts (Figures 3.3 and 3.4).

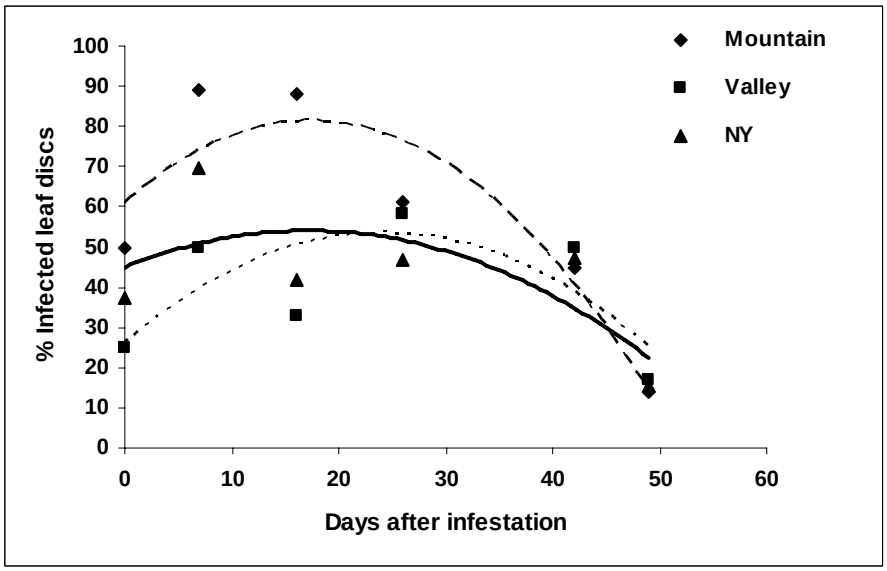

Figure 3.1. Survival of *P. infestans* sporangia as determined by the floating leaf bioassay in diverse sterile soil samples soils infested with *P. infestans* isolate MX5. Polynomial regression fitted to survival data; R^2 Mountain = 0.83 (------); R^2 Valley = 0.53 (…….); R^2 New York = 0.49 (____).

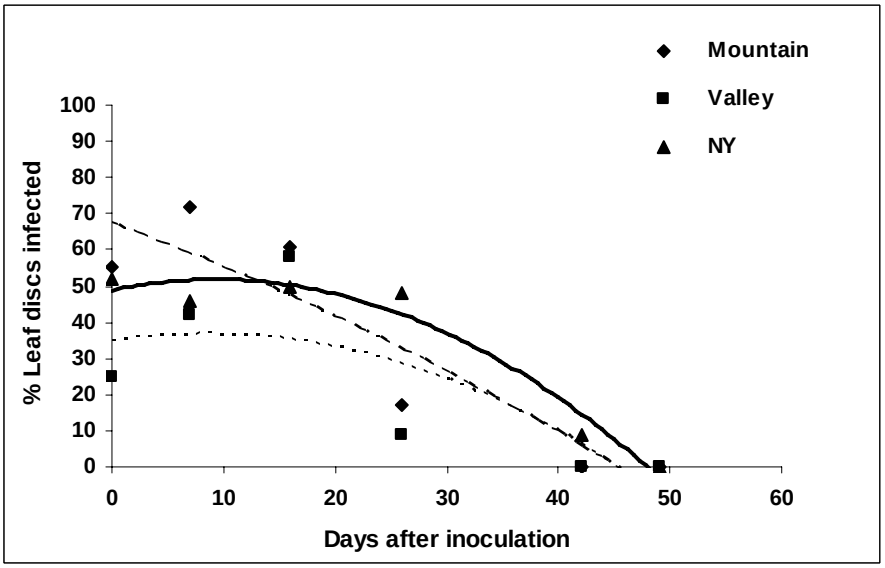

Figure 3.2. Survival of *P. infestans* sporangia as determined by the floating leaf bioassay in diverse non-sterile soil samples soils infested with *P. infestans* isolate MX5. Polynomial regression fitted to survival data; R^2 Mountain = 0.83 (----); R^2 Valley = 0.60 (…); R^2 New York = 0.95 (—).

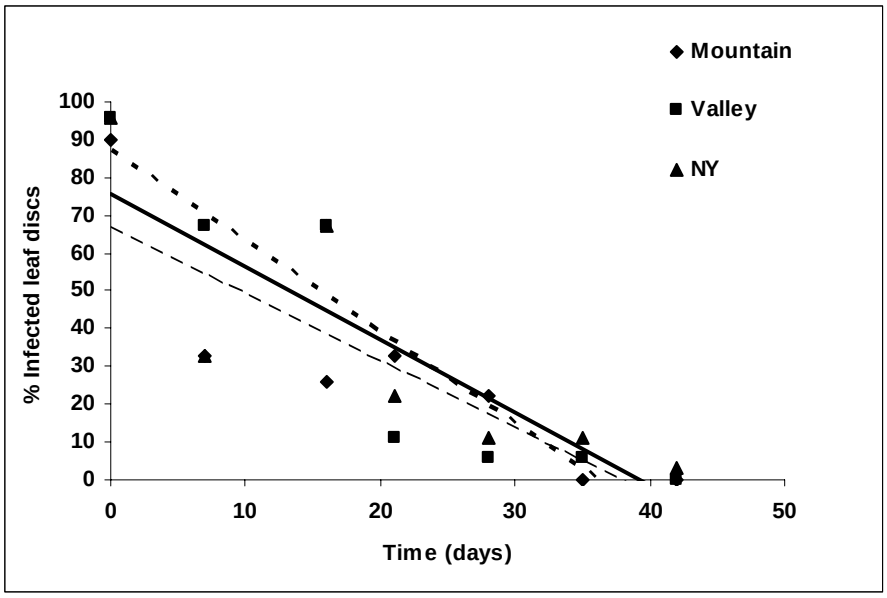

Figure 3.3. Survival of sporangia of *P. infestans* stored in sterile soil extract as determined by inoculating detached potato leaflets with the MX 5 isolate. Linear regression fitted to survival data; R^2 Mountain = 0.77 (------); R^2 Valley = 0.83 (…….); R^2 New York = 0.67(_____).

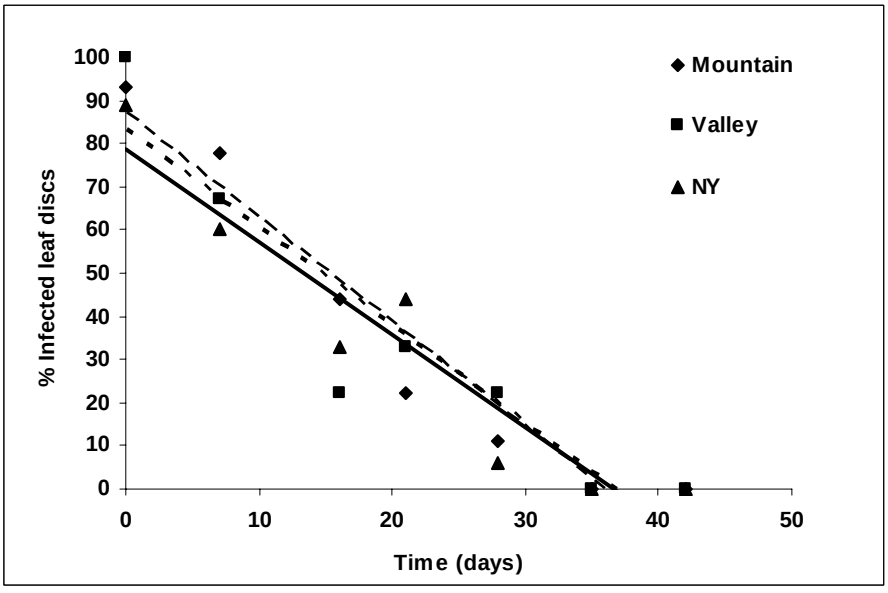

Figure 3.4. Survival of sporangia of *P. infestans* stored in non-sterile soil extract as determined by inoculating detached potato leaflets with the MX 5 isolate. Polynomial regression fitted to survival data; R^2 Mountain = 0.93 (------); R^2 Valley = 0.87 (…….); R^2 New York = 0.90 (_____).

T-RFLP bacterial diversity analysis. Hydrolysis of PCR-amplified 16S rDNA extracted from soil with restriction enzyme *Sau96*I resulted in a greater number of terminal restriction fragments (T-RFs) than with *Hha*I restriction enzyme. Results, however, from analysis of T-RF data generated through digests with each enzyme were similar in relation to species richness and diversity of bacterial communities from each sample site. Canonical correspondence analysis (CCA) of bacterial fragments with soil variables also revealed similar correlations between soil factors and bacterial community composition. Therefore data from both restriction enzyme digests were combined for analysis (see Appendix 1 for each separate enzyme analysis).

The Mexican forest soil and the Valley 1 soil had the greatest number of T-RFs (richness) from digests with *Hha*I and *Sau96*I when analyzed separately and combined, while the New York soil generated the lowest number of T-RFs (Table 3.3).

Table 3.3. Summary of richness, evenness, and diversity, of bacterial T-RF's detected from soil DNA extracted from the Mexican mountain and valley soils and the New York soil sample digested with both *Hha*I, and *Sau96*I restriction enzymes.

Soil	Richness*	Evenness**	Shannon's diversity index	Simpson's diversity index
Mt1	160	0.82	4.19	0.97
Mt2	139	0.83	4.10	0.97
Mt3 Forest	175	0.69	3.53	0.88
V1	174	0.86	4.40	0.98
V2	125	0.85	4.10	0.97
NY	119	0.86	4.09	0.98

* Richness = number of T-RFs per soil sample

** Evenness = Shannon's index/ln (richness)

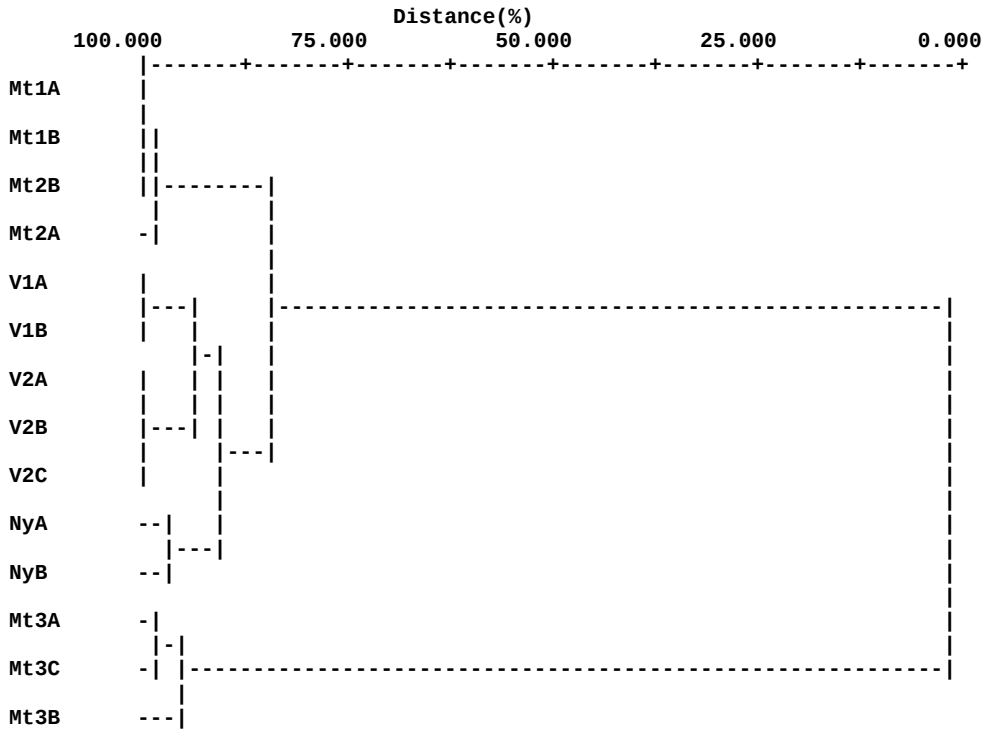

Figure 3.5. Dendrogram of the relationship between bacterial communities generated by hierarchical cluster analysis using Ward's linkage and correlation coefficient distance of T-RFLP fingerprints of 16S rDNA amplified from diverse soil samples.

All soils had somewhat uniform distributions of bacterial species as indicated by the degree of evenness, and both the Shannon-Wiener and Simpson diversity index values (Table 3.3).

Hierarchical cluster analysis of T-RFs from soils revealed that the bacterial communities present in the two mountain soils (Mt1A-B and Mt2A-B) that had recently been planted with corn in rotation with potatoes were very similar to each other (Figure 3.5). The bacterial community compositions from the two valley soils sampled were also similar to one another. Surprisingly the T-RFs detected in the New York soil were more similar to both the valley and cropped mountain soils than the mountain forest soil, which had a very different bacterial community population than all of the other soils (Figure 3.5).

Results from the CCA of T-RFs with soil variables revealed significant correlations between soil characteristics and bacterial species composition (Table 3.4). The first two ordination dimensions explained 38.3 % of the variation between bacterial profiles and environmental soil factors and the third axis explained 12.2 %, with a cumulative variation of 50.4% for the three axes (Figures 3.6 and 3.7). The bacterial R-TFs detected in the mountain forest soil (Mt3) were strongly associated with OM (%), Al and Ca concentrations, and CEC (Figure 3.6). Whereas, the other mountain (Mt1 and Mt2) soil bacterial communities were more highly correlated with soil pH (Figure 3.7). The V1 soil community composition was correlated with nitrate (NO_3^- and phosphorus (P) concentrations and the second valley (V2) and New York (NY) soil bacterial communities were correlated with soil concentrations of magnesium (Mg) (Figures 3.6 and 3.7).

