Variation in *Phytophthora infestans*

-sources and implications-

Promotor:	Prof. dr. R.F. Hoekstra, Hoogleraar in de Genetica, i.h.b. de populatie- en kwantitatieve genetica, Wageningen Universiteit
Copromotoren:	Dr. ir. L.J. Turkensteen Senior onderzoeker, Plant Research International
	Prof. dr. W.E. Fry, Plant Pathology Professor, Cornell University, Ithaca, NY, USA
Promotiecommissie:	Prof. dr. M.J. Jeger Huxley School of Imperial College, UK
	Dr. D.S. Shaw School of Biological Sciences, University of Wales, UK
	Prof. dr. ir. P. Stam Wageningen Universiteit
	Prof. dr. ir. P.C. Struik Wageningen Universiteit

Wilbert Flier

Variation in *Phytophthora infestans*

-sources and implications-

Proefschrift

Ter verkrijging van de graad van doctor
op gezag van de rector magnificus
van Wageningen Universiteit,
Prof. dr ir L. Speelman,
in het openbaar te verdedigen
op dinsdag 5 juni 2001
des namiddags om 16.00 uur in de Aula

CIP-DATA KONINKLIJKE BIBLIOTHEEK, DEN HAAG

Flier, W.G., 2001

Variation in *Phytophthora infestans,* sources and implications.
PhD thesis Wageningen University, Wageningen, the Netherlands
– With references – With summary in Dutch.

ISBN: 90 5808 420 5

Subject headings: Potato late blight / *Solanum tuberosum* / genetic variation / pathogenicity / stability of disease resistance

Typeset in 10/14pt Frutiger 45.
Printed by Ponsen & Looyen BV, Wageningen

The research described in this thesis (September 1996 – December 2000) was conducted at Plant Research International, Wageningen, the Netherlands and at PICTIPAPA (Programa Internacional Cooperativo del Tizón Tardío de la Papa), Metepec, Estado de México, México.

PLANT RESEARCH INTERNATIONAL

Stellingen

De effectiviteit van partiële resistentie in aardappelen tegen *Phytophthora infestans* wordt negatief beïnvloed door de toegenomen agressiviteit van isolaten en het optreden van ras x isolaat interacties.
dit proefschrift

In tegenstelling tot seks bij mensen leidt seksuele reproductie bij *Phytophthora infestans* tot opwinding achteraf.
dit proefschrift

Ook duurzame resistenties hebben een uiterste houdbaarheidsdatum.

In het licht van de huidige problemen met de *Phytophthora*-bestrijding kan men de biologische teelt van aardappelen beschouwen als een luxe probleem.

'Everyone is a reductionist about subjects that they do not understand'
Andrew Brown (1999). The Darwin Wars, Simon & Schuster, London, UK (p. 48)

Integrale invoering van de 'mañana[1]'-benadering in het projectmanagement kan een bedrage leveren aan het voorkomen van het zogenaamde 'burn-out syndroom'.
[1] *vrij naar Hector Lozoya-Saldaña, technisch directeur PICTIPAPA, Mexico.*

Wageningen Universiteit heeft samen met haar oude identiteit en brede vakkenpakket ook haar bestaansrecht verloren.

Het verhogen van de minimum snelheid op rijkswegen tot 100 km/uur verhoogt de veiligheid van weggebruikers.

Voor mijn ouders; Arie en Lijntje

Abstract

Flier, W.G., 2001. Variation in *Phytophthora infestans*, sources and implications. PhD Thesis, Wageningen University, the Netherlands.

The oomycete pseudofungus *Phytophthora infestans* (Mont.) de Bary, the causal organism of late blight, is considered to be one of the most devastating pathogens affecting potatoes and tomatoes worldwide. In Europe, the pathogen caused severe epidemics on potatoes after its introduction in 1845. Late blight management became much more troublesome after the introduction of another *P. infestans* population of Mexican origin. The present population of *P. infestans* in the Netherlands and an increasing number of other European countries consists of both A1 and A2 mating type isolates and sexual reproduction has been reported. The goals of this thesis were to study late blight epidemiology and population biology of the present *P. infestans* population. Therefore, sources and patterns of variation for pathogenicity in sexual *P. infestans* populations were studied and the impact of increased levels of aggressiveness on the stability of partial resistance to late blight was investigated. Results showed that considerable levels of variation for aggressiveness are maintained in regional populations of *P. infestans* in the Netherlands and that oospores are readily produced in field crops and volunteer potato plants. Oospore production and viability proved to be highly dependent on combining abilities of parental strains and there are indications that oospores are able to survive in soils for three to four years. Increased aggressiveness and cultivar-by-isolate interactions between partial resistant potato cultivars and *P. infestans* strains negatively affect the stability of resistance. Population genetic studies in its centre of origin and diversity revealed differentiation within the Toluca Valley implying host specificity. From the results presented in this thesis, it is clear that the introduction of a sexually reproducing population of *P. infestans* in the Netherlands has had a major impact on late blight epidemics and population biology of the late blight pathogen. Sexual reproduction has therefore led to a genetically more diverse population of *P. infestans* in the Netherlands that is marked by an increased adaptability to host and environment.

Contents

1

General introduction

The potato late blight pathogen

The oomycete *Phytophthora infestans* (Mont.) de Bary (de Bary, 1876), the cause of late blight in potatoes and tomatoes, is considered to be among the most important pathogens of potato crops worldwide (Hooker, 1981). The pathogen is feared by farmers around the globe through its ability to destroy entire fields of potatoes and tomatoes in a few weeks time. The pathogen affects both foliage and stems, reducing the photosynthetic capacity of the crop and therefore leading to tuber yield reduction. In addition, *P. infestans* can infect fruits and tubers, which will add up to the total losses in marketable yield.

Phytophthora infestans belongs to a select group of plant pathogens that have the dubious honour of being a direct cause of sociological and political changes in the recent history of mankind. It is thought that *P. infestans* originated and co-evolved on native wild tuber bearing *Solanum* species in the central highlands of Mexico (Reddick, 1939). Farmers in Europe and North America were unaware of its existence until the first reports of a mysterious disease of potatoes appeared in newspapers and scientific journals in Europe and northern North America. When late blight appeared in England in 1845, John Lindley (editor of the Gardener's Chronicle and Agricultural Gazette) wrote, *"...the disease consists in a gradual decay of the leaves and stem, which become a putrid mass, and the tubers are affected by degrees in a similar way. The first obvious sign is the appearance on the edge of the leaf of a black spot which gradually spreads; the gangrene then attacks the haulms, and in a few days the latter are decayed, emitting a peculiar and rather offensive odour"*. Devastating late blight epidemics swept through Europe and destroyed potato crops in Ireland during 1845 and 1846, which left the Irish population in poverty and starvation. This black episode in Irish history catalysed the mass emigration of people to North America and became known as the Irish potato famine. More recently, severe *P. infestans* epidemics destroyed potato crops in Germany in 1917 during the First World War. Priority was given to supply the German army, which at that time was engaged in a war of attrition in the trenches of Northern France. Back home in Germany, civilians starved and when news about the poor situation reached the Western front, it demoralized the German troops. The shortage of food in Germany during the winter of 1917-1918 became known as the *"Steckrübenwinter"* (Heddergott, 1957). It has been argued that the shortage in food greatly attributed to the internal instability of the German Empire in 1918 and thus accelerated the end of the Great War (Schumann, 1991).

In our days, crop losses due to late blight have been estimated to account for 10 to 15 percent of the total global annual potato production (Anonymous, 1996). The economic value of the crop lost plus the costs of crop protection amount to 3 billion US $ annually (Duncan, 1999). In the developed world, control of potato late blight is heavily dependent on the use of fungicides. Despite frequent fungicide use, late blight epidemics have proven to be increasingly more difficult to control (Goodwin *et al.*, 1995; Turkensteen *et al.*, 1997; Schepers, 2000).

Resurgence of potato late blight

The increased problem with controlling potato late blight coincides with the displacement of the US-1 clonal lineage by a new, more variable *P. infestans* population in many parts of the world (Spielman *et al.*, 1991). New populations are marked by more aggressive genotypes of the pathogen (Day & Shattock, 1997; Lambert & Currier, 1997, Turkensteen *et al.*, 1997). In regions where both mating types have been found, evidence is accumulating that sexual reproduction takes place (Drenth *et al.*, 1994; Andersson *et al.*, 1998).

An operational definition of asexual and sexual populations is used throughout this thesis. With a 'sexual population' is meant a population of *P. infestans* in which a rapid increase of infective propagules by asexual (clonal) reproduction during the epidemic is complemented by the production of functional oospores through sexual reproduction. In sexual populations, both oospores and asexual sources of inoculum (i.e. mycelium in infected tubers) serve as inoculum sources. In contrast, asexual populations are totally dependent on asexually produced inoculum to start late blight epidemics.

Prior to the 1980s, a single A1 clonal lineage of *P. infestans,* designated US-1, was spread throughout the world while the occurrence of the A2 mating type was restricted to a confined area in the highlands of central Mexico (Niederhauser, 1956). Oospores in field crops (Gallegly & Galindo, 1958) were first reported from the Toluca Valley of central Mexico, and accumulating evidence indicates that the highlands of central Mexico are indeed the centre of origin of *P. infestans* (Fry & Spielman, 1991; Goodwin *et al.*, 1992a). Populations of *P. infestans* outside central Mexico were restricted to asexual reproduction and were bound to survive during crop free periods through hibernating mycelium inside potato tubers.

During the 1980s, potato late blight became more difficult to control in Europe. Fungicide resistance against metalaxyl (which gave excellent protection against strains of the US-1 clonal lineage) developed rapidly during the 1980s (Davidse *et al.*, 1981). A few years later, the A2 mating type was first reported from Switzerland (Hohl & Iselin, 1984). These two observations led to a revival of late blight research in Western Europe. Soon, population genetic studies using allozymes (Spielman *et al.*, 1991) and DNA fingerprinting (Goodwin *et al.*, 1992b) revealed the presence of a new, genetically variable population of *P. infestans* in Western Europe.

In the Netherlands, it appeared that a genotypically diverse, sexual reproducing population had displaced the old clonal population of *P. infestans* during the 1980s and early 1990s (Drenth *et al.*, 1993). Both A1 and A2 mating types were detected in infected potato crops (Frinking *et al.*, 1987) and the presence of complex races and new virulence factors was found (Drenth *et al.*, 1994). Functional oospores were found in naturally infected potato and tomato plant tissues collected from commercial potato fields and home gardens (Turkensteen *et al.*, 1996). Oospores produced in an inoculated potato crop using Dutch A1 and A2 strains could survive in the soil under Dutch winter conditions and were able to infect potato plants in the following season (Drenth *et al.*, 1995). Recently, Zwankhuizen *et al.* (2000) studied the relative importance of different inoculum sources initiating late blight epidemics in one of the main potato growing areas in the Netherlands. Refuse piles and infected seed were identified as the principal infection source and it was argued that oospores might act as an infection source in

both commercial potato fields and home gardens but their relative contribution to initial inoculum appears to be dependent on the weather conditions.