Table 3.4. Summary of canonical correspondence analysis (CCA) of bacterial T-RFs detected from soil DNA digested with *Hha*I and *Sau96*I restriction enzyme with soil characteristics from each site. Eigenvalues (299 runs), % variance explained for each axis and Pearson's correlation values.

	Axis 1	**Axis 2**	**Axis 3**	***P* value**
Eigenvalue	0.41	0.25	0.21	0.11
% Variance explained	23.6	14.6	12.2	
% Cumulative variance explained	23.6	38.3	50.4	
Pearson's correlation	0.98	0.98	0.94	0.03

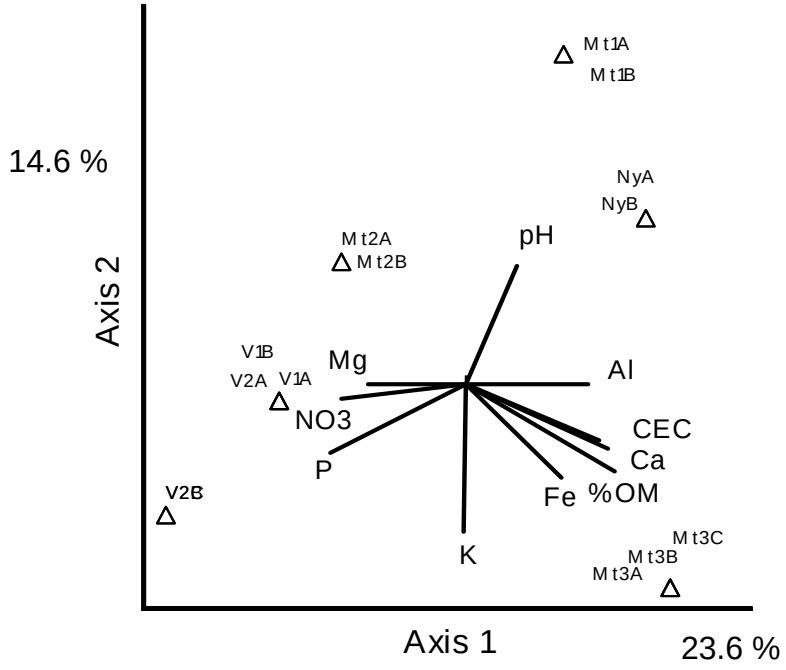

Figure 3.6. Canonical correspondence analysis ordination diagram (Axis 1 vs Axis 2) of T-RFs detected from soil DNA digested with *Hha*I and *Sau96*I restriction enzymes with soil characteristics from samples collected in Mexico and New York.

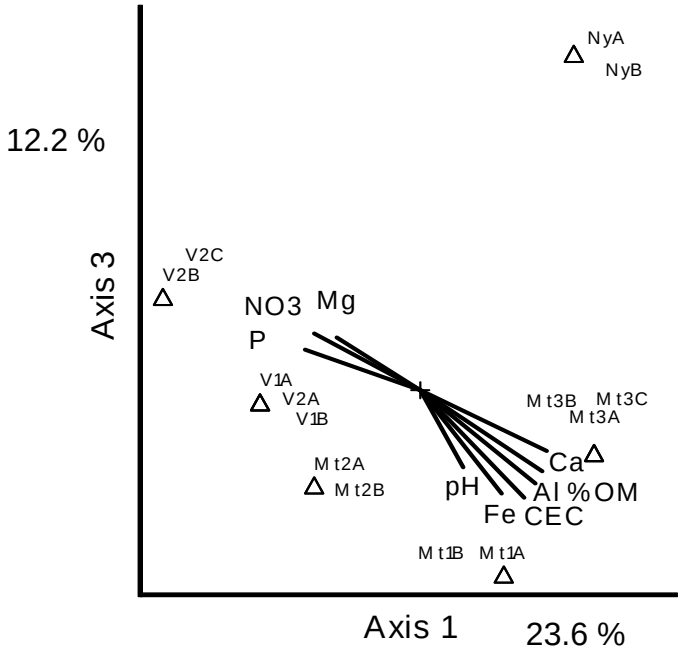

Figure 3.7. Canonical correspondence analysis ordination diagram (Axis 1 vs Axis 3) of T-RFs detected from soil DNA digested with *Hha*I and *Sau96*I restriction enzymes with soil characteristics from samples collected in Mexico and New York.

DISCUSSION

Soils collected from the central highlands of Mexico were hypothesized to be the most likely source for finding antagonists or parasites to *P. infestans* because the pathogen and potato plant have co-existed there for a very long time (Baker and Cook, 1982). The selection of soil sampling sites was based on previous research conducted by Fernandez-Pavia et al. (2003). In floating leaf bioassays, they observed that oospores germinated and had greater infectivity in specific soils. They also reported that oospores in the central highlands could survive fallow periods and be a source of primary inoculum. Several factors may be involved in the survival and infectivity of *P. infestans* in those soils. Soil texture, parent material (mineral composition), organic matter (OM) content and biological activity can all influence survival of oospores and sporangia.

Soil textures of the samples were fairly similar (Table 3.2). All soils were either sandy, fine sandy, or silt loams. The mountain samples contained higher levels of OM, (6.99, 6.26, and 14.61%) than either of the valley soils or the New York soil (2.18, 3.36, and 4.4%, respectively). Mineral soils typically have between 1.0 and 6.0% OM (Torsvik and Ovreas, 2002). Organic matter in soil is extremely important for numerous biological activities, and influences cation exchange capacity (CEC), water holding capacity, and soil structure. Soils with high CEC generally have greater quantities of essential nutrients available for use by plants and soil organisms. The mountain soils had higher CEC than the other soil samples and the forest soil sampled (Mt3) located adjacent to the other mountain fields had the highest CEC and %OM. Previous reports have shown Al to have a detrimental effect on *P. infestans* in soil (Andrivon, 1994). Although the mountain soils had high concentrations of Al, the toxic Al^{3+} ion was likely not available in solution due to the pH being above 5.9 and the CEC being greater than 25 cmol kg^{-1}. Thus, the Al concentrations measured probably did not have a detrimental effect on *P. infestans* sporangia in these bioassays.

Detached leaflet assays have been an effective method for assessing the survival of *P. infestans* in soils (Drenth et al., 1995). Based on the infestation assays, there were no differences among soils in terms of survival and infectivity of *P. infestans.* Less overall survival was observed in the soil extract bioassays where the soil mineral fraction and organic matter were removed. Porter and Johnson (2004), reported survival of sporangia of *P. infestans* to be 14-21 days in water under ambient field conditions in potato field plots in Washington State and for a longer period (25-38 days) when sporangia were incubated in water + soil in the field plots. Several researchers have reported survival of *P. infestans* in soil for greater than two months (Andrivon, 1995; Lacey, 1965). Due to the high variability of survival of *P. infestans*

sporangia, as measured by infectivity in the soil infestation and extract assays, no significant differences were observed between soils.

The advent of the molecular tools for ecological experiments in soil has greatly enhanced our ability to potentially identify particularly important species involved with control of plant pathogens. The T-RFLP method was chosen to analyze soil samples collected from Mexico and New York because it has been demonstrated to be a valid method to obtain comparative and elementary taxonomic data on soil microbial communities (Kirk et al., 2004; Osborn et al., 2000). Taxonomic classification of bacterial and fungal communities is still an arduous task due to the lack of a single definition of what constitutes a species (Hugenholtz et al., 1998; Kirk et al., 2004). Incomplete DNA extraction and digestion of soil nucleic acids, PCR bias of specific sequences and T-RF lengths that are common among bacteria also influence and may bias species identification and estimates of diversity and abundance (Osborne et al., 2006; Suzuki and Giovanni, 1996). However, despite the difficulties encountered in matching T-RF profiles with sequence databases for identifying dominant or unique species and phylogenetic groups, T-RFLP analysis is a useful first step in assessing bacterial community composition in different soils (Lukow et al., 2000; Osborn et al., 2000). Although we did not detect a suppressive effect of the soils evaluated for survival and infectivity of *P. infestans*, we were able to identify unique terminal fragments associated with the different soils and through CCA were able to correlate soil variables with bacterial community composition. Clearly, the different soils tested harbored different bacterial communities. Despite this, the soils were not suppressive to *P. infestans* when tested under laboratory conditions.

Sporangia of *P. infestans* did survive for a longer time in sterile versus non-sterile soil samples indicating a general type of biological suppression. However, no differences in survival of *P. infestans* were observed between soils, therefore, even

though the soil bacterial populations were different, no specific bacterial genera associated with survival could be identified.

These results support many similar observations that sporangia of *P. infestans* cannot survive as long as sexually produced oospores (months versus years) in soil and that survival can be influenced by soil organisms (Andrivon, 1995; Drenth et al., 1995; Lacey, 1965; Mayton et al., 2000; Peters et al., 2000). The soils collected from Mexico that were used in our experiments may have lost much of their intrinsic physical and biological qualities by removing them from their natural environment and transporting them to the laboratory. Experiments conducted by Fernandez-Pavia et. al. (2003) which showed variation in germination and infectivity of oospores were established immediately after sampling. There may be specific antagonists to *P. infestans* in soil. A search for potentially suppressive organisms should be continued. However, design and implementation of experiments with soils should be conducted with as little manipulation and disturbance of physical and environmental factors as possible to enhance the ability to identify and perhaps isolate potentially significant organisms for use in biocontrol of *P. infestans*.

APPENDIX ONE

ANALYSIS OF SOIL FROM CENTRAL MEXICO FOR SURVIVAL AND INFECTIVITY OF SPORANGIA OF *PHYTOPHTHORA INFESTANS*

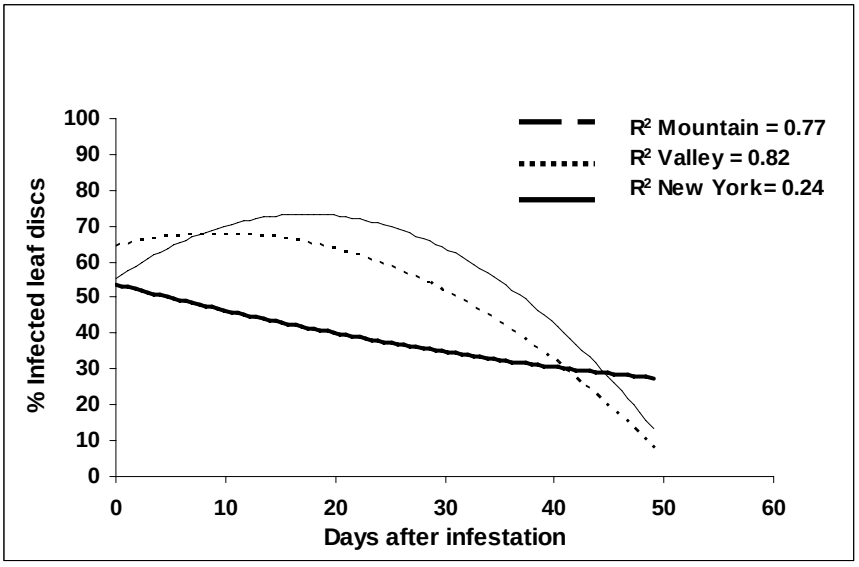

Figure A1.1. Survival of *P. infestans* sporangia as determined by the floating leaf bioassay in diverse sterile soil samples soils infested with *P. infestans* isolate US8. Polynomial regression fitted to survival data; R^2 Mountain = 0.77(------); R^2 Valley = 0.82 (…….); R^2 New York = 0.24 (____).

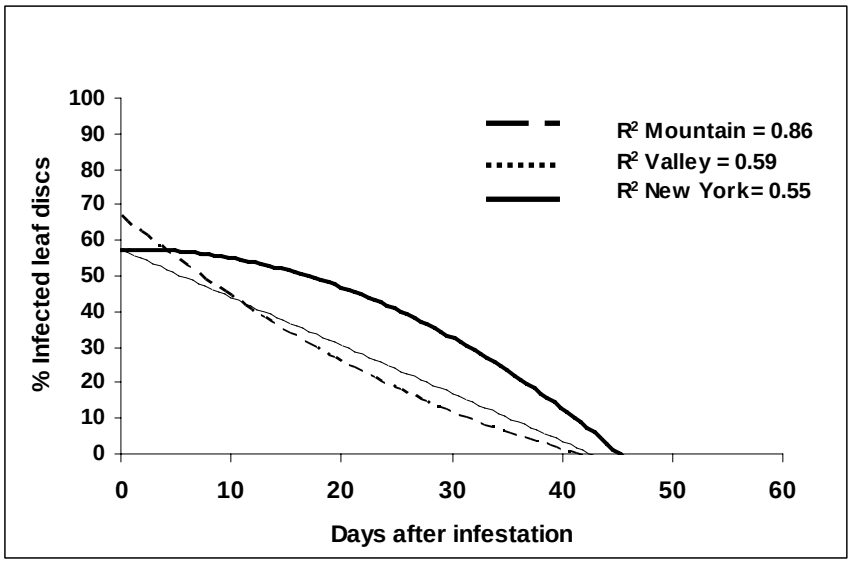

Figure A1.2. Survival of *P. infestans* sporangia as determined by the floating leaf bioassay in diverse non-sterile soil samples soils infested with *P. infestans* isolate US8. Polynomial regression fitted to survival data; R^2 Mountain = 0.86 (------); R^2 Valley = 0.59 (…….); R^2 New York = 0.55 (____).

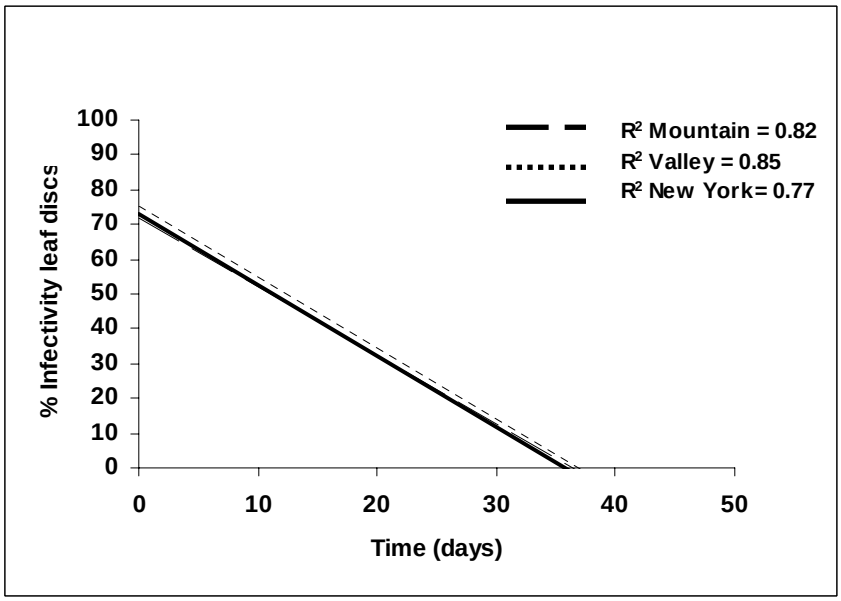

Figure A1.3. Survival of sporangia of *P. infestans* stored in sterile soil extract as determined by infectivity when the inoculated on detached leaflets with the US8 isolate. Linear regression fitted to survival data; R^2 Mountain = 0.82 (------); R^2 Valley = 0.85 (…….); R^2 New York = 0.77 (_____).

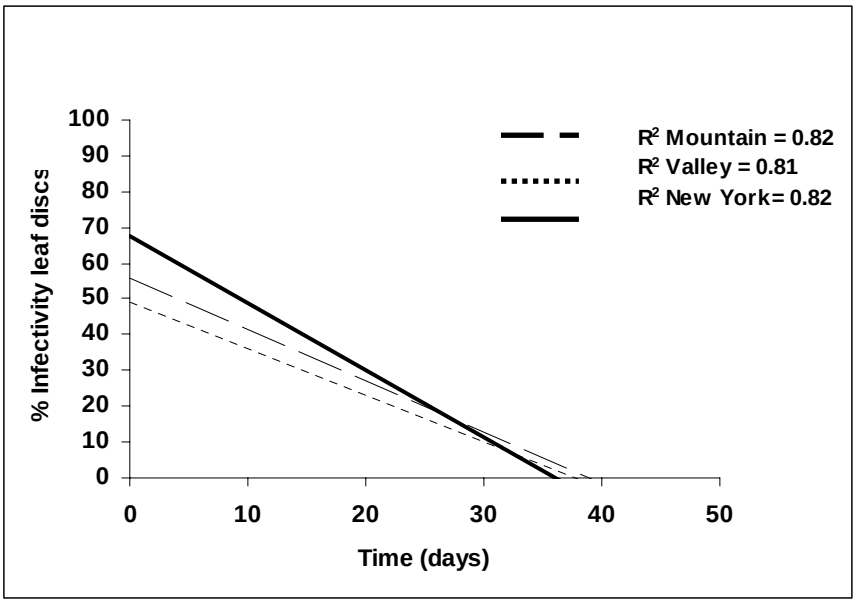

Figure A1.4. Survival of sporangia of *P. infestans* stored in non-sterile soil extract as determined by infectivity when the inoculated on detached leaflets with the US8 isolate. Linear regression fitted to survival data; R^2 Mountain = 0.82 (------); R^2 Valley = 0.81 (…….); R^2 New York = 0.82 (_____).

Table A1.1. Summary of richness, evenness, and diversity, of bacterial T-RFs detected from soil DNA extracted from the Mexican mountain and valley soils and the New York soil sample digested with *Hha*I restriction enzyme.

Soil	Richness*	Evenness**	Shannon's diversity index	Simpson's diversity index
Mt1	57	0.82	3.30	0.93
Mt2	56	0.84	3.35	0.94
Mt3 Forest	68	0.54	2.29	0.68
V1	63	0.87	3.59	0.96
V2	36	0.91	3.25	0.95
NY	23	0.93	2.87	0.93

* Richness = number of T-RFs from each soil sample

** Evenness = Shannon's index/ln (richness)

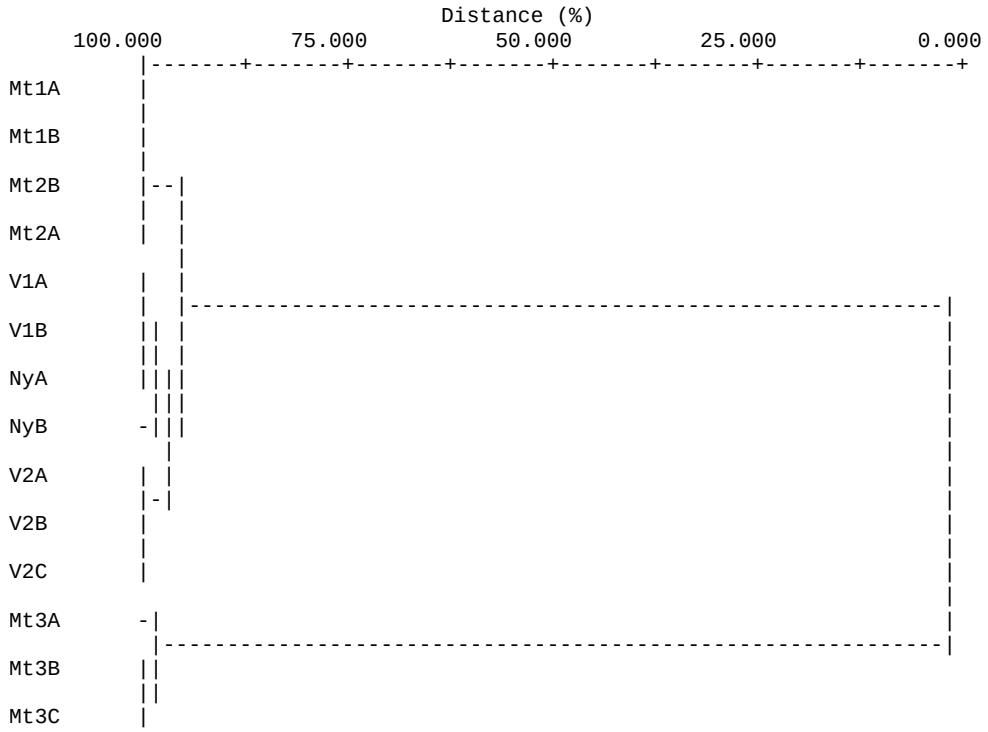

Figure A1.5. Dendrogram generated by analysis of bacterial T-RFs detected from soil DNA extracted from the Mexican mountain and valley soils and the New York soil sample digested with *Hha*I restriction enzyme.

Table A1.2. Summary of canonical correspondence analysis of bacterial T-RFs detected from soil DNA digested with *Hha*I restriction enzyme with soil characteristics from each sample location. Eigenvalues (299 runs), % variance explained for each axis and Pearson's correlation values.

	Axis 1	**Axis 2**	**Axis 3**	***P* value**
Eigenvalue	0.80	0.40	0.31	0.10
% Variance explained	29.0	14.5	11.1	
% Cumulative variance explained	29.0	43.6	54.7	
Pearson's correlation	0.96	0.98	0.98	0.02

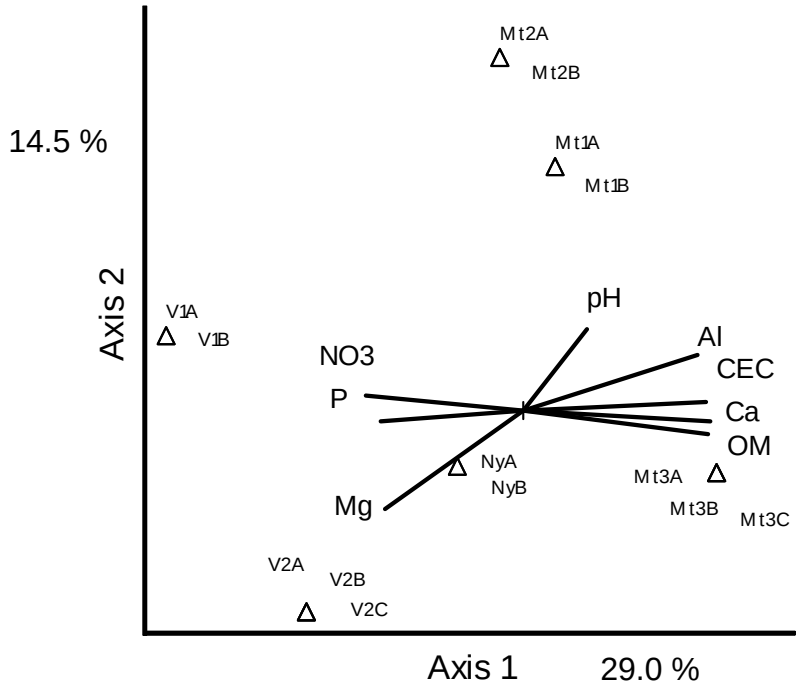

Figure A1.6. Canonical correspondence analysis ordination diagram of T-RFs detected from soil DNA digested with *Hha*I with soil characteristics from samples collected in Mexico and New York

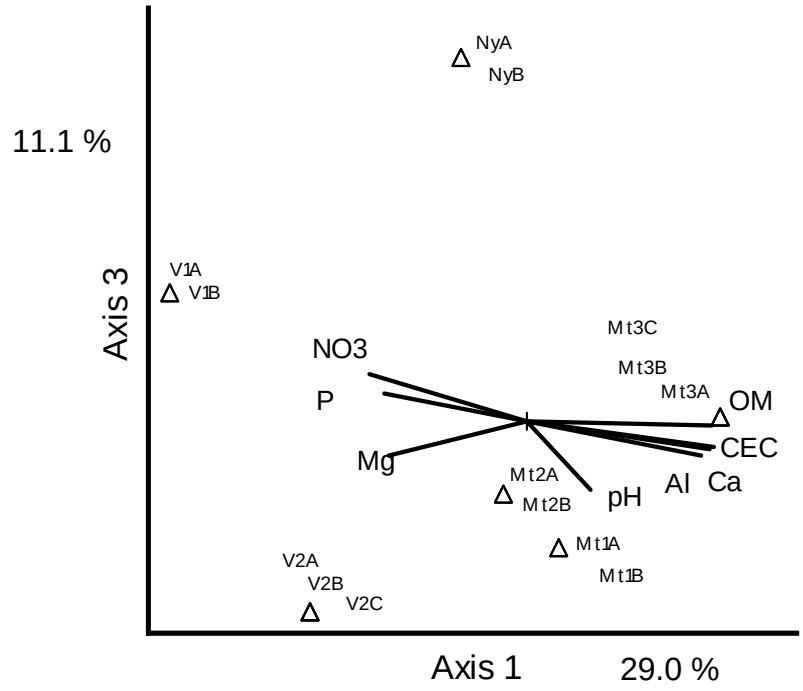

Figure A1.7. Canonical correspondence analysis ordination diagram of T-RFs detected from soil DNA digested with *Hha*I with soil characteristics from samples collected in Mexico and New York

Table A1.3. Summary of richness, evenness, and diversity, of bacterial T-RFs detected from soil DNA extracted from the Mexican mountain and valley soils and the New York soil sample digested with *Sau96*I restriction enzyme.