The need for a more integrated control of potato late blight

In the Netherlands, the number of fungicide applications to control late blight in potatoes range from an average of 7 to more than 15 applications per season (Schepers, 2000). The direct economical costs attributable to chemical late blight control sum up to approximately 55 million US $ per year (Davidse *et al.*, 1989). Due to the growing public concern about the negative side effects of pesticide use in agriculture the Dutch government launched the Multi-Year Crop Protection Plan (MJP-G) in 1991, which intended to (1) reduce the dependence on pesticides, (2) reduce the volume of pesticides used and (3) reduce the emission of pesticides. The failure to achieve the reduction in fungicide use in arable crops as proposed by the MJP-G can to a great extend be attributed to an increase in fungicide use for late blight control (Turkensteen *et al.*, 1997). With the growing public demand for crop protection methods with little or no adverse effects on public health and the environment, breeding for durable resistance against late blight has been a focus for most modern potato breeding programmes (Colon *et al.*, 1995; Inglis *et al.*, 1996; Peters *et al.*, 1999). Incorporation of host resistance in integrated late blight disease management could enable a significant reduction of fungicide use while maintaining the present yield and quality standards (Inglis *et al.*, 1996). The importance of minimising yield losses due to potato late blight by exploiting host resistance has been recognized for more than a century. In the early days of breeding for late blight resistance, complete resistance of potatoes to late blight was highly valued and the first complete resistant potato cultivars appeared in the 1930s (Müller & Black, 1953). This type of resistance was conferred mainly by introgression of dominant resistance genes, the so-called R-genes, derived from the wild *Solanum* species *S. demissum, S.* x *edinense* and *S. stoloniferum* (Toxopeus, 1964), which are endemic in central Mexico. The highlands in central Mexico are considered as a secondary centre of diversity for the genus *Solanum* (Correll, 1962) and it is plausible to assume that *P. infestans* evolved during a long period of co-evolution with *S. demissum* and *S. stoloniferum*. The hypersensitive response (HR) that is associated with the defence reaction inferred by R-genes occurs as a rapid localized cell death in incompatible interactions between plant and pathogen. Numerous attempts to achieve durable resistance in potato by the incorporation of R-genes from *S. demissum* and *S. stoloniferum* (Black *et al.*, 1953; Malcolmson & Black, 1966) proved to be unsuccessful due to rapid adaptation of the pathogen as reviewed by van der Plank, (1971) and Turkensteen (1993). This rapid adaptation of the pathogen led to the situation in which the vast majority of potato cultivars commonly grown in Western Europe (Colon *et al.*, 1995) and North America (Platt & Tai, 1998) are highly susceptible to late blight.

During the 1950s and the 1960s breeding efforts aiming at the selection for more durable forms of late blight resistance were initiated (Toxopeus, 1964; Hermsen & Ramanna 1973). These forms of resistance are often referred to as partial resistance, field resistance or quantitative resistance (Turkensteen, 1993; Colon *et al.*, 1995) and are thought to be polygenic, non-race-specific and therefore effective against all *P. infestans* genotypes by reducing the rate of the epidemic (van der Plank 1968; Parlevliet

& Zadoks, 1977). Partial resistance is thought to provide long-lasting protection against late blight (Turkensteen, 1993). In the Netherlands, the need for potato cultivars with high levels of stable forms of late blight resistance became even more urgent with the increase of organic potato production during the 1990s and the presence of a more aggressive sexual reproducing *P. infestans* population. Genetic recombination through the presence of functional oospores leads to increased levels of variation in sexual *P. infestans* populations thus potentially provoking more rapid adaptation to host resistance. Caten (1974) argued that differential interactions between strains of the pathogen and non-immune cultivars are a common feature of host-pathogen systems and he envisioned that the existence of forms of resistance totally independent of the invading strain would be extremely rare. Support for this view in the potato-late blight pathosystem is presented by Leonards-Schippers *et al.* (1994) who identified an 'isolate-specific' QTL for quantitative late blight resistance in diploid potato lines and by Vleeshouwers *et al.* (2000) who claimed a general role for the HR in resistance expression of R-gene based resistance (*sensu* Turkensteen, 1993), non-host resistance as well as partial resistance. It appears that breeders, pathologists, and potato growers alike are facing a fairly complex situation in which many of the old assumptions and dogmas about late blight biology and epidemiology have to be reconsidered.

Aims and scope of this thesis

The presence of a 'new' population of *P. infestans* that is marked by increased levels of pathogenicity and is able to reproduce sexually catalysed new late blight research initiatives. There is an unquestioned need to re-investigate late blight epidemiology and to test the stability of partial resistance. Despite extensive studies on the epidemiology and population biology of *P. infestans* in the Netherlands following the discovery of the displacement of the old A1 mating type population by new A1 and A2 mating type isolates, we only have a very limited understanding about the implications of the variation that is present in sexual reproducing populations of the pathogen. The Ministry of Agriculture, Fisheries and Nature Management of the Netherlands, aware of this lack of operational knowledge increasingly supported basic research on epidemiology of late blight and funded the late blight epidemiology group at the former Research Institute for Plant Protection (IPO) (programme DWK 337). In 1997, a joint research project was initiated by Cornell University, Ithaca, NY and IPO. The project aimed to study the epidemiology and population biology of *P. infestans* in its centre of origin in the Toluca Valley in central Mexico in collaboration with the International Collaborative Program on Potato Late Blight (PICTIPAPA), based at Metepec, Mexico. Investigating patterns of variation present in the sexual reproducing *P. infestans* population in the Toluca Valley and comparing levels of pathogenicity present in pathogen populations from central Mexico and the Netherlands help to understand and predict the evolution of *P. infestans* after its introduction in the Netherlands.

Efforts aiming to elucidate the impact of the 'new' *P. infestans* population on late blight epidemiology and population biology found concrete shape in the form of this thesis. The research described in this thesis (carried out from September 1996 until December 2000) aimed to explore the sources and patterns of variation for pathogenicity in sexual *P. infestans* populations and intended to denote possible

implications for the stability of partial resistance to late blight. This thesis can be divided into four sections based on the different themes that are addressed.

Section I describes the variation for aggressiveness present in populations of *P. infestans*. The variation for several pathogenicity factors associated with aggressiveness to the potato haulm and tubers was assessed for three regional populations of *P. infestans* in the Netherlands in order to investigate the overall level and variation in aggressiveness (Chapter 2 & 3).

Section II aims to elude some aspects related to the ecology of oospores. The role oospores play in the epidemiology of late blight is poorly understood (Andrivon, 1995). Since the work of Pittis and Shattock (1994) and Drenth and co-workers (Drenth *et al.*, 1995) only few efforts have been made to clarify the role oospores play in initiating late blight epidemics. For example, it is not known whether oospore germination is confined to one single "burst" of zoospore release or whether germination takes place throughout a period of favourable environmental conditions. Formation, survival and infectivity of oospores of *P. infestans* were studied under field conditions and in laboratory experiments. The relationship between the number of lesions per leaflet and oospore incidence was explored using a simple mathematical model (Chapter 4). Several aspects of the ecology of oospores were studied in the highlands of central Mexico, the centre of origin of the late blight pathogen. The effect of population diversity and parental genotype on oospore production and fecundity was evaluated by means of in vitro matings between Mexican isolates of *P. infestans*. In addition, the formation of oospores in blighted leaflets of the native host plant species *Solanum demissum* was studied (Chapter 5).

Section III is denoted to the impact of the presence of the new *P. infestans* population on the stability of partial resistant potato cultivars. Stability of partial resistance was evaluated using field experiments and bioassays in combination with highly aggressive isolates of *P. infestans*. Specificity in tuber blight resistance was explored using whole tuber inoculations and tuber slice assays (Chapter 6). The differential interaction between *P. infestans* isolates and potato cultivars with varying levels of partial resistance in the foliage and the tubers was quantified (Chapter 7).

Section IV elucidates the population biology of *P. infestans* in the Toluca Valley and reports on a new *Phytophthora species*, closely related to *P. infestans*. The population structure of *P. infestans* in the Toluca valley, Central Mexico was adopted as a model system in which the influence of host plant species and crop management on population diversity and population differentiation in a sexual population of the late blight pathogen was studied (Chapter 8 & 9). The possibility of ongoing speciation in a variable group of highly specialized plant pathogens in a diverse environment was explored using *Phytophthora* species in the Toluca Valley as a model. The Toluca Valley was monitored for the presence of new, adapted forms of Phytophthora. In 1999, a homothallic *Phytophthora* species was found on blighted foliage of *Ipomoea longipedunculata*, a morning glory species native to the highlands of central Mexico. It

was concluded that the isolates obtained belonged to a new *Phytophthora* species, based on host range, morphology, allozyme marker alleles, mitochondrial DNA haplotypes and rDNA sequences. A species description was prepared and a name *Phytophthora ipomoeae* Flier *et* Grünwald proposed (Chapter 10).

Finally, the results are summarised and discussed (Chapter 11).

Section I

2

Variation in tuber pathogenicity of *Phytophthora infestans* in the Netherlands

W. G. Flier, L. J. Turkensteen and A. Mulder

Potato Research (1991) **41**: 345-354

Summary

Variation in aggressiveness to tubers among isolates of *Phytophthora infestans* sampled from three potato growing regions in the Netherlands was compared. Variation in the ability to infect tubers of cv. Bintje was found between isolates of each of the three regional populations. The most aggressive isolate of the old population matched the average level of the new population in its ability to infect tubers. As a consequence, the commonly used reference isolate VK 6C can no longer be considered to be representative for the present population of *P. infestans*. Therefore it is recommended that testing tuber resistance for the official list of potato cultivars with this isolate should be discontinued.

Tuber infection and subsequent spread of the fungus in the tuber tissues were not found correlated. The components of tuber pathogenicity studied were not correlated to pathogenicity factors in the foliage, as measured under growth chamber conditions.

Introduction

The devastating effect of a late blight epidemic caused by the oomycete pseudofungus *Phytophthora infestans* (Mont.) de Bary on the potato crop is well known. Yield losses are attributable to both premature death of the foliage and diseased tubers. The annual yield loss in developing countries is estimated at nearly 3 billion US Dollars (Mackay, 1996). One of the most promising strategies for a more reliable and sustainable potato production is the introduction of durable resistance in future potato cultivars (Mackay, 1996; Wastie, 1991).

Historically, research has been focussed on increasing the level of resistance to late blight in the foliage. Tuber resistance has been somewhat neglected by most potato breeders (Umaerus & Umaerus, 1994), perhaps because it is expected that high levels of resistance in the foliage will decrease the amount of inoculum to which the tubers are exposed, and therefore minimise the risk of tuber blight (Wastie, 1991). However, on such cultivars *P. infestans* may produce inoculum during a longer period than on a more susceptible one, and so prolong the period during which tuber infections may occur (Schwinn & Margot, 1991; Toxopeus, 1958). Moreover, in years with excessive rainfall during the growing period, fungicide application can be hampered and a serious late blight outbreak may occur. Potato crops which were treated with a contact fungicide and in which low levels of blight were tolerated during the growing season showed high levels (up to 20%) of blighted tubers (Schwinn & Margot, 1991). In the Netherlands, where *P. infestans* is in general well controlled by numerous applications of protective fungicides, commercial potato growers occasionally have experienced unexpectedly high yield losses due to severe tuber blight attack.

Recently there has been a striking displacement of the old population of *P. infestans* by a new one (Spielman *et al.*, 1991), probably originating from Mexico (Niederhauser, 1991). The old population was marked by a low level of genetic variability as few virulence patterns were found and only one RFLP clone appeared to be present (Spielman *et al.*, 1991). The new population is marked by many virulence

patterns as well as by many RFLP lineages. Hence, the new population must be genetically highly variable.

Caten (1974) reported differences between six isolates in their ability to infect tubers. Though significant, these differences were relatively small. These isolates were all representatives of the old population, and are only rarely found at present. No information has been available on level and variation in aggressiveness to tubers of the new population.