Soil	Richness*	Evenness**	Shannon's diversity index	Simpson's diversity index
Mt1	106	0.8	3.68	0.96
Mt2	83	0.8	3.47	0.95
Mt3 Forest	108	0.8	3.73	0.96
V1	98	0.8	3.80	0.96
V2	87	0.8	3.56	0.95
NY	112	0.8	3.66	0.96

*Richness = number of T-RFs from each soil sample

** Evenness = Shannon's index/ln (richness)

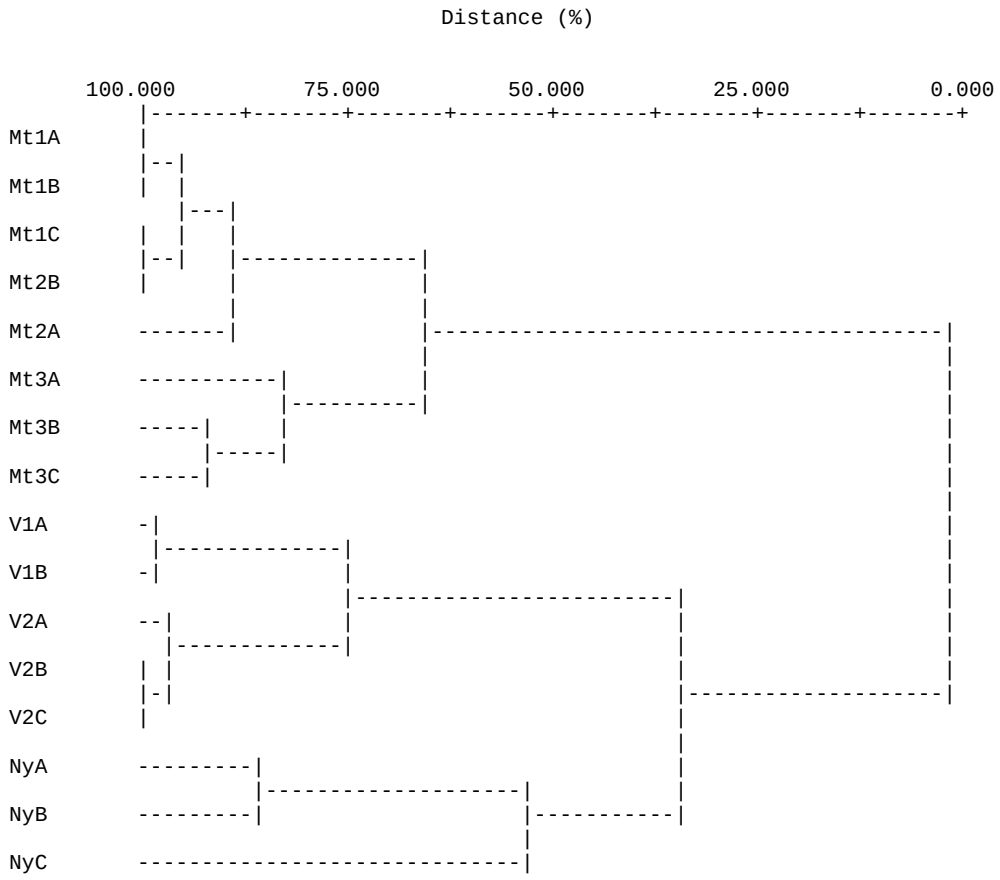

Figure A1.8. Dendrogram generated by analysis of bacterial T-RFs detected from soil DNA extracted from the Mexican mountain and valley soils and the New York soil sample digested with *Sau96*I restriction enzyme.

Table A1.4. Summary of canonical correspondence analysis of bacterial T-RFs detected from soil DNA digested with *Sau96*I restriction enzyme with soil characteristics from each site. Eigenvalues (299 runs), % variance explained for each axis and Pearson's correlation values.

	Axis 1	**Axis 2**	**Axis 3**	***P* value**
Eigenvalue	0.50	0.30	0.20	0.09
% Variance explained	24.0	14.5	9.9	
% Cumulative variance explained	24.0	38.5	48.4	
Pearson's correlation	0.98	0.98	0.94	0.007

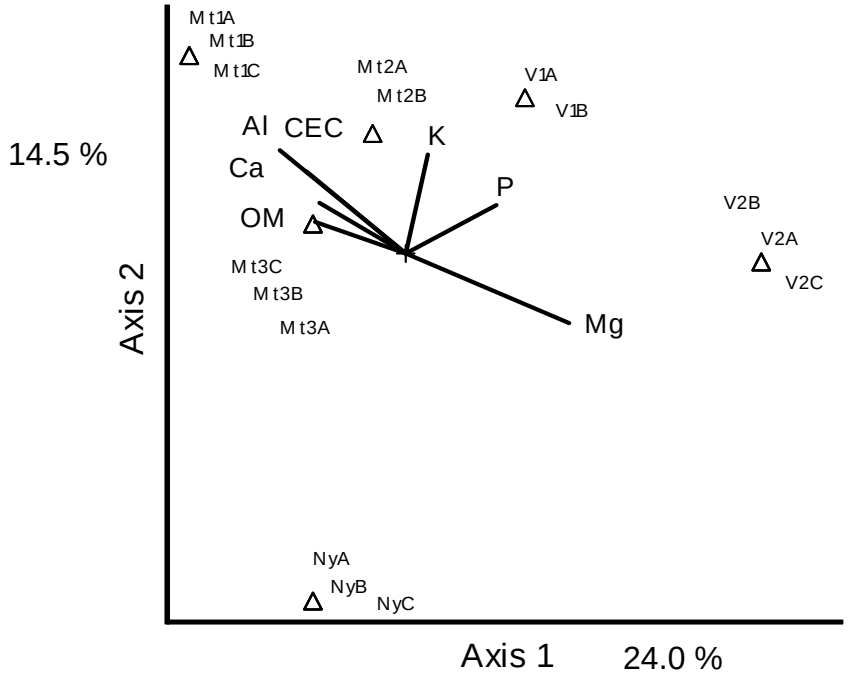

Figure A1.9. Canonical correspondence analysis ordination diagram of T-RFs detected from soil DNA digested with *Sau96*I with soil characteristics from samples collected in Mexico and New York

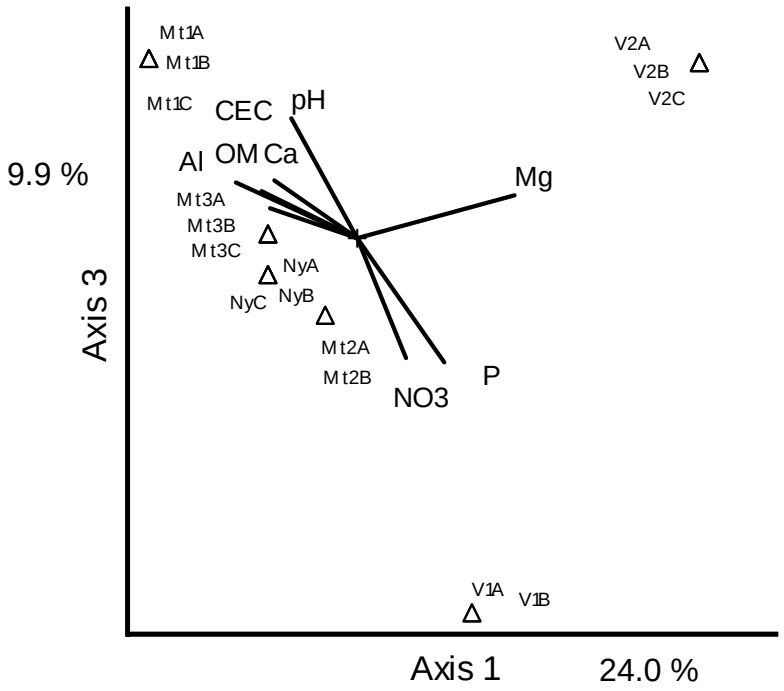

Figure A1.10. Canonical correspondence analysis ordination diagram of T-RFs detected from soil DNA digested with *Sau96*I with soil characteristics from samples collected in Mexico and New York

LITERATURE CITED

Andrivon, D. 1994. Fate of *Phytophthora infestans* in a suppressive soil in relation to pH. Soil Biology & Biochemistry 26:953-956.

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

Baker, R. 1991. Diversity in Biological Control. Crop Protection 10:85-94.

Baker, R.K., and R.J. Cook. 1982. Biological control of plant pathogens, p. 433. The American Phytopathological Society, St. Paul, MN.

Blackwood, C.B., T. Marsh, S.-H. Kim, and E.A. Pau. 2003. Terminal restriction fragment length polymorphism data analysis for quantitative comparison of microbial communities. Applied and Environmental Microbiology 69:926-932.

Clement, B.G., L.E. Kehl, K.L. Debord, and C.L. Kitts. 1998. Terminal restriction fragment patterns (TRFRs), a rapid, PCR-based method for the comparison of complex bacterial communities. Journal of Microbiological Methods 31:135-142.

Daniel, R. 2005. The metagenomics of soil. Nature 3:470-478.

Dorrance, A.E., and D.A. Inglis. 1998. Assessment of laboratory methods for evaluating potato tubers for resistance to late blight. Plant disease 82:442-446.

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

Erwin, D.C., and O.K. Ribeiro. 1996. *Phytophthora* Diseases Worldwide. The American Phytopathological Society, APS Press, St. Paul, MN.

Fernandez-Pavia, S.P., N.J. Grunwald, M. Diaz-Valasis, M. Cadena-Hinojosa, and W.E. Fry. 2003. Soilborne oospores of *Phytophthora infestans* in central Mexico survive winter fallow and infect potato plants in the field. Plant Disease 88:29-33.

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

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

Gallegly, M.E., and J. Galindo. 1957. Mating types and oospores of *Phytophthora infestans* in nature in Mexico. Phytopathology 48:274-277.

Garbeva, P., J.A. van Veen, and J.D. van Elsas. 2004. Microbial diversity in soil: Selection of microbial populations by plant and soil type and implications for disease suppressiveness. Annual Review of Phytopathology 42:243-270.

Gilreath, J.P., M.S. Beilinski, T.N. Motis, J.W. Noling, and J.M. Mirusso. 2005. Methyl bromide alternatives for nematode and *Cyperus* control in bell pepper (*Capsicum annum*). Crop protection 24:903-908.

Goodwin, S.B., L.S. Sujkowski, A.T. Dyer, B.A. Fry, and W.E. Fry. 1995. Direct detection of gene flow and probable sexual reproduction of *Phytophthora infestans* in northern North America. Phytopathology 85:473-479.

Grunwald, N.J., and W.G. Flier. 2005. The biology of *Phytophthora infestans* at its center of origin. Annual Review of Phytopathology 43:171-190.

Haas, D., and G. Defago. 2005. Biological control fo soil-borne pathogens by fluorescent pseudomonads. Nature Reviews-Microbiology 10:1-13.

Hill, G.T., N.A. Mitkowski, L. Aldrich-Wolfe, L.R. Emele, D.D. Jurkonie, A. Ficke, S. Maldonado-Ramirez, S.T. Lynch, and E.B. Nelson. 2000. Methods for assessing the composition and diversity of soil microbial communities. Applied Soil Ecology 15:25-36.

Hornby, D. 1983. Suppressive soils. Annual Review of Phytopathology 21:65-85.

Hugenholtz, P., B.M. Goebel, and N.R. Pace. 1998. Impact of culture-independent studies on the emerging phylogenetic view of bacterial diversity. Journal of bacteriology 180:4765-4774.

Kirk, J.L., L.A. Beaudette, M. Hart, P. Moutoglis, J.N. Klironomos, H. Lee, and T. Trevors. 2004. Methods of studying soil microbial diversity. Journal of Microbial Methods 58:169-188.

Lacey, J. 1965. The infectivity of soils containing *Phytophthora infestans*. Annuals of Applied Biology 56:363-380.

Liu, W.-T., T.L. Marsh, H. Cheng, and L.J. Forney. 1997. Characterization of Microbial diversity by determining terminal restriction fragment length polymorphisms of genes encoding 16S rRNA. Applied and Environmental Microbiology 63:4516-4522.

Lukow, T., P.F. Dunfield, and W. Liesack. 2000. Use of the T-RFLP technique to assess spatial and temporal changes in the bacterial community structure within an agricultural soil planted with transgenic and non-transgenic potato plants. FEMS Microbiology Ecology 32:241-247.

Mayton, H., C.S. Smart, B.C. Moravec, E.S.G. Mizubuti, A.E. Muldoon, and W.E. Fry. 2000. Oospore survival and pathogenicity of single oospore recombinant progeny from a cross involving US-17 and US-8 genotypes of *Phytophthora infestans*. Plant Disease 84:1190-1196.

Mazzola, M. 2002. Mechanisms of natural soil suppressiveness to soilborne diseases. Antonie Van Leeuwenhoek 81:557-564.

Moeseneder, M.M., J.M. Arrieta, G. Muyzer, C. Winter, and G. Herndl. 1999. Optimization of terminal-restriction fragment length polymorphism analysis for complex marine bacterioplankton communities and comparison with denaturing gradient gel electrophoresis. Applied and Environmental Microbiology 65:3518-3525.

Nelson, E.B. 1996. Maximizing disease control with fungicide applications: The basics of turfgrass fungicides part two: Behavior in soil. Turfgrass trends 5:5-11.

Osborn, A.W., E.R.B. Moore, and K.N. Timmis. 2000. An evaluation of terminal-restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. Environmental Microbiology 2:39-50.

Osborn, M., and S. J. 2005. Molecular Microbial Ecology. Taylor and Francis, New York, NY.

Osborne, C.A., G.V. Rees, Y. Berstein, and P.H. Janssen. 2006. New threshold and confidence estimates for terminal restriction fragment length polymorphism analysis of complex bacterial communities. Applied and Environmental Microbiology 72:1270-1278.

Peters, R.D., H.W. Platt, and R. Hall. 2000. Infectivity of asexual propagules of *Phytophthora infestans* in sterile peat exposed to various temperature regimes. Phytoprotection 81:123-128.

Porter, L., and D.A. Johnson. 2004. Survival of *Phytophthora infestans* in surface water. Phytopathology 94:380-387A.

Suzuki, M., and S.J. Giovanni. 1996. Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. Applied and Environmental Microbiology 62:625-630.

Torsvik, V., and L. Ovreas. 2002. Microbial diversity and function in soil: from genes to ecosystems. Current Opinion in Microbiology 5:240-245.

Zan, K. 1962. Activity of *Phytophthora infestans* in soil in relation to tuber infection. Transactions British Mycological Society 45:205-221.