In the Netherlands, several ways of potato growing may be distinguished. In this study, three of such systems have been considered. First, the growing of seed and ware potatoes as practised in Southern Flevoland on clay soils. The cultivars used are in general very susceptible and spraying is intensive to prevent any risk of tuber infection. Consequently, very little late blight is present in the farmers' fields. The second system is found in the starch potato growing area in the northeastern part of the Netherlands on sandy and reclaimed peat soils. In this region, predominantly moderately resistant cultivars are grown with relatively high levels of tuber resistance. Spraying is more relaxed and low levels of late blight attack are permitted. The third system is to be found in an allotment garden complex at Ede on a sandy soil, remote from potato growing areas. In this garden complex, late blight is thought to start from oospores, considering mating type ratios and the high level of genetic variation of *P. infestans* collected over the years.

In the Netherlands, tuber blight resistance of advanced breeding clones and novel potato cultivars is routinely tested in field experiments using the Dutch *P. infestans* tester isolate (VK 6C). Considering the displacement of the old population represented by VK 6C, the question arose whether this isolate is still representative for the current population of the pathogen.

The objectives of this study were to evaluate the variation in tuber pathogenicity of the present population of *P. infestans,* and to compare the performance of a number of reference isolates with this random sample of new population genotypes.

Materials and methods

Origins of P. infestans isolates

In 1995, isolates of the fungus were collected in the following three regions: 1) A main starch potato growing region in the south-east of Drenthe on sandy peat soils. 2) A ware and seed potato production area in Southern Flevoland on clay soils. 3) An allotment garden complex at Ede on a sandy soil. The isolates collected in Southern Flevoland were kindly provided by Ir. M. Zwankhuizen of the Department of Phytopathology, Wageningen Agricultural University, the Netherlands. From each region, 12 isolates were selected that appeared to be genetically different from each other on the basis of race determination (Ede and Drenthe) or RG 57 Fingerprint pattern (Flevoland).

In addition, the following four reference isolates were selected. Isolate VK 6C had been collected in 1958 (Mooi, 1964) and is moderately aggressive to tubers. This isolate is commonly used for tuber blight resistance testing (including tests for the annual lists of recommended cultivars) in the Netherlands. Isolate VK1-3-4 had also been collected in 1958 and is considered to be highly aggressive toward tubers. The isolate US1 is a race 0 strain used for resistance testing and was kindly provided by Prof. W. E. Fry, Cornell University, USA. Isolate I82001 served as the reference isolate for the assessment of foliage blight resistance. This isolate is marked by a low level of aggressiveness to tubers. All reference isolates except isolate I82001 belong to the old population of *P. infestans*. Information on virulence factors, mating type and origin of the isolates used is presented in Table 2-1.

Inoculum production

Isolates were cultured on leaflets of cv. Bintje, which were placed with the abaxial side up in 9 cm Petri dishes containing 10 ml 2% water agar. After drop inoculation of the leaflets, the Petri dishes were kept in a climate chamber at 13 °C with a 16 hours light period using fluorescence tubes type 33 at an intensity of 12 Wm^{-2}. After seven days, zoosporangial inocula were prepared by gently shaking the heavily sporulating leaflets in 25 ml of tap water followed by two wash steps using 15 µm nylon filter cloth. Inoculum concentration was adjusted to 10.000 sporangia ml^{-1} using a Coulter Counter Z1 (Coulter Electronics Inc.).

Tuber blight assessment

The tuber blight assay was mainly adopted from Bjor (1987) and Mooi (1964) with some minor modifications. Tubers of cv. Bintje grown on a light clay soil were harvested by hand to minimise wounding and immediately transferred to the laboratory. Undamaged tubers were placed in plastic seed tuber crates with their rose end facing upwards, about 80 tubers per crate. Three replicates per isolate (one crate per replicate) were used. Tubers received 25 ml of inoculum per crate, sprayed with a low-pressure nozzle at a pressure of 300 kPa. Inoculated tubers were incubated in the dark at 15°C for 14 days. During the first 24 hours, the inoculated tubers were kept moist by enclosing the crates in plastic bags, and then they were incubated at 80% RH.

Individual tubers were scored for the presence or absence of tuber blight symptoms and the average percentages of infected tubers per crate determined (**IF**). In addition, 10 randomly selected diseased tubers per crate were cut longitudinally and disease development was scored according to a 1 to 5 disease severity scale and a severity index was calculated (**SI**) (Table 2-2).

To estimate the number of infections from the percentage infected tubers, Gregory's multiple infection transformation was applied (Gregory, 1948). The transformed data can be interpreted as a relative measurement of infection efficiency (**IE**)

(Zadoks & Schein, 1979). Finally, a tuber blight index (**TI**) was calculated as TI = - Log_e (IE x SI).

Statistical analysis

All statistical analyses were performed using Genstat 5 version 3.1 (Payne *et al.*, 1993). A mixed model for analysis of variance was performed on IF, SI, IE and TI. Isolates within regions were assumed to represent a random sample of genotypes of the underlying population from each sampled region concerned. Restricted Maximal Likelihood (REML) was used to obtain estimates for variance components and mean values of the parameters concerned. The ratio between the average estimated variance components due to genotypic variation between isolates (s^2_A) and random error components (s^2_e) was used to examine the presence of significant genotypic variation in the populations studied (Searle *et al.*, 1992). Wald tests (Payne *et al.*, 1993) were used for evaluating fixed model terms. Spearman rank correlation coefficients were calculated between the assessed aggressiveness components IF, SI, IE, TI and mating type as well as the number of virulence factors in order to search for correlations between pathogenicity related factors.

Results

Within the three local populations and the group of the four reference isolates, substantial and significant ($P \leq 0.001$) variation in the pathogenicity components tuber blight incidence, disease severity, infection efficiency and tuber blight index was found (Table 2-2). As an analysis of the residuals did not show an irregular distribution for any of those components, no further data transformation was applied. According to the Wald test no significant differences were found between the average levels of tuber pathogenicity components of the three populations sampled in 1995. However, the group of four reference isolates showed a significantly lower ($P = 0.01$) mean disease severity value (Table 2-3).

Significant genotypic variation was present in the following pathogenicity components among the isolates collected in 1995; tuber blight incidence, infection efficiency and tuber blight index. No significant variance component was detected in disease severity (Table 2-4). Genotypic variation with respect to disease severity was exclusively found when REML analysis was performed with a pooled set of data including both reference isolates and isolates collected in 1995. The presence of genetic variation in disease severity could be attributed to the exceptionally low invasive capacity of isolate I82001.

For all three populations, the tuber blight index was found significantly ($P < 0.01$) correlated with disease incidence and infection efficiency (Table 2-5). Both tight correlations can be traced back to the intrinsic relation between the two pairs of pathogenicity components.

Table 2-1. Virulence pattern and mating type of 36 isolates of *Phytophthora infestans* originating from three potato growing regions in The Netherlands.

Southern Flevoland			***Allotment gardens Ede***			**Drenthe**		
Isolate	**Race**	**Mating type**	**Isolate**	**Race**	**Mating Type**	**Isolate**	**Race**	**Mating Type**
F95100	1,3,4,7,10,11	A1	I95004	3,4,10,11	A1	I95117	3,4	A1
F95104	1,3,4,10,11	A1	I95018	1,2,3,4,5,7,10,11	A1	I95130	3,4,7,10,11	A1
F95130	1,3,4,7,10,11	A1	I95023	1,3,4,5,6,7,10,11	A2	I95137	1,3,4,6,7,10	A2
F95162	1,2,3,4,7,11	A1	I95024	1,4,7,10,11	A2	I95138	1,1	A1
F95301	1,2,3,4,6,7,10,11	A1	I95032	3	A1	I95145	1,3,7,10,11	A2
F95305	1,3,10,11	A1	I95039	1,3,4,7,8,10,11	A2	I95150	1,3,4,7,10,11	A1
F95516	1,3,4,7,10,11	A1	I95050	1,3,4,5,7,8,10,11	A2	I95161	1,3,4,7,8,10,11	A1
F95528	1,3,4,7,10,11	A1	I95052	1,3,4,5,6,7,8,10,11	A2	I95175	1,3,4,6,7,8,10,11	A1
F95555	3,4,7,11	A1	I95054	4	A2	I95181	1,3,4,6,7,8,10,11	A2
F95573	1,3,4,7,10,11	A1	I95057	1,3,4,7,8,10,11	A2	I95185	3,4,7,10,11	A1
F95587	1,2,3,4,6,7,10,11	A1	I95062	1,3,4,7,10,11	A1	I95188	1,3,4,6,7,10,11	A2
F95624	1,2,3,4,10,11	A2	I95066	1,3,4,7,11	A1	I95197	1,4,10,11	A1

Reference isolates:

I82001, race 1,2,3,4,5,6,7,10,11; A2; new population *P. infestans*

Fry US 1, race 0; A1; old population *P. infestans*

VK 6C, race 1,4; A1; old population *P. infestans*

VK 1.3.4, race 1,3,4; A1; old population *P. infestans*

Table 2-2. Tuber pathogenicity of *P. infestans* isolates originating from three different potato growing regions in the Netherlands and four reference isolates, as assessed with a bioassay using tubers of cv. Bintje.

Flevoland					Allotment gardens Ede					Drenthe				
Isolate	IF[1]	SI[2]	IE[3]	TI[4]	Isolate	IF	SI	IE	TI	Isolate	IF	SI	IE	TI
F95001	52.4	3.7	0.7	1.0	I95004	14.5	2.7	0.2	-0.9	I95117	45.2	5.0	0.6	1.1
F95104	69.5	3.7	1.2	1.5	I95008	85.8	3.7	2.0	2.0	I95130	29.2	4.7	0.4	0.5
F95130	64.1	3.3	1.0	1.2	I95018	36.9	4.0	0.5	0.6	I95137	38.8	4.3	0.5	0.8
F95162	75.1	2.3	1.4	1.2	I95023	39.8	3.7	0.5	0.6	I95138	46.5	4.0	0.6	0.9
F95301	10.6	3.7	0.1	-0.9	I95024	67.6	5.7	1.1	1.9	I95145	23.4	3.0	0.3	-0.2
F95305	66.6	4.3	1.1	1.6	I95039	44.1	4.3	0.6	0.9	I95150	78.2	4.3	1.5	1.9
F95516	34.5	2.7	0.4	0.1	I95050	21.6	4.3	0.2	0.1	I95161	51.3	4.0	0.7	1.1
F95528	32.8	4.0	0.4	0.5	I95052	67.3	4.0	1.1	1.5	I95175	14.1	4.3	0.2	-0.4
F95555	66.1	4.3	1.1	1.5	I95054	38.8	4.7	0.5	0.8	I95181	77.4	4.0	1.5	1.8
F95573	76.5	5.0	1.5	2.0	I95057	76.8	4.3	1.5	1.9	I95185	38.4	5.0	0.5	0.9
F95587	37.8	3.3	0.5	0.5	I95062	52.7	5.0	0.8	1.3	I95188	52.8	4.0	0.8	1.1
F95624	71.9	3.0	1.3	1.3	I95066	71.3	3.0	1.3	1.3	I95197	56.6	3.3	0.8	1.0
Reference isolates														
VK 6C	38.6	2.7	0.5	0.3										
US 1	33.3	4.0	0.4	0.5										
VK 1.3.4	59.0	4.3	0.9	1.4										
I82001	26.5	1.3	0.3	-0.9										
I82001[5]	68.1	3.0	1.1	1.2										
LSD (0.95)	16.8	1.6	0.4	0.9										

[1] IF = disease incidence
[2] SI = disease severity
[3] IE = infection efficiency
[4] TI = tuber blight index
[5] = inoculation with 80.000 zoosporangia.ml^{-1}

Table 2-3. Average tuber pathogenicity of *P. infestans* isolates for the three sampled potato-growing regions in The Netherlands and four reference isolates.

Region	Pathogenicity component			
	IF[1)]	SI[2)]	IE[3)]	TI[4)]
Flevoland	54.8[a*]	3.6[a]	0.9[a]	0.9[a]
Ede	51.4[a]	4.1[a]	0.9[a]	1.0[a]
Drenthe	46.0[a]	4.2[a]	0.7[a]	0.8[a]
Reference isolates	45.1[a]	3.1[b]	0.7[a]	0.4[a]

[1]IF = disease incidence [2]SI = disease severity
[3]IE = infection efficiency [4]TI = tuber blight index
*) Figures with different letters are significantly different (LSD) at P=0.01.

A low, but significant (P = 0.05) correlation existed between tuber blight severity and the tuber blight index. For the Flevoland samples, a significant (P = 0.01) negative correlation was found for the number of virulence factors (VIR) and the tuber blight index. Tuber blight severity was not correlated with disease incidence, nor with infection efficiency or the number of virulence factors.

No correlation was found between the tuber pathogenicity components and mating type of the tested isolates. Using a subset of 33 isolates tested also for aggressiveness to foliage, no correlations were found between parameters of tuber pathogenicity and various foliar pathogenicity parameters (data not shown).

Table 2-4. Variance components for tuber pathogenicity components using Restricted Maximal Likelihood (REML). Variance components are based on 36 isolates collected from three potato growing regions in the Netherlands.

Pathogenicity component	Variance component		
	$\sigma^2_{isolates}$	σ^2_{error}	$\sigma^2_{isolates}/\sigma^2_{error}$
IF	403.7	113.5	3.6**
SI	0.2	0.98	0.2
IE	0.21	0.06	3.5**
TI	0.58	0.29	2.0**

**) Variance ratio significantly (P < 0.01) larger than one, based on a Fisher distribution with 35 and 72 degrees of freedom respectively.

Table 2-5. Spearman Rank Correlation Coefficients for tuber pathogenicity and number of virulence factors of the isolates tested.

	Region			
Components[1]	**Flevoland**	**Ede**	**Drenthe**	**Total**
IF - SI	0.07	0.08	-0.28	0.08
IF - Vir	-0.45	-0.11	0.1	-0.06
IF - IE[2]	1	1	1	1
IF - TI	0.81***	0.96***	0.90***	0.95***
SI - Vir	-0.42	0.07	-0.11	-0.15
SI - IE	0.07	0.08	-0.28	0.08
SI - TI	0.55	0.25	-0.01	0.33*
Vir - IE	-0.45	-0.11	0.1	-0.06
Vir - TI	-0.74**	-0.15	0.05	-0.14
IE - TI	0.81***	0.96***	0.90***	0.95***

[1] Significance level of correlation per region (Flevoland, Ede en Drenthe) and pooled data, using an Exact test with 10 degrees of freedom and the Student approximation with 39 degrees of freedom respectively. Vir = number of virulence factors expressed in the isolates tested.

[2] Components intrinsically correlated.

*) Correlation significant at $P = 0.05$.

**) Correlation significant at $P = 0.01$.

***) Correlation significant at $P < 0.01$.

Discussion

Isolates of *P. infestans* originating from three potato growing regions in the Netherlands strongly differed in their ability to induce tuber blight symptoms in a susceptible potato cultivar. However, no differences in tuber pathogenicity were found between regional populations of *P. infestans*, suggesting that the average pathogenicity levels of the populations tested were comparable.

No correlation was found between the assessed components of pathogenicity. For the farmer, the number of infected tubers is much more important than the level of disease development after infection. Therefore, in screening for tuber resistance, it is important to select a tester strain with a higher capability to infect than to invade. To represent the most dangerous clones of *P. infestans* in the field, a tester isolate should have a high IF value. In this respect, isolates, I95008, F95573, I95057, I95150 and I95181 were good candidates of whom I95008 was by far the most infective one and F95573 the most invasive one. Therefore, the tuber resistance tester isolate VK 6C cannot be considered to be representative of the present population of *P. infestans*. The

use of this isolate to test tuber resistance for the official list of potato cultivars should be discontinued.

In the past, representatives of the old population of *P. infestans* showed only limited variation in tuber aggressiveness. As the late blight fungus was totally dependent on surviving the winter in infected tubers, one might suggest that the parasitic fitness of a strain should be heavily dependent on the ability to colonise tubers. Intermediate aggressive isolates would tend to survive best, in contrast to both highly aggressive strains, which would probably kill their host before sprouting, or weakly aggressive strains which would either fail to infect tubers or be inactivated by the phenomenon of lesion arrest (Wastie, 1991). In the past, variation in tuber pathogenicity in asexual reproducing populations of *P. infestans* was very limited. This general experience might be the principal reason that information on the tester isolate used is lacking in most papers on tuber blight assessment (Pietkiewicz & Jellis, 1976; Lapwood, 1977; Stewart *et al.*, 1983; Bjor, 1987; Wastie *et al.*, 1987). Considering the variation in aggressiveness of recently introduced populations of the fungus (this paper; Lambert & Currier, 1997), the choice of the test isolate is of key importance for tuber blight assessment. Therefore, it is strongly recommended to use well-defined tester isolates, which should be representative of the present population of *P. infestans* in growers' fields.

Tuber infection and the subsequent spread of the fungus in the tissues were not correlated in this research. The results suggest that these components of aggressiveness were controlled by different mechanisms. With respect to mating type, no correlation between components of tuber pathogenicity and mating type was found, suggesting that the level of pathogenicity to tubers is not being influenced by mating type in *P. infestans*.

The strong negative correlation (-0.74) that was found between increasing race complexity (virulence) and tuber pathogenicity in the Flevoland population could be explained by stabilising selection in *P. infestans* (Van der Plank, 1968). According to this hypothesis, unnecessary virulence reduces the pathogenic fitness of the pathogen so that selection operates in favour of races of the pathogen without unnecessary virulence. In most years, late blight epidemics in Southern Flevoland are strongly dependent on tuber borne inoculum, mainly through refuse piles (Zwankhuizen & Frinking, 1996). Hence, the ability to survive in tubers must have a strong impact on the fitness of the isolates concerned. As most potato cultivars grown in Southern Flevoland are fairly susceptible and chemical control is stringent, the accumulation of unnecessary virulence factors in the pathogen might reduce its fitness on tubers. However, this intriguing phenomenon was not found to be present for the other two regional populations sampled.

The components of tuber pathogenicity studied were not correlated to foliar pathogenicity factors, as measured under growth chamber conditions. This strongly supports the view of Holden (1977) who suggested that late blight in tubers and the foliage should be treated as different diseases.

Acknowledgements

We would like to thank Marieke Förch, Petra Horsten, Marjon Krijger, Els Verstappen and Anneke Wolters of the Resistance & Virulence section at IPO-DLO for all their help during the experiments and Pieter Vereijken (GLW-DLO) for assistance with the statistical analysis and Prof. W.E. Fry (Cornell University) for valuable comments on the manuscript. This work was financially supported by the Dutch Potato Association (NAA).

3

Foliar aggressiveness of *Phytophthora infestans* in three potato-growing regions in the Netherlands

W. G. Flier and L. J. Turkensteen

European Journal of Plant Pathology (1999) **105**: 381-388

Summary

Thirty-six isolates of *Phytophthora infestans* originating from three different potato growing regions in the Netherlands were tested for their aggressiveness to leaves of potato cultivar Bintje under controlled conditions. Measurements of latent period, maximal growth rate, infection efficiency and sporulation intensity were made and a composite aggressiveness index was calculated. Large variation in aggressiveness was present among isolates for each regional *P. infestans* population studied. The three populations differed significantly in latent period, but not for maximal growth rate and infection efficiency. Phenotypic variation existed for all components of aggressiveness and the aggressiveness index in *P. infestans* from each regional source. No association was found between mating type and aggressiveness. It is concluded that high levels of variation for aggressiveness are being generated and maintained through sexual reproduction in *P. infestans* strains from regional potato growing practices.

Introduction

Late blight, caused by the oomycete *Phytophthora infestans* (Mont.) de Bary, is one of the most devastating threats to the potato crop in the world. Crop losses due to late blight have been estimated to account for 10 to 15 percent of the total global annual potato production (Anonymous, 1996). The economic value of the crop lost and the costs of crop protection amount to 3 billion US $ annually (Anonymous, 1996). In The Netherlands, each year large amounts of fungicides, with a total value of approximately 55 million US $ are applied to control the disease (Davidse *et al.*, 1989). With increasing public concern about the environmental consequences of pesticide use, more sustainable and consumer friendly disease control measures are desired.

For more than a century, the genetic structure of *P. infestans* in Western Europe was highly uniform with only limited race diversity. As only A1 mating type strains were present, the fungus was forced to propagate asexually by means of sporangia and zoospores during the potato growing season, while its survival during the winter depended entirely on hibernating mycelium in potato tubers (van der Zaag, 1956). As a consequence, only a few closely related strains of the fungus were found (Drenth *et al.*, 1994).

The situation became different with the introduction of a new population of *P. infestans* to Western Europe (Spielman *et al.*, 1991; Fry *et al.*, 1991, 1993; Drenth *et al.*, 1994). Most likely this new population originated from Mexico and was introduced to the European mainland shortly before 1980 (Spielman *et al.*, 1991). An impressive increase in genetic variation was demonstrated at the molecular level (Drenth *et al.*, 1993a), and this was accompanied by an increase in race diversity and the appearance of 'new' virulence factors in the Netherlands (Drenth *et al.*, 1994). However, only a little attention has been paid to assessing the level of variation of aggressiveness in the current population of *P. infestans* (Day & Shattock, 1997). The old population of *P. infestans* showed some variation in aggressiveness (Caten, 1970). However, no significant differences were found when *P. infestans* isolates from a Mexican population were compared with isolates from the United States and Wales (Tooley *et al.*, 1986).

The objective of this study was to compare aggressiveness of *P. infestans* isolates sampled from three regions of The Netherlands with typical potato growing practices by means of a bioassay and to determine the variation amongst isolates and between regional sources of the pathogen.

Materials and methods

Origins of isolates

In 1995, Isolates from single lesions were sampled from the following three regions; (a) the main starch potato growing region in the south-east of the Province of Drenthe on sandy peat soils, (b) the ware and seed potato production area in Southern Flevoland on clay soils and (c) an allotment garden complex at Ede on sandy soil. The three regional sources are isolated from each other by spatial effects, potato growing practice and potato cultivars. The isolates collected in Flevoland were kindly provided by Dr. M. Zwankhuizen of the Department of Phytopathology, Wageningen University, the Netherlands. Since this study deals with variation in aggressiveness between isolates within regions and variation between regions, 12 non-identical isolates were randomly selected from each of the three potato growing regions. For Drenthe and Ede, this selection was made based on physiological race and mating type. For the isolates collected in Southern Flevoland, isolate selection was based on RG57 RFLP fingerprint patterns (Zwankhuizen *et al.*, 1998). In Table 3-1, a list of the isolates used in this study is given.

Culturing and preparation of inoculum

Isolates were stored in a liquid nitrogen storage system, for which purpose sporangium suspensions from single lesions were prepared in a 15% dimethylsulphoxide solution. Spore suspensions were transferred into 1.8 ml cryovials, cooled down to -40 °C at a rate of approximately 0.5 °C min^{-1} using a Neslab CC 60 II immersion cooler and an alcohol bath and transferred to liquid nitrogen.