CHAPTER FOUR

FOLIAR AND TUBER LATE BLIGHT RESISTANCE IN A TETRAPLOID *Solanum tuberosum* POTATO MAPPING POPULATION

ABSTRACT

Foliar and tuber resistance to *Phytophthora infestans* were evaluated in a mapping population (n = 94) developed between two *Solanum tuberosum* breeding lines, NY121 x NY115. Foliar disease severity of the progeny clones was measured by the area under the disease progress curve (AUDPC) in field tests in 2004 and 2005. Correlation analysis of AUDPC values with amplified fragment length polymorphism (AFLP) molecular markers revealed that 56 %, 53 %, and 52 % of the quantitative phenotypic variance for resistance were associated with markers PCTMATC 184, PATMATA 171, and PATMACT 236, respectively, located on a chromosome V homolog derived from NY121 ($p < 0.00001$). In 2005, an additional 126 clones of the same population, not used in the initial marker analysis, were evaluated in the field and it was confirmed that these markers were associated with about 50 % of the phenotypic variance ($p < 0.00001$) for foliar disease resistance. The genetic component of tuber blight variance was much smaller than the foliar blight component, but still these three markers were correlated with tuber blight resistance ($p < 0.01 – 0.10$) and explained 3-8 % of the variance. Most markers associated with foliar resistance were not associated with tuber resistance.

INTRODUCTION

Late blight, caused by the oomycete *Phytophthora infestans* (Mont.) de Bary, is one of the most devastating diseases of potato. Both the belowground (tubers) and above ground (foliar) parts of the plant can be affected. Because late blight of tubers is

often associated with soft rotting bacteria and other secondary pathogens an almost insignificant amount of tuber blight (2-5%) can lead to complete loss of potatoes in storage (Dorrance and Inglis, 1998). When environmental conditions favor the pathogen there are currently no available methods to completely control late blight once it has become established within a field. Similarly, when infected tubers are stored at high humidity and moderate temperature, little can be done to prevent rot. Infected asymptomatic tubers can harbor the pathogen and when planted can be the source of primary infections.

Host resistance is recognized as potentially the most effective and environmentally acceptable method to control late blight. Unfortunately, most commercially cultivated potato varieties, have moderate to little resistance to both foliar and tuber blight. Resistance to late blight is conferred by both dominant single (vertical, R-gene, monogenic, qualitative) and multiple minor genes (horizontal, polygenic, quantitative). The introgression of dominant alleles (R-genes) from wild *Solanum* species to the cultivated *Solanum tuberosum* for resistance has not been a durable approach for disease management. This is because R-gene resistance in potato is easily overcome by the appearance of new races of the pathogen *P. infestans* (Niederhauser and Mills, 1953). In addition, R-genes which confer resistance to *P. infestans* in the foliage, do not always impart resistance to the tubers (Roer and Toxopeus, 1961). Because of the lack of success in controlling late blight with R-genes, a more intensive effort has been initiated to produce potato cultivars with horizontal or polygenic resistance.

Selection of resistant germplasm has been focused almost exclusively on foliar blight. As with R-gene resistance polygenic resistance in the foliage does not always correlate well with tuber resistance (Rasmussen et al., 1998; Stewart et al., 1992). For example, in one study, Stewart et al (Stewart et al., 1992), found a negative correlation

between foliar and tuber resistance and concluded that different genes may be involved with control of both phases of resistance. However, in a later study Stewart observed that quantitative resistance in foliage and tubers were correlated (Stewart et al., 1994). Oberhagemann et al. (Oberhagemann et al., 1999) analyzed, through quantitative trait loci (QTL) analysis, populations derived from crosses between *Solanum tuberosum tuberosum* and four wild *Solanum* species (*S. vernei, S. kurtzianum, S. stenotum, and S. chacoense*) for resistance to both foliar and tuber blight. A QTL associated with both tuber and foliar resistance was identified on chromosome V, however, the phenotypic traits were negatively correlated: the QTL reportedly conferred a decrease in resistance to tuber blight and an increase in resistance to foliar blight (Oberhagemann et al., 1999).

A likely explanation for these data is that the relationship between genetic loci conferring resistance or susceptibility to foliar or tuber blight is dependent on the plant genotype evaluated. In addition, genes associated with resistance may be present but differentially expressed in different plant organs or tissue. Potato tubers and leaves differ in phytoalexin production (Rohwer et al., 1987). The role of phytoalexins in disease resistance is unclear, however, production is stimulated by pathogen elicitors. Infected tubers have been shown to accumulate the phytoalexins, rishitin, lubimin, phytuberin and solavetivone, but no phytoalexins were found in infected leaf tissue in a study conducted by Rohwer et al. (Rohwer et al., 1987).

A tetraploid population developed by the Cornell University potato breeding program appeared particularly promising for identifying genetic loci involved with both foliar and tuber blight resistance. The breeding line NY121 which exhibits high levels of field resistance to foliar late blight as well as resistance to races of the golden cyst nematode, potato virus Y, and common scab was crossed with the blight susceptible breeding clone NY115. The origin of resistance in the NY121 clone is

unclear, although it may descend from blight resistant *Solanum andigena* in its ancestry. The immediate parents of NY121 are no longer available and neither was ever tested for reaction to late blight. This population is especially useful for this study because the parental lines, specifically the NY121 parent are currently being used to develop breeding populations. The progeny of this cross exhibit attractive round, white skin tubers with acceptable vine maturity and yield. Most mapping studies used to identify quantitative resistance or quantitative trait loci associated with late blight resistance have been conducted with diploid populations of *S. tuberosum* crossed with wild species resulting in progenies with non uniform growth and development of tubers and poor yields (Collins et al., 1999; Ewing et al., 2000; Gebhardt and Valkonen, 2001; Oberhagemann et al., 1999).

A high level of foliar resistance may extend the duration of a late blight epidemic and lead to significant amounts of tuber blight if the tubers are very susceptible. Blight free tubers are the goal of any potato production system. Therefore identifying genetic loci conferring resistance to tubers is essential for potato breeding. The objective of this research was to identify genetic loci associated with both foliar and tuber blight resistance to late blight. The results will be useful in marker assisted selection (MAS) for both traits in breeding for resistance.

MATERIALS AND METHODS

Plant material. The Cornell breeding clone NY121 which exhibits high levels of field resistance was crossed with blight-susceptible clone NY115 (as the male parent) to create population Y36. A total of 1872 seedlings of Y36 were grown in pots in 1999. After practicing mild selection for tuber type and appearance after harvest, four tubers were saved from each of 1622 of the progeny. These clones were replanted as four-hill plots in breeding plots near Ithaca, NY in 2000. Further selection for tuber

appearance and yield at harvest reduced the Y36 population to a total of 313 clones. Ninety-four of these clones were selected for initial DNA marker analyses, based primarily on reaction to race Ro2 of the golden cyst nematode (*Globodera rostochiensis*), and to a lesser degree based on reaction to late blight. Forty-one of the 94 clones were chosen because they had exhibited resistance to race Ro2 of the golden cyst nematode in an initial screen, while 35 were chosen because they had tested susceptible to Ro2. The remaining 18 were chosen because they exhibited some degree of resistance to late blight in greenhouse trials, as well as an intermediate reaction to Ro2. In a second round of marker analysis, 126 additional Y36 progeny, randomly selected from the 219 clones not used in the initial marker analysis, were evaluated with five polymorphic markers that appeared to be linked to blight resistance in the first 94 progeny evaluated in the field in 2004. The markers associated with resistance are derived from the NY121 parent (Table 4.2).

Marker development. Genomic DNA was isolated from leaves of NY121, NY115 and Y36 clones with a DNeasy kit (Qiagen), following manufacturer's instructions. DNA was prepared for AFLP analysis by digestion with *Pst* I and *Mse* I, ligation of adaptors, and preamplification with nonselective Pst and Mse primers, as described by Vos et al (1995). A total of 57 Pst-Mse primer pairs was used for final amplification. Selective Pst primers were labeled with ^{33}P prior to amplification. Products were separated on a denaturing polyacrylamide gel, which was then dried and exposed to film. Amplification products that segregated in the progeny were manually scored for presence or absence in parents and progeny. Genomic DNA was also evaluated for polymorphism with 13 SSR primer pairs (STM003, STM007, STM1003, STM1005, STM1024, STM1029, STM1064, STI006, STI017, STI018, STI028, STI049, STI059) (Milbourne et al. 1998, Feingold et al. 2005).

Late blight field evaluations. All field experiments were conducted at a site adjacent to the Cornell University Homer C. Thompson Research Farm located in Freeville, NY. In 2004, the original 94 clones and parents of the Y36 population selected in 2000 were established on 20 June 2004. In 2005, the original population (n=94) and an additional 126 randomly selected clones, was planted 28 June 2005. In both years, two plant plots of each clone were planted 23 cm apart within a row and 82 cm between rows in a complete randomized block design with three replications. Each year at planting, a 13-13-13 blend of nitrogen, phosphorus and potassium (N-P-K) fertilizer was applied at the rate of 167 kg/ha of each element. Weeds in both years were controlled by pre-emergent applications of Lorox (Linuron) (DuPont, Wilmington, DE) and post emergent applications of Dual II Magnum (metalochlor, Syngenta, Greensboro, NC) applied at labeled rates. In 2005, insects were controlled by applications of Endosulphan 50 WP, Provado 1.6 F (imidacloprid, Bayer Crop Science, Research Triangle Park, NC). Reglone (diquat, Syngenta, Greensboro, NC) was applied for vine kill in 2005.

Due to the very conducive weather for late blight development and disease in the surrounding area in 2004, late blight was observed in the experiment on 13 August. Infected leaflets obtained from the field were used to isolate the late blight pathogen, *P. infestans*. This isolate designated (US040009) is an A2 mating type and belongs to the US-8 clonal lineage, which is the predominant lineage that has been found recently on potatoes in the USA. The US040009 isolate was maintained on rye agar media and detached leaflets and was used for laboratory tuber blight assessments and also to inoculate the field research plots in 2005.

In 2005, plants were inoculated on the evening of 25 August with 10 ml per two-plant plot of a suspension containing 500 sporangia/ml with sporangia obtained from sporulating lesions from leaflets of the susceptible potato cultivar Katahdin. The

inoculum was applied with a hand-held sprayer. Research plots were sprinkler irrigated for two hours (equivalent to ca 0.2 inches of rainfall) before inoculation and as needed to encourage epidemic development throughout the season.

Foliar disease, as a percentage of total foliage, was evaluated every three days between 15 August and September 30 in 2004 and 31 August and 2 October in 2005. The area under the disease progress curve for each genotype (AUDPC) was calculated as described by Shaner et al. (Shaner and Finney, 1977). Tubers were harvested from the experimental plots at the conclusion of the foliar field trials. In each year, total yield for each two-plant plot was measured by weight and tuber blight (detected visually) was recorded as a percentage (by weight) of the total yield.

Maturity. Maturity of each of the original 94 clones, the parents, and the additional 126 clones was evaluated in a separate blight free field in 2005. Five plant plots spaced 23 cm apart within rows and 39 cm between rows were planted with whole “B” size tubers on17 May 2005 at the Cornell University Mt. Pleasant Research Farm, Ithaca, NY. At planting, fertilizer (10-20-20 N-P-K) was applied to the field at a rate of about 179 kg of N per hectare. Admire (imidachloprid, Bayer Crop Science, Research Triangle Park, NC), and Quadris (azoxystrobin, Syngenta, Greensboro, NC) were applied at planting, followed by Sencor (metribuzin, Bayer Crop Science, Research Triangle Park, NC) and Dual (metalochlor, Syngenta, Greensboro, NC) for weed control prior to emergence. All pesticides were applied at labeled rates. Weekly rotation sprays of various fungicides and insecticides began in early July. Plant maturity was rated on a 1-5 (1 = very early, 5= very late) scale beginning on 2 August with the last rating on 27 August, 2005.

Laboratory tuber blight evaluations. During the spring of 2005, both whole tubers and tuber slices of the original 94 clones and parents of the Y36 population harvested from a blight free field in 2004 were assessed for tuber blight resistance in

the laboratory. Intact tubers were surface sterilized in 0.5% bleach solution, rinsed three times with tap water and were air dried for 24 h for both whole tuber and tuber slice evaluations. For the whole tuber assay three tubers of each progeny clone were spray inoculated with 5 ml of a sporangial suspension of *P. infestans* (isolate US040009) consisting of 10,000 sporangia/ml. Tubers were then placed in plastic mesh bags and were incubated in a complete randomized design at 15° C in the dark at 90-100 % relative humidity for 4 weeks. After the incubation period, tubers were assessed visually for tuber blight. The experiment was repeated. Tubers for the second experiment were inoculated two weeks after the initiation of the first experiment.

Resistance of the inner medullary potato tissue was assessed with a tuber slice assay. The tuber slice assays were conducted with four tuber slices cut from one surface sterilized tuber of each progeny clone. The four tuber slices from each genotype were placed into petri plates containing 1.5 % water agar. Each slice was then inoculated with 1 ml of a sporangial suspension containing 15,000 sporangia/ml. Mini-moist chambers were incubated at 15°C for 4-7 days in the dark. Tuber slices were rated visually for percentage of infected surface area. A second trial of the experiment was conducted, and was initiated one week after completion of the first trial.

Data analysis. Disease severities (as measured by the area under the disease progress curve (AUDPC) of foliar epidemics) of individual progeny were used to correlate molecular markers with disease phenotype. Because late blight epidemics in the field differed in duration in 2004 and 2005 standardized (sAUDPC) values were used to correlate foliar and tuber blight data. Standardized AUDPC's were calculated by dividing AUDPC values by the duration (measured in days) of the epidemic in each year. Marker correlation analysis for tuber blight severity in the field and laboratory

was conducted with values of the percentage of tuber blight severity assessed in the field and laboratory experiments.