Isolates taken from long-term storage were first cultured on tuber slices of the general susceptible potato cultivar Bintje by incubation in the dark at 15 °C for 5 to 7 days. When sporulating mycelium was present, small pieces of mycelium were placed on the lower epidermis of leaflets of cultivar Bintje placed with the abaxial side up in 9 cm Petri dishes containing 10 ml 2% water agar. Inoculated leaflets were kept in a climate chamber at 15 °C with a 16 hours light period by means of fluorescence tubes type 33 at an intensity of 12 W m^{-2}. Leaflets densely covered with sporulating mycelium were obtained after seven days of incubation.

Sporangial inoculum was prepared by washing these leaves in 20 ml of tap water. The crude suspension was washed and collected on a 15 µm nylon filter cloth. After re-suspension, the concentration was adjusted to 20.000 sporangia per ml using a

Coulter Counter Z1 (Coulter Electronics Inc.) and kept at 18 °C. The suspensions were used as inoculum within 30 minutes after preparation.

Race and mating type determination

The R-gene differential set of potato clones for race identification consisted of: r0 (Bintje), R1, R2, R3, R4, R5, R6, R7, R8, R9, R10, R11, R1R2, R1R3, R1R4, R2R3, R2R4 and R1R2R3 (Black *et al.*, 1953; Malcolmson & Black, 1966). Potato tubers of each differential line were planted in 12 l plastic pots containing loam-based compost. Plants were grown under greenhouse conditions with at least 16 hours light a day augmented with Philips Son-T Agro illumination. The greenhouse temperature was kept at 18 °C with a RH of 80%.

For race determination, detached leaflets of the differentials were placed abaxial side up in 9 cm diameter plastic Petri dishes containing 10 ml 2% water agar. The leaflets were sprayed with a sporangial suspension (10^4 sporangia ml^{-1}) by means of a spraying nozzle at a pressure of 0.5 kg m^{-2} until runoff. Inoculated leaflets were incubated in a growing chamber at 15 °C in the dark for 24 hours. Subsequently the remaining fluid was allowed to evaporate by placing the Petri dishes without lids in a laminar flow cabinet for 30 minutes. Incubation was continued in a climate chamber at 15 °C with a photoperiod of 16 hours provided by fluorescence tubes type 33 (Philips) at an intensity of 12 W m^{-2}. At day 4, 5 and 6 development of lesions was scored. When sporulation was clearly visible on a R-gene differential leaflet the disease reaction was considered compatible.

Mating type of the isolates was determined by pairing with the A1 and A2 tester strains 80029 and 88133, respectively (Drenth *et al.*, 1994), on 14 mm leaf discs of cultivar Bintje, placed in 9 cm Petri dishes on 2% water agar. Leaf discs were inoculated with the isolate to be tested and one of the tester strains by placing small mycelial plugs in 10 µl droplets of tap water, 7 mm apart. Leaf discs were incubated at 15 °C with a photoperiod of 16 hours for two weeks after which the leaf tissue was decolourised in 70% ethanol at 50 °C and microscopically examined for the presence of oospores.

Aggressiveness assessment; bioassay

The bioassay comprised three experiments and each experiment involved thirteen isolates, i.e. twelve isolates from one of the three regions sampled, and the reference isolate I82001. The aggressiveness assay for each isolate was performed using three replicates, each replicate (experimental unit) consisting of 50 leaf discs of cultivar Bintje with a diameter of 14 mm. A single leaf disc was placed abaxial side up in each cell of two 25-cell replicate plates (Greiner no. 638102) supplied with 1 ml 2% water agar per cell. Leaf discs were cut by means of a cork borer, using only young full-grown leaflets of plants grown under the greenhouse conditions described before. Thus, a total of 5850 leaf discs were inoculated.

Table 3-1. Virulence and mating type of 36 isolates of *Phytophthora infestans* originating from three different potato growing regions in the Netherlands.

Southern Flevoland			Allotment gardens Ede			Drenthe		
Isolate	**Race**	**Mating type**	**Isolate**	**Race**	**Mating Type**	**Isolate**	**Race**	**Mating Type**
F95100	1.3.4.7.10.11	A1	I95004	3.4.10.11	A1	I95117	3.4	A1
F95104	1.3.4.10.11	A1	I95018	1.2.3.4.5.7.10.11	A1	I95130	3.4.7.10.11	A1
F95130	1.3.4.7.10.11	A1	I95023	1.3.4.5.6.7.10.11	A2	I95131	1.3.4.6.7.10	A2
F95162	1.2.3.4.7.11	A1	I95024	1.4.7.10.11	A2	I95138	1.1	A1
F95301	1.2.3.4.6.7.10.11	A1	I95032	3	A1	I95146	1.3.7.10.11	A2
F95305	1.3.10.11	A1	I95039	1.3.4.7.8.10.11	A2	I95150	1.3.4.7.10.11	A1
F95516	1.3.4.7.10.11	A1	I95050	1.3.4.5.7.8.10.11	A2	I95155	3.4.7.10.11	A1
F95528	1.3.4.7.10.11	A1	I95052	1.3.4.5.6.7.8.10.11	A2	I95161	1.3.4.7.8.10.11	A1
F95555	3.4.7.11	A1	I95054	4	A2	I95175	1.3.4.6.7.8.10.11	A1
F95573	1.3.4.7.10.11	A1	I95057	1.3.4.7.8.10.11	A2	I95181	1.3.4.6.7.8.10.11	A2
F95587	1.2.3.4.6.7.10.11	A1	I95062	1.3.4.7.10.11	A1	I95188	1.3.4.6.7.10.11	A2
F95624	1.2.3.4.10.11	A2	I95066	1.3.4.7.11	A1	I95197	1.4.10.11	A1

Reference isolate I82001, race 1.2.3.4.5.6.7.10.11; A2 mating type.

[1]) Physiological race determined with R-gene differentials R1 to R11.

[2]) Mating type assessed after pairing of the isolates with tester strains 80029 (A1) and 88133 (A2) on leaf discs of cultivar Bintje.

Inoculation was performed by placing a 10-µl droplet of a sporangial suspension of 2.0 x 10^4 sporangia ml^{-1} at the centre of each disc. The plates with the inoculated leaf discs were placed in plastic trays, which then were enclosed in a transparent polythene bag to avoid desiccation. The trays were placed in the dark in a climate chamber at 15 °C. After 24 hours, droplets were removed with the help of filter paper and incubation was continued at 15 °C and 16 hours photoperiod for 8 days. Readings started 72 hours after inoculation and were repeated with intervals of approximately 12 hours. Leaf discs were examined for the presence or absence of sporangiophores until sporulation was present. The fraction of sporulating leaf discs for each experimental unit was calculated. In addition, a random sample of 6 sporulating leaf discs was taken from each experimental unit (18 discs for each isolate) when those discs had been sporulating for 72 hours. These samples were subsequently checked for sporulation density. The sporangia were released from the sporangiophores by shaking the leaf discs in 1 ml of Isoton II electrolytic buffer (Coulter Electronics Inc.), supplemented with 1% formaldehyde, using a vortex mixer. After dilution, spore density was measured in three sub samples of 0.5 ml by means of a Coulter counter.

Aggressiveness assessment; statistical analysis

All statistical analyses were performed using Genstat 5 version 3.1 (Payne *et al.*, 1993). For each experimental unit the fraction of sporulating leaf discs was fitted to the Gompertz growth model to find parameter estimates. Three growth-defining parameters were calculated from the curve equations. The latent period (LP10) was calculated as the period measured in hours after inoculation in which 10 % of the total sporulating discs became apparent. In addition, LP50 and LP90 values were calculated but appeared to be less informative and therefore excluded. The maximal curve growth rate (MGR) was calculated as the first derivative of the fitted curve at the point of inflexion. This parameter is a derived measure for the speed with which the maximum number of infections was achieved. The infection efficiency index (IEI) was calculated as the fitted asymptotic value of leaf discs with the pathogen sporulating after 8 days. Sporulation intensity (SPOR) was calculated as the natural logarithm of the average number of sporangia per cm^2 infected leaf tissue, based on 18 affected leaf discs per isolate. A composite aggressiveness index (AI) was calculated for each isolate using the formula AI = 1/LP10 x MGR x IEI x SPOR x 10^4. The aggressiveness index in this form combines information about infection efficiency, latent period, speed of appearance of sporulating lesions and sporulation density in a single value. This index presents the epidemiological potential of an isolate concerned. The multiplication factor 10^4 was introduced for scaling purposes. Similar pathogenicity/aggressiveness indices have been used for this and other pathogens (Crute *et al.*, 1987; Day & Shattock, 1997; Thakur & Shetty, 1993).

A mixed model analysis of variance was performed on the assessed parameters as well as sporulation intensity and aggressiveness index. Isolates within regions were assumed to be a random sample of the underlying population of genotypes of each

sampled region. Restricted Maximal Likelihood (REML) analysis was used to obtain estimates for variance components and mean values of the parameters concerned for the three regional populations of the pathogen. The ratio between the average estimated variance components due to phenotypic variation between isolates (σ^2_A) and random error components (σ^2_e) was used to examine whether significant phenotypic variation for a given component existed in the regional populations (Searle *et al.*, 1992). Wald tests (Payne *et al.*, 1993) were used for testing contrasts between fixed model terms. Spearman rank correlation coefficients were calculated between the assessed components LP10, MGR, IEI, SPOR, AI and mating type as well as the number of virulence factors.

Results

Phenotypic variation for the assessed aggressiveness components and the aggressiveness index was found to be present (Table 3-2). Remarkable differences in infection efficiency, latent period and maximal growth rate were detected when individual isolates were compared but the average values for the aggressiveness components of the regional populations were similar for most components except LP10 (Table 3-2). A significant difference ($P = 0.05$) between regional populations could only be detected for the latent period. On average, isolates originating from the allotment gardens at Ede showed a longer average latent period when compared to isolates from Drenthe and Southern Flevoland. Furthermore, results indicate that the average aggressiveness index of the Ede population is slightly lower compared to the other regional populations. A marginally significant difference ($P = 0.10$) was found when the t test approximation was used (Table 3-2). The more appropriate Wald test statistic did not detect significant contrasts.

The populations did not differ significantly for the MGR, as defined by the first derivative of the fitted curve at the point of inflexion, the infection efficiency index or sporulation density (Table 3-2). Substantial variation for traits related to aggressiveness was detected within the three regional populations of *P. infestans*. The estimated variance components for LP10, MGR, IEI, SPOR and AI are presented in Table 3-3. The ratio $\sigma^2_{average}/\sigma^2_e$ was calculated to measure the relative amount of variability due to phenotypic differences between the pooled group of isolates assessed. The phenotypic variation for all assessed aggressiveness components was found to be significantly ($P < 0.001$) larger than the matching random error component of variance (Table 3-3).

Rank correlation coefficients were calculated among the aggressiveness components, aggressiveness index, mating type and the number of virulence factors in order to determine whether associations existed between the parameters assessed (Table 3-4). As the sampled *P. infestans* populations from the three regions concerned did not seem to differ much either with respect to their average value of aggressiveness components nor components of variance, correlation's were calculated with the pooled data set of the three sampled populations.

Table 3-2. Estimated components of aggressiveness and composite aggressiveness index of *P. infestans* isolates originating from three different potato growing regions in the Netherlands.