RESULTS

Disease development. Disease progressed more slowly in 2004 than in 2005. A natural influx of inoculum of *P. infestans* initiated the epidemic in 2004 and no irrigation was used to enhance disease development. In contrast, all experimental plots were inoculated and irrigated at least once a day for two hours in 2005. Regardless of the source and temporal dynamics of initial inoculum, disease resistance/susceptibility was consistent between years (Figure 4.1). The most susceptible clones were completely dead by 3 September, 2004, 21 days after inoculation (DAI), and by 10 September, 2005 (16 DAI). The most resistant clones had 34% disease by the conclusion of the season in 2004 and 5% disease in 2005.

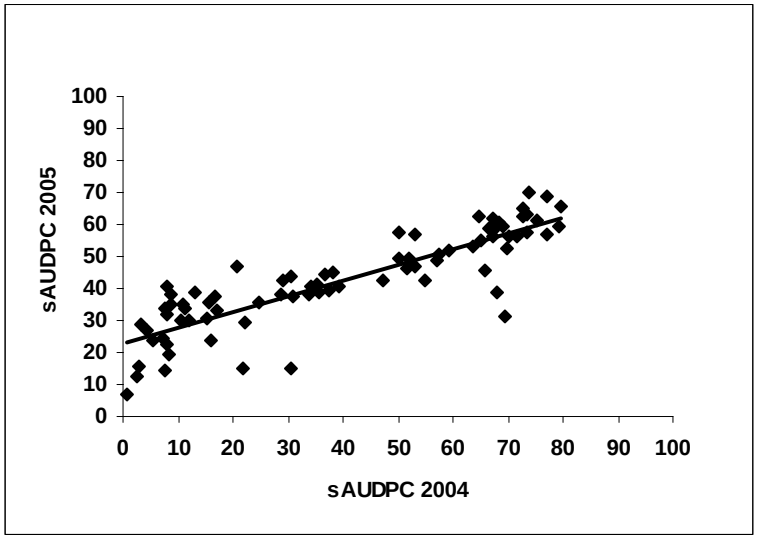

Figure 4.1. Correlation of standardized values of the area under the disease progress curve (sAUDPC) for foliar disease severity in 2004 to those in 2005.

Foliar blight. Transgressive segregation for foliar blight resistance was observed in the Y36 population with some clones exhibiting significantly higher or

significantly lower disease resistance than either parent (Figure 4.2). Correlation analysis of AUDPC values from the 2004 field trial of the original 94 clones and parents with AFLP molecular markers revealed that 56%, 53%, and 52% of the quantitative phenotypic variance for resistance was significantly ($p < 0.00001$) associated with markers PCTMATC 184, PATMATA 171, and PATMACT 236, respectively. These markers are located on a chromosome V homolog (Table 4.1). Several other highly significant markers were also identified and were located on linkage group V (Table 4.1). In contrast to data from other QTL evaluations for foliar blight, which identified genetic loci important for disease resistance dispersed through out the potato genome, all of the highly significant markers in this population were located on chromosome V.

Table 4.1. Correlation of foliar blight severity with AFLP markers in 2004 and 2005. The correlation analysis was performed with AUDPC values in the original population (n=94).

	Foliar blight 2004				**Foliar blight 2005**	
Marker	**LG***	**R^2****	***P* <**		**R^2****	***P* <**
PCTMATC 184	V	56	0.00001	(-)***	51	0.00001 (-)***
PATMATA 171	V	52	0.00001	(-)	45	0.00001
PATMACT 236	V	53	0.00001	(-)	45	0.00001
PCTMATC 217	V	24	0.00001	(-)	24	0.00001
PAGMCCG 333	V	14	0.00002	(-)	13	0.00003
PATMCAT 152	V	25	0.00001	(-)	26	0.00001
PACMATT 275	V	25	0.00001	(-)	26	0.00001
PAGMCAG 286	V	23	0.00001	(-)	23	0.00001

* LG = linkage group

**R^2 = percentage of phenotypic variance of markers associated with resistance in 2004 and 2005 field trials

*** (-) = decrease in disease severity in both years

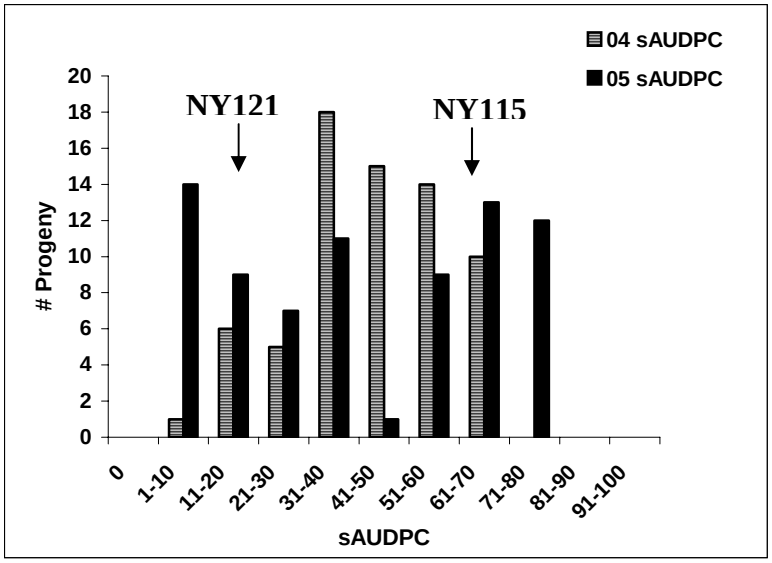

Figure 4.2. Frequency distribution of foliar late blight severity measured by standardized relative area under the disease progress curve (sAUDPC) of Y36 progeny and parents (NY121 and NY115) in the field 2004-2005 (n=94).

In 2005, markers PCTMATC 184, PATMATA 171, and PATMACT 236 were again strongly associated with resistance in 2004 (Table 4.1). Each of these markers was associated with about 50% of the phenotypic variance for foliar disease. Additionally, in 2005, 126 progeny clones not used in the initial marker analysis were evaluated, using the five most significant polymorphic markers identified in the original population (Table 4.2). Again, each of these markers was highly correlated with resistance (Table 4.2) (Figure 4.3). Foliar blight disease severity in 2005 with the expanded population showed a bimodal distribution (Figure 4.4).

Table 4.2. Correlation of foliar blight resistance with AFLP markers using the expanded population (n=126) in 2005. The correlation analysis was performed with AUDPC values.

Foliar blight 2005 expanded population			
Marker	**LG***	**R^2****	***P* <**
PCTMATC 184	V	47	0.00001***
PATMATA 171	V	49	0.00001
PATMACT 236	V	48	0.00001
PCTMATC 217	V	26	0.00001
PAGMCCG 333	V	23	0.00001

* LG = linkage group

** R^2 = percentage of phenotypic variance of markers associated with resistance in 2004 and 2005 field trials

*******All markers were associated with a decrease in disease severity

In most cases where markers PAGMCCG 333 and PCTMATC 217 were absent, and PCTMATC 184, PATMATA 171, and PATMACT 236 were present, clones were resistant to *P. infestans*. Conversely, if PCTMATC 184, PATMATA 171, and PATMACT 236 were absent, in most cases, the clones were more susceptible (Table 4.3). The presence of PCTMATC184 appears to be most closely correlated with foliar resistance.

Maturity. Ninety days after planting (DAP) only a handful of the clones were completely dead. In contrast, at 102 DAP, more than 50 % of the progeny clones were dead. The majority of the remaining clones were yellowing and visibly senescent, while only five clones were still very lush and green at 102 DAP. Maturity was not correlated with either foliar or tuber disease severity (Figure 4.5).

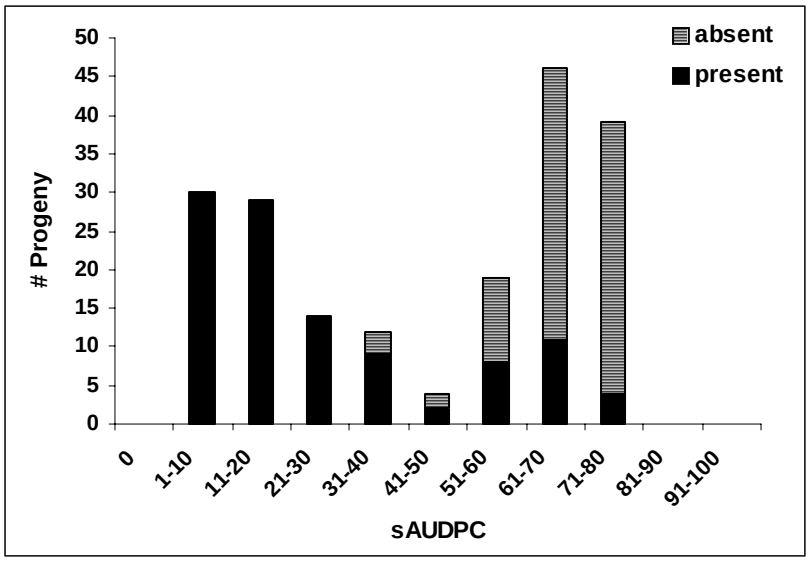

Figure 4.3. Frequency distribution of foliar late blight sAUDPC values of the Y36 expanded population progeny clones (n=126) evaluated in 2005 with and without the marker PCTMATC184.

Table 4.3. Presence (1) and absence (0) of markers in parents NY121 and NY115 and six progeny clones and AUDPC values from foliar field trial 2005.

Source	AUDPC*	PAGMCCG 333	PCTMATC 217	PCTMATC 184	PATMACT 236	PATMATA 171
NY121	365	1	1	1	1	1
NY115	1724	0	0	0	0	0
Y36-247	1691	1	1	0	0	0
Y36-280	1628	1	1	0	0	0
Y36-143	224	1	1	1	0	0
Y36-239	261	0	0	1	1	1
Y36-289	111	0	0	1	1	1
Y36-245	251	0	0	1	0	0
	P* < *	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**
	R^2	**14**	**24**	**56**	**53**	**52**

*****Low AUDPC = more resistant; high AUDPC = more susceptible
***P* value and R^2 = percentage of phenotypic variance of markers associated with resistance in 2005 field trial

Tuber blight in the field. The incidence of tuber blight was higher in 2004, than in the 2005 field season (Figure 4.6). In 2004, markers PCTMATC 184, PATMATA 171, and PATMACT 236 that were highly correlated with foliar resistance were also associated with resistance to tuber blight (Table 4.4). However, these markers explained only a small proportion of the total phenotypic variance (Table 4.4). In 2005, markers PCTMATC 184, PATMATA 171, and PATMACT 236 were not significantly associated with tuber blight.

The correlation between foliar and tuber blight was poor in both years (Figures 4.7, 4.8). However, a few markers were significantly associated with tuber blight in both 2004 and 2005. In contrast to the large (50%) proportion of the phenotypic variance associated foliar blight in both years the markers that were significantly correlated with tuber blight explain only a small portion of the phenotypic variance (Table 4.5).

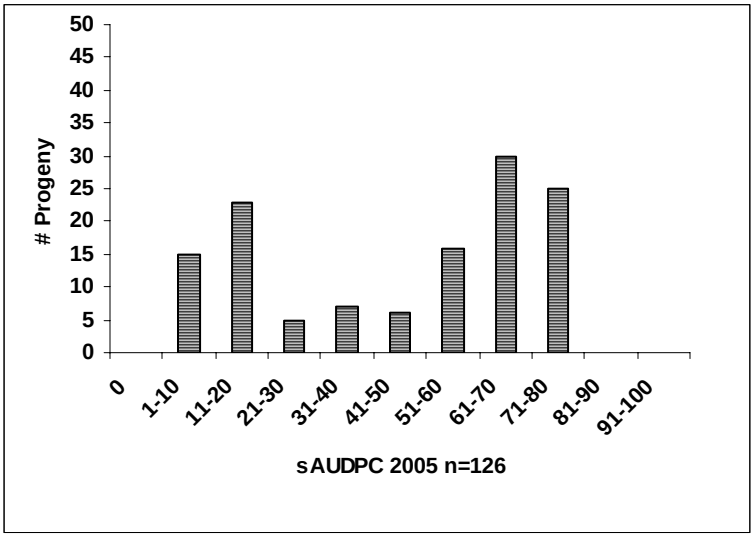

Figure 4.4. Frequency distribution of foliar late blight severity measured by standardized area under the disease progress curve (sAUDPC) of Y36 progeny in the field 2005 (expanded population, n = 126).

Table 4.4. Most significant markers associated with tuber blight disease severity from the field trials in 2004 and 2005 (original population n = 94).

Marker	Tuber blight 2004		Tuber blight 2005	
	R^2*	*P* - value =	R^2 *	*P* - value =
PATMACC181	4	0.05 (-)**	22	0.00001 (-)
PATMATA 181	13	0.0003 (+)***		NS
PACMATT 171		NS****	8	0.006 (-)
PATMCAT 181	9	0.0003 (+)	9	0.0003 (+)
PCTMAAC 159	11	0.001		NS
PAGMACT 232	9	0.004		NS
PAGMAAG 253	10	0.002 (+)		NS
PAGMATA 244	8	0.007 (+)	4	0.062 (+)
PCAMCAT 380	10	0.003		NS
PCTMAAC 159	11	0.001		NS
STM 1003169	10	0.002 (+)		NS
PCAMACT 209	7	0.011 (-)		NS
PACMAGT 164	7	0.01 (-)	7	0.007 (-)
PACMAGT 167	12	0.005 (-)	7	0.012 (-)
PAGMCAA 311	5	0.03 (-)	8	0.006 (-)
PATMAGT 205		NS	12	0.002 (+)
PCAMACT 285		NS	10	0.001 (+)
PATMATG 414		NS	8	0.007 (+)

* R^2 = percentage of phenotypic variance of markers associated with resistance in 2004 and 2005 field trial

** (-) = decrease in disease severity

*** (+) = increase in disease severity

**** NS = not significant

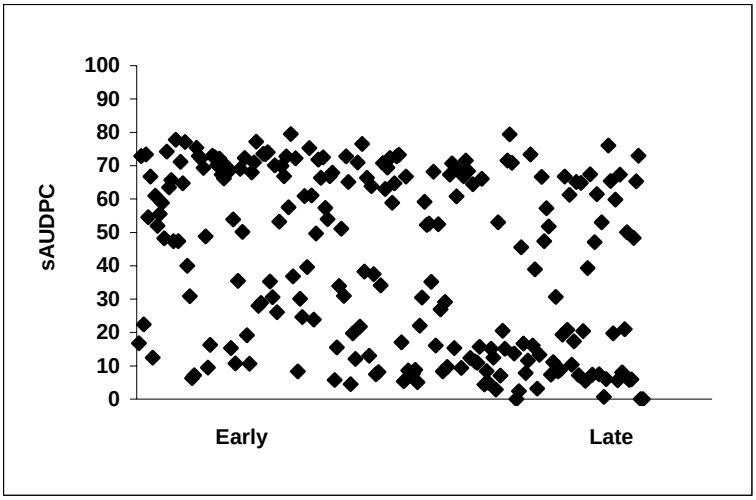

Figure 4.5. Comparison of foliar disease severity (sAUDPC) with plant maturity. Data were acquired in 2005 and are based on the total (original {n=94} plus expanded {n=126}) population.

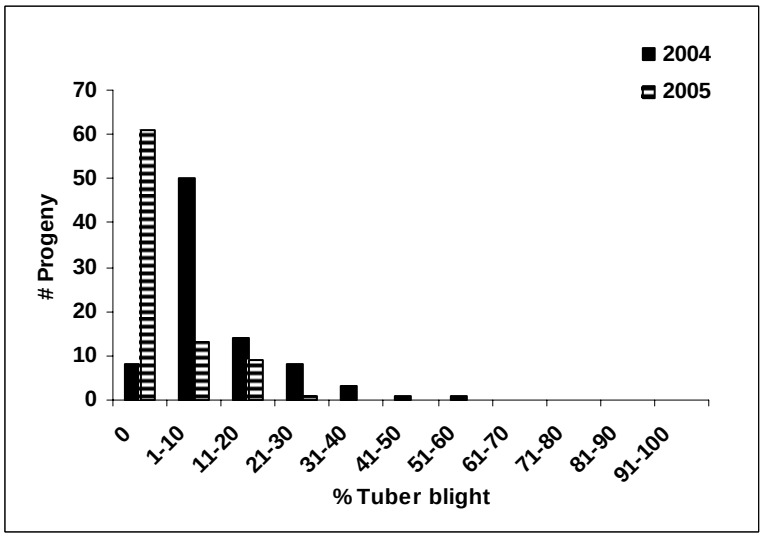

Figure 4.6. Comparison of the frequency distribution of the % tuber blight severity among the Y36 progeny assessed in the field 2004 and 2005.

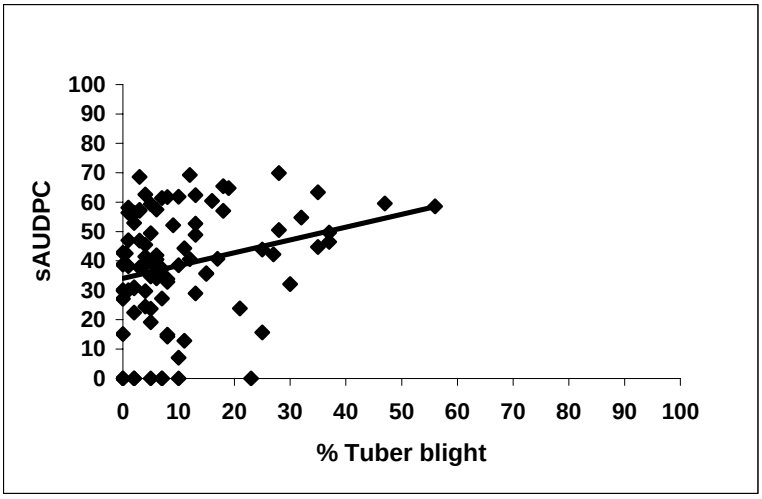

Figure 4.7. Correlation of foliar disease severity (as measured by sAUDPC) with tuber blight severity (%) in 2004 (n=94).

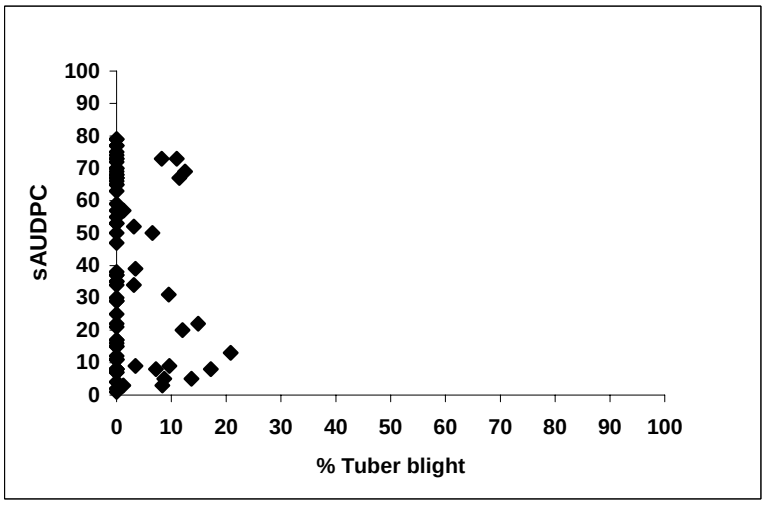

Figure 4.8. Correlation of foliar disease severity (as measured by sAUDPC) with tuber blight severity (%) in 2005 (n=94).

Laboratory assessment of tuber blight resistance. In general tuber slices were more prone to disease than were whole tubers (Figure 4.9). Many inoculated whole tubers failed to show symptoms after incubation for 4 weeks in both experiments. In contrast, almost all tuber slices exhibited some infection after inoculation. Clone Y36-114 was an exception and was not infected in either the whole

tuber or tuber slice assays. This clone was also very resistant in the field. Tuber resistance phenotypes in the laboratory assays were only marginally correlated with tuber blight incidence in the field. Few markers were found significantly associated with a decreased incidence in both field foliar, tuber and laboratory analyses (Table 4.5).

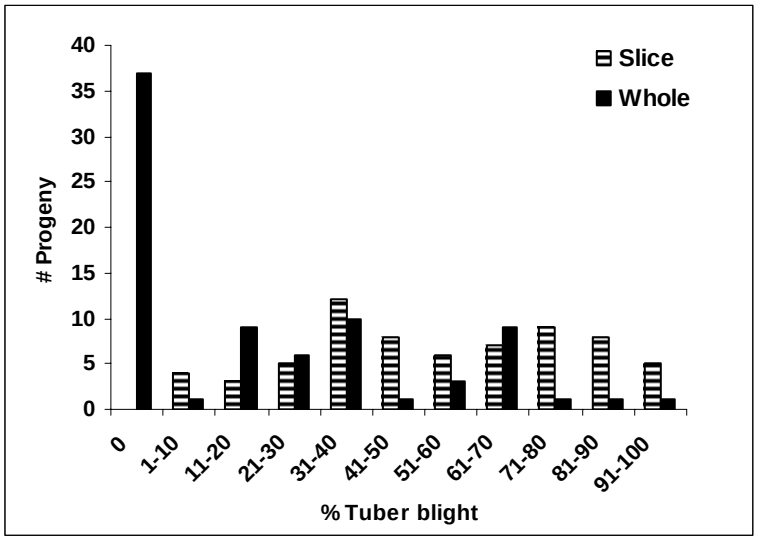

Figure 4.9. Tuber blight severity of progeny clones from laboratory inoculations with sporangial suspensions of *P. infestans* as determined by the tuber slice or whole tuber method.

Table 4.5. Markers associated with foliar blight in the field, tuber blight in the field or tuber blight in the tuber slice or whole tuber assay.

Marker	Source	Effect	Foliar	Field tuber	Slice	Whole tuber
PCTMATC 184	NY121	(-)	****	*	*	**
PATMATA171	NY121	(-)	****	**	*	**
PATMACT 236	NY121	(-)	****	*	*	**
PCTMATC 217	NY121	(-)	****			
PAGMCCG 333	NY121	(-)	****			
PATMATA181	NY121	(+)		***		**
PATMCAT 181	NY121	(+)		***		**
PACMAGT 164	Both	(-)		**		*
PACMAGT 167	Both	(-)		**		
PAGMATA 244	NY115	(+)		**	**	***
PACMCAA 232		(-)			*	**
PAGMATG 244		(-)			*	***
PCAMACT 209	Both	(-)		**	***	***
PACMAAC 272		(-)			*	***
PAGMCCT 134		(+)			***	
PCAMAAC 205		(+)			***	
PATMACT 323		(-)			**	**
PATMATA 320						***
PCTMAAC 198	NY121	(-)			*	***

* Significance* $\boldsymbol{P} < 0.10$; ** $\boldsymbol{P} < 0.01$; *** $\boldsymbol{P} < 0.001$; **** $\boldsymbol{P} <= 0.0001$

DISCUSSION

Numerous QTL analyses for late blight resistance have been conducted on mapping populations of wild Solanum species crossed with *S. tubersoum* or with other tuber-bearing Solanum species in an effort to identify genetic loci that can be introgressed either through genetic engineering or conventional breeding into cultivated potato (Bonierbale et al., 1994; Bormann. et al., 2004; Collins et al., 1999; Gebhardt and Valkonen, 2001; Helgeson et al., 1998). Analyses of mapping populations for foliar late blight resistance have consistently identified QTL with large phenotypic variances associated with resistance using both detached leaf and field experiments (Ewing et al., 2000; Leonards-Schippers et al., 1994). In contrast, reliable

phenotypic data for tuber blight in the laboratory and field, have been more difficult to obtain (Darsow, 1987; Dorrance and Inglis, 1998; Doster et al., 1990).

The study reported here is unusual in that a tetraploid mapping population was used. The progeny were from a cross between a resistant *S. tuberosum* NY121 and a susceptible *S. tuberosum* parent. NY115 and were used to identify QTL associated with foliar and tuber late blight resistance. For tuber blight assessments, I used data from the field and laboratory. The laboratory data included whole tuber and tuber slice evaluations and were conducted in an effort to reduce variability and improve the accuracy in identification of significant genetic loci involved with tuber blight resistance.

Several markers on chromosome homologue V (descending from NY121) accounted for both foliar and tuber resistance. These markers explained about 50% of the genetic variance in foliar resistance. Foliar disease data from 2004 and 2005 were very consistent, despite dramatically different field seasons (2004 being quite wet, and 2005 being quite dry). In contrast the tuber blight data in the field were much less consistent, and these markers explained less than 10% of the genetic variance in those years that they were significant. These results are consistent with the majority of previous studies that have found the potato linkage group V to be been associated with late blight resistance (Bormann. et al., 2004; Collins et al., 1999; Ewing et al., 2000; Gebhardt and Valkonen, 2001; Leonards-Schippers et al., 1994).

Analysis of an expanded population n=126 in the field in 2005 confirmed the hypothesis that markers on a chromosome V homolog were particularly important for foliar disease resistance. Additionally, the particular combination of markers appears to be important. In some cases clones, which had only PCTMCAT 184 or a combination of PCTMATC 184, PATMATA 171, and PATMACT 236, without markers PCTMATC 217, PAGMCCG 333, had some resistance (Table 4.4). No

clones were resistant without at least some combination of these markers. In contrast, there were a few cases where progeny had all five of the most significant markers and were moderately to highly susceptible to foliar blight. A bimodal distribution of foliar disease resistance was observed when foliar data were grouped for the presence or absence of marker PCTMATC 184 (Figure 4.3) which is indicative of a dominant allele or closely linked alleles responsible for observed resistance. Chromosome V harbors a cluster of R-genes as well as genes associated with plant defense (Collins et al., 1999; Ewing et al., 2000; Gebhardt and Valkonen, 2001; Leonards-Schippers et al., 1994). Three of these markers (PCTMATC 184, PATMATA 171, and PATMACT 236) were marginally associated with tuber blight resistance.

Recently, Simko et al (Simko et al., 2006), working with a diploid population of *S. phureja* X *S. stenotomum* found that all QTLs associated with tuber blight resistance were distinct from those associated with foliar blight resistance (Simko et al., 2006). We also found that the majority of the significant markers involved with foliar disease resistance in the Y36 population were distinct from those associated tuber blight resistance.

In contrast to the location and source of QTL associated with foliar resistance, the QTL associated with tuber blight resistance (or susceptibility) were dispersed throughout the genome and segregated from either or both parents. For example, PATMATA 181, PATMCAT 181, both linked to chromosome homolog XII were significantly associated with an increase in disease severity of tuber blight in the field and in whole tuber laboratory but not in tuber slice assays. A resistance gene cluster containing an R-gene for nematode resistance is also located on chromosome XII. The marker loci PACMAGT164, and PACMAGT167, present in both parents contributed to a decrease in tuber blight severity in both the field and whole tuber experiments. However, markers 164 and 167 were not associated with tuber resistance in laboratory

slice assays. This indicates some aspect of the periderm may play a role in resistance. Several significant markers were detected only with the tuber slice assay. Overall, QTL from eight genomic regions were detected as significantly associated with resistance in at least one of the three tuber blight evaluation assays (Table 4.6). With the exception of the three mostly highly significant markers correlated with foliar blight resistance (PCTMATC 184, PATMATA 171, and PATMACT 236), no other markers were associated with both foliar and tuber blight.