		Component				
Region	**Isolate**	**LP10**	**MGR x 10^3**	**IEI**	**SPOR**	**AI**
Southern Flevoland	F95100	106.8	8	0.44	4.95	1.6
	F95104	107.1	16.2	0.77	5.16	2.7
	F95130	121.7	4.8	0.12	5.23	0.2
	F95162	89.3	16.2	1	5.59	10.3
	F95301	94.7	41.2	0.99	5.21	22.6
	F95305	110.6	22.5	0.72	5.16	7.6
	F95516	85.9	20.3	0.92	5.19	11.2
	F95528	81.5	24.5	1	4.83	14.5
	F95555	88.6	13.6	0.48	4.84	3.6
	F95573	84.4	15	0.97	5.01	8.6
	F95587	106.5	4.7	0.4	5.18	0.9
	F95624	130.1	5.8	0.3	5.12	0.7
	Average	**100.6**	**15.3**	**0.68**	**5.14**	**7**
Allotment gardens Ede	I95004	136.9	22.8	0.56	5.23	4.9
	I95018	113.4	34.8	0.77	6.27	14.8
	I95023	99	26.2	0.98	5.12	13.4
	I95024	92.1	2	0.17	5.16	0.2
	I95032	106.2	17.6	0.55	5.29	4.8
	I95039	94.7	19.5	0.8	4.85	8
	I95050	115.9	5.8	0.11	4.95	0.3
	I95052	98.2	18.7	0.74	4.94	6.9
	I95054	123.6	10.3	0.37	5.05	1.5
	I95057	116.7	5.5	0.25	5.07	0.6
	I95062	125.8	11.7	0.24	5.05	1.1
	I95066	144.5	18.3	0.72	4.87	4.4
	Average	**113.9**	**16.1**	**0.52**	**5.23**	**5.1**
Drenthe	I95117	92	23.4	0.97	5.51	13.6
	I95130	85.7	28.3	0.98	5.46	17.7
	I95131	115.3	5.1	0.18	4.78	0.4
	I95138	109.2	4.4	0.15	4.87	0.3
	I95146	102.2	21.7	0.83	5.06	9
	I95150	96.5	9.6	0.34	5.44	1.8
	I95155	91	4.4	0.31	5.26	0.8
	I95161	81.2	31.4	0.99	5.38	20.7
	i95175	100.8	17.4	0.69	4.91	5.9
	I95181	119.6	45.5	0.78	4.89	14.4
	I95188	87.4	22.6	0.96	4.85	12.1
	I95197	77.2	27.8	0.95	4.87	16.7
	Average	**96.5**	**20.1**	**0.68**	**5.17**	**9.5**
Reference strain	I82001	109.7	10.7	0.71	4.72	3.6
SED	Average	6	4.5	0.13	0.12	2.6

[1] LP10 = latent period, MGR = maximal growth rate, IEI = infection efficiency index, SPOR = sporulation intensity, AI = aggressivity index; AI = 1/LP10 x MGR x IEI x SPOR x 10^4.

Table 3-3. Variance components of aggressiveness components using Restricted Maximal Likelihood (REML) estimators. Variance components are based on a sample of 12 isolates from three regional populations of *P. infestans*.

	Component[1]				
Region	**LP10**	**MGR x 10^5**	**IEI x 10^3**	**SPOR**	**AI**
Drenthe	158	10.3	110.6	0.08	52.2
Flevoland	224	14.7	97.4	0.03	44.2
Ede	261	7.8	81.1	0.14	21.6
$\sigma^2_{average}$[2]	214	10.9	96.4	0.08	39.3
σ^2_e[3]	60	3.8	6.1	0.03	8.2
$\sigma^2_{average}/\sigma^2_e$	3.6*	2.9*	15.8*	2.9*	4.8*

*) variance ratio significantly ($P < 0.001$) larger than one, based on a F distribution with 33 and 72 degrees of freedom respectively.

[1]) LP10 = latent period, MGR = maximal growth rate, IEI = infection efficiency index, SPOR = sporulation intensity, AI = aggressivity index; AI = 1/LP10 x MGR x IEI x SPOR x 10^4.

[2]) Average aggressiveness variance component.

[3]) Random error variance component.

Table 3-4. Spearman rank correlation coefficients among aggressiveness components, composite aggressiveness index, mating type and the number of virulence factors for isolates sampled.

	Component[1]				
	LP10	**MGR**	**IEI**	**SPOR**	**AI**
MGR	-0.33*				
IEI	-0.61**	0.81**			
SPOR	-0.12	0.14	0.13		
AI	-0.55**	0.95**	0.93**	0.17	
MT	-0.11	-0.01	-0.03	-0.19	0
VIR	0.16	-0.04	-0.05	-0.34*	-0.03

*) denotes a significant (unequal to zero) correlation at $P = 0.05$ with 37 degrees of freedom when applying the *t* approximation method.

**)denotes a significant (unequal to zero) correlation at $P < 0.01$ with 37 degrees of freedom when applying the t approximation method.

[1]) LP10 = latent period, MGR = maximal growth rate, IEI = infection efficiency index, SPOR = sporulation intensity, AI = aggressivity index; AI = 1/LP10 x MGR x IEI x SPOR x 10^4.

A negative correlation was found between the latent period and the maximum growth rate ($P \approx 0.05$) and infection efficiency index ($P < 0.01$). A positive correlation was calculated between maximum growth rate and infection efficiency index ($P < 0.01$). Mating type was neither correlated with any of the aggressiveness components

assessed, nor with race complexity. A small, yet significant (P = 0.05) negative rank correlation was found between race complexity and sporulation density.

Discussion

The bioassay used to estimate the components of aggressiveness in *P. infestans* isolates is characterised by an extremely low level of environmental variation. Accurate measurement of aggressiveness under controlled conditions is very useful in ecological and genetic studies since it by-passes many problems encountered when measuring relative fitness between isolates under field conditions (James & Fry, 1983; Tooley & Fry, 1985).

From our data, it can be concluded that a large amount of variation for aggressiveness to the foliage is present in regional *P. infestans* populations. Regional populations of *P. infestans* differed significantly for the latent period but not for maximal growth rate or infection efficiency. Isolates from the allotment garden complex at Ede showed a longer average latent period compared to isolates originating from Drenthe and Southern Flevoland. Only a marginally significant difference was found for the composite aggressiveness index, the latter difference could be assigned, to a large extent, to the difference in average latent period. The difference in latent period between regions might be explained by the presence of "tomato-adapted types" (Legard *et al.*, 1995) at Ede, whereas isolates from the other two regions were "tomato-unadapted".

Variation for aggressiveness seems evenly distributed over regional populations of the pathogen since variation in aggressiveness is present amongst isolates in each regional population, whilst no significant differences in average variance components between regional populations were detected. The presence of high levels of variation for aggressiveness in local populations of *P. infestans* is most likely to be credited to the occurrence of sexual reproduction. The aggressiveness related factors, latent period, maximum growth rate and the infection efficiency index were correlated and a small, but significant association (-0.34) was found between sporulation density and race complexity. However, the relevance of the association between spore production and race complexity is doubtful since the value of the calculated rank correlation coefficient is rather small. It is therefore concluded that no clear indications for fitness costs related to the accumulation of unnecessary virulence factors are present as no association between race complexity and aggressiveness was found to be present. Our study, in concordance with many previous studies, did not find evidence to support the concept of "stabilising selection" (van der Plank, 1968).

No association was found between any component of aggressiveness or the aggressiveness index and mating type. The absence of a specific association between mating type and aggressiveness in the Dutch population of *P. infestans* might be explained by the presence of sexual reproduction of the pathogen in the Netherlands (Drenth *et al.*, 1994). The process of random assortment during meiosis guarantees that any association between mating type and aggressiveness will be broken after a few

cycles of sexual reproduction, provided that this association is not based on close genetic linkage.

Large differences for aggressiveness were found to be present between isolates. These factors are clearly associated with parasitic fitness (Nelson, 1979), as they influence the relative ability of a genotype to persist successfully over time. Aggressiveness is typically involved in epidemiological success, a case of short-term parasitic fitness. However, short-term reproductive success is not necessarily equivalent to long-term parasitic fitness, which reflects the ultimate reproductive success and survival over a longer period of time. For asexually reproducing populations of *P. infestans* in the Netherlands, hibernation in infected tubers during the crop-free season is the sole mechanism for survival and, consequently, a principal factor involved in long-term parasitic fitness (Shattock, 1977). As components of aggressiveness to the foliage were not found associated with either the rate of tuber infection or the successive invasion of tuber tissue (Flier *et al.*, 1998), long-term reproductive success and survival appears to be a complex trait, being influenced by many biological and environmental factors. The relative importance of oospores as an alternative means of survival in partially sexual populations of *P. infestans* is still not well established (Andrivon, 1995). Viable oospores of *P. infestans* were collected from allotment gardens in 1992 and from farmers' fields in the starch potato region in Drenthe in 1993, 1994 and 1998 (Turkensteen *et al.*, 2000). These observations, combined with observed mating type ratios and the amount of variation in aggressiveness present in regional populations of the pathogen suggest that oospores play a considerable role in late blight epidemics in the Netherlands. Sexual reproduction also serves as the prime process for recombination of existing genetic variation for parasitic fitness, thus leading to maximal variability and aggressive strains. However, whether the more aggressive isolates may also serve as superior mates for maximal oospore production is to be questioned.

Taking into account the small sample size in this study, the most aggressive strain is not necessarily present within this sample. The components of aggressiveness were found to be only weakly to moderately correlated (Table 3-4), which implicates that any combination of such components may occur in nature. Combining components with the highest aggressiveness values in a single strain of *P. infestans* appears to be possible. However, the discrepancy between short-term epidemiological success and long-term reproductive success and survival makes it extremely difficult to predict the possibility of adaptation of highly aggressive strains of *P. infestans* to partially resistant potato cultivars. Moreover, erosion of quantitative resistance is not the action of increased levels of aggressiveness against all cultivars, but based on differential interactions with certain components of resistance in the host (van der Plank, 1968).

Taking into account that we have only tested a limited sample of isolates from three regional populations, we conclude that large amounts of variation for aggressiveness are being maintained in regional populations of *P. infestans* in the Netherlands. Thus, adaptation of the pathogen to partially resistant potato varieties cannot be

excluded. Our knowledge about both short-term and long-term parasitic fitness, and their interactions with resistant hosts and the environment is poor. More information is needed to provide reliable model based predictions on the stability of partial resistance against late blight.

Acknowledgements

We thank Marieke Förch and Els Verstappen for carrying out much of the experimental work, Pieter Vereijken of the Centre for Biometry Wageningen for his invaluable help with the statistical analysis. The useful comments on the manuscript given by Prof. Rolf Hoekstra (WAU), Prof. William E. Fry (Cornell University), Dr. F. Govers (WAU) and two anonymous reviewers are highly appreciated. The Dutch Potato Industry and the Dutch Ministry of Agriculture, Fisheries and Nature Management (Programme DWK 337) supported this work.

Section II

4

Production, survival and infectivity of oospores of *Phytophthora infestans*

L. J. Turkensteen, W. G. Flier, R. Wanningen and A. Mulder

Plant Pathology (2000) **49**: 688-696

Summary

The formation of oospores of *Phytophthora infestans* in potato crops and volunteer plants under field conditions and in laboratory tests with leaf discs of potato cultivars differing in their level of race non-specific resistance was studied. Oospores were readily detected in blight affected tomato leaflets and fruits and in leaflets of field crops and volunteer potato plants. Oospores extracted from blighted potato leaflets yielded thirteen oospore derived progeny. Oospores were also produced following inoculation of leaf discs of eight potato cultivars expressing different levels of race-nonspecific resistance with a mixture of sporangia of A1 and A2 isolates. Highest numbers of oospores were produced in cultivars Bintje (susceptible) and Pimpernel (resistant), and the lowest in Nicola (intermediate resistance).

The relationship between lesions per leaflet and oospore incidence, influenced by varying A1:A2 ratios, was explored using a simple mathematical model and validated by comparing actual oospore production in leaflets with multiple lesions of the race-nonspecific resistant potato clone Lan 22-21 with the predictions generated by the model.

Survival of oospores was investigated after their incorporation in either a sandy or a light clay soil in buried clay pots exposed to the local weather conditions. Over six years these soils were regularly assessed for their infection potential using floating leaflets in a spore-baiting bioassay. Both sandy and clay soil remained infectious for 48 and 30 months when oospore contaminated soils were flooded. Infections of floating potato leaflets occurred within 84-92 hours and ceased 11 days. Soil samples remained infective if dried and re-flooded on two, but not more occasions.

Introduction

Late blight is considered to be a disease of re-emerging importance in potato and tomato crops worldwide (Fry & Goodwin, 1997). *Phytophthora infestans*, the causal organism of potato late blight, is heterothallic and produces oospores when compatible strains of opposite mating types A1 and A2 interact (Galindo & Gallegly, 1960). In central Mexico, the presumed centre of diversity of *P. infestans*, oospores are abundantly formed in the field and were found to be infective for up to two years (Niederhauser, 1991).

Before 1984, the A2 mating type was not found outside Mexico in spite of an extensive survey coordinated by the International Potato Center (CIP) during 1974 until 1977 on isolates from Costa Rica, Germany, Japan, Mexico, Peru, Sweden, The Netherlands and the USA (Turkensteen, unpublished). All European isolates tested were collected before 1976, the year in which a new population of *P. infestans* may have been introduced in Europe (Niederhauser, 1991).

Recent introductions of new strains into the United States and Western Europe, include the introduction of the A2 mating type (Spielman *et al.*, 1991; Drenth *et al.*, 1993b) and has given the pathogen the opportunity to reproduce sexually and, as a consequence, to produce oospores. However, the role of oospores in the epidemiology of late blight is poorly understood (Andrivon, 1995). Since the work of Drenth *et al.* (1995), only few efforts have been made to clarify the role, which oospores play in

initiating late blight epidemics (Pittis & Shattock, 1994; Andersson *et al.*, 1998). For example, it was not known whether oospore germination is confined to one single "burst" of zoospore release or whether germination takes place throughout a period of favourable environmental conditions.

This study describes observational and experimental studies on the presence of oospores in field crops, on the formation of oospores in relation to field resistance, and, during a six year period, survival and infection potential of oospores of *P. infestans* under field conditions.

Materials and methods

Sampling and sources of P. infestans

From 1986 till 1994 small experimental plots with R-gene differentials of potato and tomato were laid out to monitor the race structure of the *P. infestans* population at sites near Zeewolde, Renkum and Wageningen (all in the central part of the Netherlands). In September 1992, green fruits of tomato accession West Virginia (W.Va.) 63 (Gallegly, 1964), showing two or more lesions were collected from these plots and examined for the presence of oospores using several techniques, described below. In addition, blight affected leaflets and stems of potato cultivar (cv.) Bintje and tomato W.Va. 63 with two or more lesions were collected at the Wageningen, Renkum and Zeewolde site. In addition, blighted tomato fruits of various, unidentified cultivars were sampled at an allotment garden complex near Ede (Turkensteen *et al.*, 1996).

In September 1993 and 1994, late blight was commonly found in the starch potato growing area in the northeastern part of The Netherlands. From each of 20 commercial potato crops, five leaflets each with two lesions were collected each year. The incubated leaflets were non-destructively examined for the presence of oospores and oospore-derived offspring were established.

In 1994, the experimental potato clone Lan 22-21 (Turkensteen, 1993) was grown in an allotment garden complex near Ede. Lan 22-21 is an experimental CIP clone with a high level of race-nonspecific resistance (Table 4-3). At the end of September, leaflets of this clone with two or more distinctly visible lesions per leaflet were collected and examined for the presence of oospores.

In 1998, extremely high incidences of volunteer potato plants were observed in farmers' fields. High levels of late blight developed on these unprotected plants during the growing season. During July, August and September 6 such fields (3 wheat, 2 sugar beet and flax) were visited in the starch potato growing area. Leaflets with two or more lesions were collected and examined for the presence of oospores as described below.

Detection and viability of oospores in leaf tissues

Immediately after collection, every leaflet was placed in a 9 cm diameter Petri dish containing 10 ml 2% water agar. Leaflets were incubated in a climate chamber at 15

°C exposed to a 16 h photoperiod at a light intensity of 12 Wm^{-2} for 10-14 days. After a two-week incubation period at 20 °C in the light, the leaflets were kept for two weeks in the dark at ambient temperature to decompose. Then the leaflets were microscopically examined for the presence of oospores. Samples of affected leaf tissues were cleared by boiling them in 96% ethanol for 10 minutes, mounted in glycerine and microscopically examined.

Where oospore viability was to be maintained, the leaflets concerned were left to decompose for two weeks at 15 °C in the dark before microscopic observation. Oospore-derived offspring were obtained from these leaflets. For this purpose, twelve oospore containing leaflets were frozen overnight at –20 °C in order to avoid infection from mycelial fragments or sporangia and each leaflet subsequently homogenized for 60 seconds at 20,000 rpm using an IKA T20 homogeniser with S20 probe (IKA, Staufen, Germany). The homogenate was separated by means of a 100 and 50 µm filter and the oospore containing filtrate spread on 0.8% water agar plates containing ampicillin (200 mg l^{-1}) and PCNB (75 % WP, 67 mg l^{-1}) and incubated at 15 °C in the dark for 10 days. Viability of oospores was determined by collecting germinating oospores and transferring them to tuber slices of cv. Bintje cut from surface sterilized tubers. When sporulating mycelium was present after an incubation period of five to seven days at 15 °C in the dark, small pieces of mycelium were placed on the lower epidermis of leaflets of cv. Bintje placed with the abaxial side up in 9 cm Petri dishes containing 10 ml 2% water agar. Inoculated leaflets were kept in a climate chamber at 15 °C with a 16 hours light period by means of fluorescence tubes type 33 (12 W m^{-2}). Leaflets densely covered with sporulating mycelium were obtained after seven days of incubation. Oospore derived offspring was cultured and subsequently identified as *P. infestans* based on colony morphology of cultures grown on rye A agar (Caten & Jinks, 1968). Mating type was determined by pairing isolates individually with tester strains F80029 (A1 mating type) and F88133 (A2 mating type) on Rye A agar according to standard procedures (Forbes, 1997).

For long term storage, sporangium suspensions were prepared in a 15% Dimethylsulphoxide solution, transferred into 1.8 ml cryovials, cooled down to -40 °C at a rate of approximately 0.5 °C min^{-1} using a Neslab CC 60 II immersion cooler (Neslab Instruments Inc., Portsmouth, USA) and an alcohol bath and stored in liquid nitrogen.

Relationship between lesions per leaflet and oospore incidence

The relationship between ratio of A1 and A2 mating type isolates in samples from a location and the percentages of leaflets with multiple lesions in which oospores are formed was examined in isolates from allotment gardens at Ede in 1994. A total of 168 leaflets, each with two or more lesions were collected by the end of September and examined for the presence of oospores. Oospores were detected in 56 blighted leaflets.

In this case, the mating type ratio can be derived from the proportion of leaflets in which oospores are formed. Let p and q represent the frequencies of *P. infestans*

strains of A1 and A2 mating type respectively, ranging from 0.0 to 1.0. In the case of two lesions per leaflet, the probability of oospore formation is estimated as $2pq$ and the probability of absence of oospores as p^2+q^2. The probability of oospore production in leaflets with more than two lesions is $1-(p^n + q^n)$ in which n represents the number of distinct lesions per leaflet. The expected fraction of leaflets with oospores was calculated for 2 to 10 lesions per leaflet for a number of mating type ratios and the results of this simulation are presented in Figure 4-1.

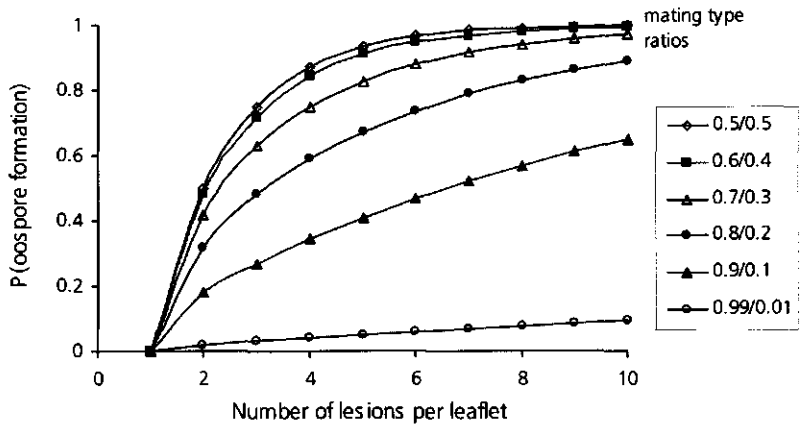

Figure 4-1. Probability of oospore formation by *Phytophthora infestans* in an infected potato leaflet based on the theoretical relationship between mating type ratios and the number of lesions per blighted leaflet. Data derived from the equation $P_{(oospore\ formation)}=1-(p^n + q^n)$ in which p and q represent the frequencies of *P. infestans* strains of A1 and A2 mating type and n represents the number of distinct lesions per leaflet.

These calculations were made under the following assumptions; 1) each individual fungal isolate in the allotment garden has an equal chance of establishing a new lesion (i.e. no founder-effects or preferential infection), 2) each pair of isolates with opposite mating type is able to form oospores. Even so, the described model can at its best only give an indication of the chance that oospores will be formed in infected leaflets. No inferences are made about the number of oospores being formed. The validity of the oospore formation model was evaluated by comparing actual oospore production in leaflets with multiple lesions of potato clone Lan 22-21 with the model-predicted probability of oospore formation.

First, the estimated proportion of oospore containing leaflets was calculated for 2,3 and 4 lesions per leaflet for a number of mating type ratios. These fractions were compared to the observed proportions and the goodness of fit for mating type ratios was determined by means of contingency tables in GENSTAT (Payne *et al.*, 1993) see Figure 4-2.

Oospore formation in race-nonspecific susceptible and resistant potato cultivars

The potential oospore production in late blight affected potato cultivars with different levels of race-nonspecific resistance was assessed in inoculated leaf discs of seven cultivars and one single breeding clone (Table 3). Leaflets were produced by planting seed tubers in 10 l plastic containers containing steamed potting soil and maintained in a greenhouse at 20/15 °C day/night temperature, 16 h light yielding a light intensity of 18 W m^{-2} and a relative humidity of 80%. Two isolates, F80029 (A1) and F88133 (A2) (Drenth *et al.*, 1995) were retrieved from liquid nitrogen storage and recovered on tuber slices of the susceptible cv. Bintje by incubation in the dark at 15 °C for 5 to 7 days. When sporulating mycelium was present, small pieces of mycelium were placed on the lower epidermis of leaflets of the same cultivar placed with the abaxial side up in 9 cm Petri dishes containing 10 ml 2% water agar, and maintained at 15 °C with a 16 hours light period at 12 Wm^{-2}. Sporangial inoculum was prepared by dipping sporulating inoculated leaflets in 10 ml of tap water, which was then filtered through a 50µ filter. Finally, sporangia were collected on a 15µ filter, re-suspended and the concentration adjusted to 2 x 10^4 sporangiospores ml^{-1} using a Coulter Counter Z1 (Beckman Coulter B.V., Mijdrecht, The Netherlands). Then the two inocula were mixed.