In my study, the proportion of the genetic component of variance explained by markers associated with foliar resistance was much higher than that explained by markers correlated with tuber resistance. Preliminary analysis had indicated that the foliage of NY121 was very resistant while the foliage of NY115 was very susceptible to late blight. Tuber blight analyses had not been as conclusive, in that both parents exhibited moderate resistance to late blight. Therefore it was not surprising that we did not detect large QTL for tuber blight resistance.

Tuber quality, shape, appearance, color and taste drive the selection process in potato breeding programs with little or no selection for resistance to tuber blight. Identification of genetic loci correlated with tuber blight resistance/susceptibility is a critical step that can facilitate the development of resistant tuber blight phenotypes through conventional, MAS breeding, or genetic engineering. Data from this mapping population have shown that while a few QTL are associated with both foliar and tuber resistance, most QTL are associated with either foliar or tuber resistance. Because tuber blight resistance is so difficult to detect, it has not been selected. Foliar blight resistance is much easier to detect, and there may has been selection or at least detection. It may be that persistent selection for factors other than tuber blight resistance has led to a loss of genetic loci involved with tuber blight resistance.

The results of this study can aid potato breeding. Both NY121 and NY115 have characteristics that are useful in breeding programs. The identification of markers associated with foliar and tuber resistance can facilitate the production of cultivars with these traits. However, because the levels of tuber resistance are low, additional approaches seem necessary. Further investigation of progeny from crosses involving breeding lines differing significantly in tuber blight resistance should be initiated. If that approach does not yield good levels of tuber blight resistance, investigation of wild species for high levels of tuber resistance may be necessary. Success in this approach will contribute significantly to the incorporation of tuber blight resistance into commercial cultivars – a very important component to solving the late blight problem.

LITERATURE CITED

Bonierbale, M.W., R.L. Plaisted, O. Pineda, and S.D. Tanksley. 1994. QTL analysis of trichome-mediated resistance in potato. Theoretical and applied genetics 87:973-987.

Bormann., C.A., A.M. Rickert, R.A.A. Ruiz, J. Paal, J. Lubeck, J. Strahwald, K. Buhr, and C. Gebhardt. 2004. Tagging quantitative trait loci for maturity-correlated late blight resistance in tetraploid potato with PCR-based candidate gene markers. Molecular plant microbe interactions 17:1126-1138.

Collins, A., D. Milbourne, L. Ramsay, R. Meyer, C. Chatot-Balandras, P. Oberhagemann, W. De Jong, C. Gebhardt, E. Bonnel, and R. Waugh. 1999. QTL for field resistance to late blight in potato are strongly correlated with maturity and vigour. Molecular Breeding 5: 387-398.

Darsow, U. 1987. Long-Term Results of a Tuber Slice Test for Relative Resistance to Late Blight. Potato Research 30:9-22.

Dorrance, A.E., and D.A. Inglis. 1998. Assessment of laboratory methods for evaluating potato tubers for resistance to late blight. Plant disease 82:442-446.

Doster, M.A., M.G. Milgroom, and W.E. Fry. 1990. Quantification of factors influencing potato late blight suppression and selection for metalaxyl resistance in *Phytophthora infestans*: a simulation approach. Phytopathology 80:1190-1198.

Ewing, E., I. Simko, C.S. Smart, M. Bonierbale, E.S.G. Mizubuti, G.D. May, and W.E. Fry. 2000. Genetic mapping from field tests of qualitative and quantitative resistance to *Phytophthora infestans* in a population derived from *Solanum tuberosum* and *Solanum berthaultii*. Molecular breeding 6:25-36.

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

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

Leonards-Schippers, C., W. Gieffers, R. Schafer-Pregl, E. Ritter, S.J. Knapp, F. Salamini, and C. Gebhardt. 1994. Quantitative resistance to *Phytophthora infestans* in potato: a case study for QTL mapping in an allogamous plant species. Genetics 137:67-77.

Niederhauser, J.S., and W.R. Mills. 1953. Resistance of Solanum species to *Phytophthora infestans* in Mexico. Phytopathology 43:456-457.

Oberhagemann, P., C. Chatot-Balandras, R. Schafer-Pregl, D. Wegener, C. Palomino, F. Salamini, E. Bonnel, and C. Gebhardt. 1999. A genetic analysis of quantitative resistance to late blight in potato: towards marker-assisted selection. Molecular Breeding 5:399-415.

Rasmussen, J.O., J.P. Nepper, H.G. Kirk, K. Tolstrup, and O.S. Rasmussen. 1998. Combination or resistance to potato late blight in foliage and tubers by intraspecific dihaploid protoplast fusion. Euphytica 102:363-370.

Roer, L., and H.J. Toxopeus. 1961. The effect of R-genes for hypersensitivity in potato-leaves on tuber resistance to *Phytophthora infestans*. Euphytica 10:35-42.

Rohwer, F., K.H. Fritzemeier, D. Scheel, and K. Hahlbrock. 1987. Biochemical Reactions of Different Tissues of Potato (Solanum- Tuberosum) to Zoospores or Elicitors from Phytophthora- Infestans - Accumulation of Sesquiterpenoid Phytoalexins. Planta 170:556-561.

Shaner, G., and R.E. Finney. 1977. The effect of nitrogen fertilization on the expression of slow-mildewing resistance in Knox wheat. Phytopathology 67:1051-1056.

Simko, I., S. Costanzo, V. Ramanjulu, B.J. Christ, and G.H. Haynes. 2006. Mapping polygenes for tuber resistance to late blight in a diploid *Solanum phureja* x *S. stenotomum* hybrid population. Plant Breeding and Seed Science 125.

Stewart, H.E., J.E. Bradshaw, and R.L. Wastie. 1994. Correlation between resistance to late blight in foliage and tubers in potato clones from parents of contrasting resistance. Potato Research 37:429-434.

Stewart, H.E., J.E. Wastie, J.E. Bradshaw, and J. Brown. 1992. In heritance of resistance to late blight in foliage and tubers of progenies from parents differing in resistance. Potato Research 35:313-319.

CHAPTER FIVE

SUMMARY

The soil phase is arguably the most important aspect of late blight yet, it is the least studied and least understood part of the disease cycle. Tubers infected in soil during the growing season are the cause of the social and economic misery and suffering associated with late blight. The disease cycle in most textbooks begins with an infected tuber because until recently tubers were the primary source of inoculum in most potato growing regions of the world (Erwin and Ribeiro, 1996). Changes in the population biology of *Phytophthora infestans* in many locations worldwide have changed the epidemiology of this pathogen (Goodwin et al., 1998). The recent distribution of both mating types (A1 and A2) has created the possibility that sexual reproduction will occur and result in the production of oospores (which can reside in soil) (Erwin and Ribeiro, 1996). The survival and infectivity of oospores and sporangia in soil are still poorly understood.

An additional poorly understood soil component of late blight is the infection of tubers in the field. At the present time we have no definitive data on the infection process of tubers in soil. For now, that process is a "black box." Control of foliar epidemics through intensive fungicide applications and host resistance are the primary methods used to prevent and limit tuber infections in soil. Problems associated with these approaches are that the fungicides currently labeled for late blight do not provide protection to the tubers and the correlation between foliar and tuber resistance for any given cultivar or mapping population of potatoes is not predictable.

This thesis investigated three aspects of the soil phases associated with late blight: 1) sexual reproduction and survival of oospores in different environments over

time 2) survival and infectivity of sporangia in different soil types and 3) identification of genetic loci correlated with both foliar and tuber blight resistance.

Data presented in this thesis demonstrated that isolates of opposite mating type of *P. infestans* collected from New York can produce viable recombinant progeny that can infect both tomato and potato. Oospores buried in soil exposed to natural field conditions survived for > 1 8 months and remained infective to both tomato and potato. Although a "super pathogen" was not observed in the progeny produced through crosses in the laboratory the possibility for the introduction of isolates with new aggressive traits that can infect both potato and tomato is real. Contamination of production fields with oospores could be devastating and extremely difficult to manage. Primary infections from soil-borne oospores have been reported from Northern Europe, and the northeastern USA (Gavino et al., 2000; Stromberg et al., 1999). Additional research on the ecology of *P. infestans* sexual reproduction and activity in soil is necessary for development of effective cost efficient management strategies.

The second objective of this thesis was to investigate soil samples collected from the Mountains in central Mexico for their potential to suppress survival and infectivity of *P. infestans*. The highlands of central Mexico are the regions with the greatest diversity of *P. infestans* and it is also believed that *P. infestans* co-evolved with wild *Solanum* species in that part of the world (Fry et al., 1993). I explored the possibility that these soils in which the pathogen had co-evolved might harbor microbial antagonists to *P. infestans*. Unfortunately, the soils from the central highlands of Mexico did not have any specific suppressive influence on survival of sporangia. Analyses of survival, as measured by infectivity bioassays, confirmed that *P. infestans* sporangia are fairly short lived but can survive for longer periods of time in sterile versus non-sterile soil. These data and others indicate that, while there are

probably biological factors in soils that limit survival of *P. infestans*, soils from the mountains of central Mexico don't appear to have any factors (antagonists) that are particularly more effective than in soils from upstate New York.

My third and final objective was to identify genetic loci associated with both foliar and tuber blight resistance to late blight in a tetraploid mapping population of a cross between two *S. tuberosum* breeding lines NY121 and NY115 (Y36). Data from analysis of the Y36 mapping population have shown that while a few quantitative trait loci (QTL) are associated with both foliar and tuber resistance, most QTL are associated with either one or the other type of resistance. Three markers (PCTMATC 184, PATMATA 171, and PATMACT 236) located on a chromosome homolog V (descending from NY121) were significantly associated with both foliar and tuber resistance. These markers explained approximately 50% of the phenotypic variance associated with foliar resistance, but less than 10% of tuber blight resistance. Clearly the genes involved in defense associated with the markers on chromosome V do not contribute as much protection to the tubers as for the foliage. No QTL in the Y36 population that were significantly associated with tuber blight resistance had as large an effect as markers identified for foliar blight resistance. There is a great need to identify regions in the potato genome that are specifically involved with tuber blight resistance.

Foliar resistance has been much easier to select in mapping populations because the screening process is straight-forward and consistent between years and locations. Tuber blight resistance in breeding populations has been much more difficult to assess. Many environmental and biological factors are involved with tuber resistance/susceptibility. Many methods, similar to the methods used in this thesis which included inoculation of whole tubers, tuber slices, and tubers infected during epidemics in the field, are necessary for identification of genetic loci associated with

resistance to late blight of tubers. Further investigation of progeny from crosses involving breeding lines differing significantly in tuber blight resistance should be initiated.

Future management strategies for late blight must include the soil component of the disease. There are no fungicides available that can suppress infection of tubers and plant tissue from soil-borne oospores. In addition, we need to have a more complete understanding of the infection and survival mechanisms of both oospores and sporangia in the soil environment in order to develop integrated management strategies for all of the components of late blight.

FUTURE RESEARCH—NEXT STEPS

In the United States, the majority of potatoes are grown in three very different climates all with different soil types. The largest production areas are located in the northwest (Idaho, Oregon, Washington) followed by the northern mid-western states (Wisconsin, North Dakota, Minnesota, Michigan) and California. Another important production area for winter table stock is Florida. Additional studies on survival of progeny oospores in the field from crosses produced from isolates of *P. infestans* collected from those regions should be conducted. Both sporangia and oospore survival and infectivity are influenced by soil type, moisture, and temperature. Data obtained from survival experiments can then be used to predict the potential short and long-term risks for fields infested with oospores. Without these data, management of infested fields will be difficult and may ultimately affect export of potatoes from those regions.

Developments in molecular methods for the study of *P. infestans* may help to increase our knowledge of the germination, growth, survival and infectivity of *P. infestans* in soils. Preliminary studies with green fluorescent protein (GFP) and beta-

glucuronidase (GUS) transformed isolates of *P. infestans* conducted in our lab have been somewhat successful in allowing for the observation of the pathogen in soil. Unfortunately the problems involved with the transformation process of *P. infestans* have not yet been solved. Once these transformed (GFP and GUS) isolates become available experiments can be designed to gain knowledge of *P. infestans* in the soil environment.

Greater emphasis should be placed on the development of methods to control tuber blight. The employment of host resistance is the most economically and environmentally sustainable approach for control of both foliar and tuber late blight. Therefore crosses of NY121 with germplasm conferring quantitative resistance to tuber blight should be initiated and the progeny analyzed for resistance. Because the QTL located on chromosome V is so effective in controlling foliar blight and because this QTL also has some influence on tuber blight, further investigations of that region of the chromosome should be undertaken.

Lastly, the development of a model which can predict the incidence and severity of tuber infections within infected fields and also on an area-wide basis would provide growers and researchers with information that can be used to manage late blight epidemics.

LITERATURE CITED

Erwin, D.C., and O.K. Ribeiro. 1996. Phytophthora diseases worldwide The American Phytopathological Society, APS Press, St. Paul, MN.

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

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

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

Stromberg, A., L. Persson, and M. Wikstrom. 1999. Infection of potatoes by oospores of *Phytophthora infestans* in soil. Plant Disease 83:876.