Leaf discs (14 mm diameter), from fully grown leaflets of the 4th or 5th leaf layer from the top of 8 to 10-week-old plants were placed abaxial side up in 9 cm diameter Petri dishes filled with 10 ml of 2% water agar. Four replicates of three leaf discs per cultivar were inoculated with 10 µl droplets of the mixed A1/A2 sporangial suspension at the centre of each disc. Dishes with the inoculated leaf discs were placed in plastic trays, enclosed in transparent polythene bags to inhibit desiccation and incubated for 14 days (15 °C) at a light intensity of 12 Wm^{-2}, 16 h light per day. The experiment was repeated.

Leaf discs were clarified in ethanol, as described previously and mean numbers of oospores per disc were assessed using bright field illumination. An oospore index was defined in terms of oospore formation using a scale from 0 to 13: 0 = 0 oospores cm^{-2}, 1 = 1-5 oospores cm^{-2}, 2 = 6-10 oospores cm^{-2}, 3 = 11-50 oospores cm^{-2}, 4 = 51-100 oospores cm^{-2}, 5 = 101-500 oospores cm^{-2}, 6 = 501-1000 oospores cm^{-2}, 7 = 1001-2000 oospores cm^{-2}, 8 = 2001-3000 oospores cm^{-2}, 9 = 3001-4000 oospores cm^{-2}, 10 = 4001-5000 oospores cm^{-2}, 11 = 5001-10,000 oospores cm^{-2}, 12 = 10,000-15,000 oospores cm^{-2}, 13 = > 15,000 oospores cm^{-2}. Analysis of variance was performed by Fisher's least significant difference test at the 5% probability level using GENSTAT 5 version 3.2.1 (Payne *et al.*, 1993). The relationship between the level of race-nonspecific host resistance and oospore production, expressed as an index value, was evaluated by means of a polynomial regression model.

Oospore survival under field conditions

Oospores, formed in potato leaves after inoculation with a mixture of isolates F80029 and F88133, were mixed with river sand or light clay (825 oospores per cm^3 of soil) and

put in 800 ml clay pots as previously described by Drenth *et al.*, (1995). Pots were buried in a field on November 9th, 1992. From November 16th 1992 until June 10th 1998, soil infectivity was assessed by means of the spore baiting bioassay (Drenth *et al.*, 1995). Pot contents (approximately 800 cm^3) were placed in a freezer (-20 °C) for 24 h in order to eliminate contamination by sporangia or mycelium of *P. infestans*. After thawing, the soil was transferred to a plastic container with a transparent lid and mixed with 2 L of tap water. After two days of incubation at 15 °C at a light intensity of 12 Wm^{-2}, 16 h per day, 15-20 greenhouse-grown leaflets of cultivar Bintje were floated abaxial side up on the water and incubated for 2 weeks. Developing lesions were counted and from which isolations were made by placing pieces of infected leaf tissue under potato tuber slices which were incubated at 17-19 °C for 5 days until sporulation appeared. An inoculation needle was used to transfer sporangia from the tuber slice to Rye A agar (Caten & Jinks, 1968) supplemented with ampicillin (200 mg l^{-1}), Benlate (50% WP, 100 mg l^{-1}), PCNB (75% WP, 67 mg l^{-1}), polymixin B (50 mg l^{-1}) and rifampicin (20 m l^{-1}) and incubated at 20 °C for 1-2 weeks. Subsequently, small pieces of selective medium containing actively growing *P. infestans* hyphae were transferred to Rye A agar plates. Isolates were maintained on Rye A agar at 20 °C and subsequently stored in liquid nitrogen.

Table 4-1. Incidence of oospores of *Phytophthora infestans* in blighted green tomato fruits, September 1992.

Source	Cultivar	Number of tomato fruits	
		with blight	with oospores
Wageningen, Gelderland	W.Va. 63	12	8
Renkum, Gelderland	"	7	2
Zeewolde, Flevoland	"	7	0
Allotment gardens, Ede	Moneymaker and others	9	2

Infectivity of oospore contaminated soils

The minimum response time for oospores to germinate in flooded soil, release zoospores and produce sporulating lesions on floating leaflets was established and the longevity of soil infectivity after flooding was also determined. For this purpose, 10 clay pots containing sandy soil containing oospores (as described above) were taken at random on 28 February 1995. These soil samples had been exposed to local weather conditions since November 1992. Ten simultaneous spore-baiting bioassays were initiated (as described earlier) to test the infection potential of a sample of 200 g sandy soil. For this purpose, 20 leaflets of cultivar Bintje were placed on the water surface at the start of each bioassay and replaced by fresh ones twice a day during the first 10 days of the experiment. After 10 days, the leaflets were replaced every 5 days until day 25. Each of the exposed leaflets was placed in separate Petri dish and incubated at 15

°C and 16 h light (12 Wm^{-2}) per day. After 5-7 days, the leaflets were examined for the presence of lesions.

In order to determine the germination potential of oospores in a sandy soil, a sequential soil infectivity test was performed by alternately flooding and drying the soil samples. A sample of ca. 500 g sandy soil from the survival experiment was used. The experiment started at October 28th, 1994 (day 0) with bioassay 1. At day 3 of bioassay 1, 20 leaflets of cultivar Bintje were placed on the water surface and were subsequently replaced by new ones every 3 days. The exposed leaflets were incubated and examined as described above. On November 16th, bioassay 1 was terminated and the water was poured off. The soil was air dried at room temperature (15-20 °C) and then divided into three equal soil portions. One portion (A) was frozen at -20 °C for 24 h in order to eliminate viable sporangia and zoospores, which might still be present in the soil sample. The other soil samples (B and C) were stored at 5 °C and subsequently frozen (-20 °C) before the following sequential bioassay was initiated. Bioassay 2 started on November 25th when 1.5 L water was added to soil sample (A). Bioassay 2 was terminated on December 8th, as new lesions ceased to appear. The water was poured off and the soil of sample (A) was air-dried and h on December 21st frozen for 24 hours. The second sequential bioassay (3) with soil sample A started at December 22nd. In total, the sequential testing procedure was carried out 8 times in 7 months. Soil sample A was tested in all sequential bioassays, sample B in assay No.: 1, 4, 5, 6, 7 and 8 while sample C was tested in assay No.: 1, 7 and 8.

Results

Oospore formation under natural conditions

In 1992, all four sites showed blight affected tomatoes and potatoes at the time when samples were collected. Oospores of *P. infestans* were detected in 12 out of 35 affected green tomato fruits collected at three sites in the central part of the Netherlands (Table 4-1). At the Wageningen site, oospores were found in 8 out of 20 leaflets of tomato line W.Va. 63 and in 4 out of 60 leaflets of cv. Bintje, showing two or more lesions per leaflet. An additional four oospore-containing tomato fruits were collected at Renkum and Ede. Oospores, however, were not observed in 310 infected stems of cv. Bintje with coalescent lesions sampled at Wageningen nor where they observed in potato and tomato leaflets with multiple lesions collected at Renkum and Zeewolde.

The following two years in the starch potato region in the Province of Drenthe potato leaflets with two blight lesions yielded 7 out of 98 and 5 out of 100 oospore containing leaflets, respectively. Oospores were extracted from each oospore-containing leaflet and oospore derived progeny was established. A total of thirteen *P. infestans* cultures could be established from two (1993) and one (1994) leaflet respectively. The A1:A2 ratio of isolates originating from each leaflet was 5:1 and 0:3 (1993 sample) and 1:3 (1994 sample), leading to a pooled A1:A2 ratio of 6:7.

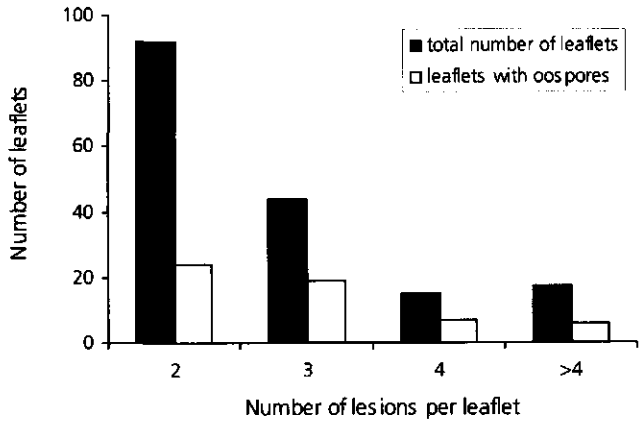

Figure 4-2. Incidence of oospores of *Phytophthora infestans* in naturally infected leaflets of clone Lan 22-21 with two or more lesions growing in an allotment garden at Ede in 1994.

In September 1994, oospores were found in leaflets with multiple lesions collected from potato clone Lan 22-21, in an allotment garden complex at Ede. A total of 168 leaflets with two or more lesions were examined for the presence of oospores. Oospores were found to be present in 56 leaflets (Figure 4-2). The percentage of oospore containing leaflets ranged from 26% to 47% for leaflets with two and four lesions respectively. However, no significant differences in the likelihood of oospore formation was observed when the probability of oospore formation for leaflets with either two, three, four or more than four lesions per leaflet were compared according to the contingency test ($\chi^2_{[3]} = 3.55$; $P = 0.314$) (Table 4-2).

Calculations based on the postulated relationship between lesions per leaflet and oospore incidence using the oospore incidence data from potato clone Lan 22-21 at Ede in 1994 predicted a mating type ratio at that location of 1:5, based on the goodness of fit test statistic ($\chi^2_{[2]} = 0.20$; $P = 0.906$). The actual (observed) mating type ratio in the same allotment garden complex in July 1994 was 1:3, based on mating type determination of 78 isolates.

Oospores were detected in 61% of the multiple-lesion carrying leaflets collected from volunteer potato plants in six commercial fields in 1998 (n=462) (Table 4-3). The percentage of leaflets with oospores present ranged from 33.3% to 87.0% for the localities of Velingerveen and Rolde respectively, the proportion of leaflets containing oospores being higher in the samples that were collected in September compared to the leaflets that were collected in July/August.

Table 4-2. Contingency test of observed and expected numbers of leaflets containing oospores of *Phytophthora infestans* in the race-nonspecific breeding clone Lan 22-21 growing in an allotment garden at Ede in 1994.

Lesions per leaflet	Total number of leaflets	Leaflets with oospores		Test statistic $(O-E)^2/E$
		Observed (O)	Expected (E)	
2	92	24	30.7	1.45
3	44	19	14.7	1.28
4	15	7	5.0	0.80
>4	17	6	5.7	0.02
Total	168	56	56	3.55[a]

[a] $\chi^2_{(3)} = 3.55$; $P = 0.314$ (n.s.).

Table 4-3. The presence of oospores of *Phytophthora infestans* in leaflets with multiple lesions sampled from volunteer potato plants in the starch potato region of the Netherlands in 1998.

Locality	Sampling date	Crop	Potato cultivar (volunteers)	Total number of leaflets	% leaflets with oospores
Muntendam A	July 29	Winter wheat	Karnico	101	35.6
Muntendam B	August 4	"	Karnico	54	35.2
Velingerveen	August 4	"	Kartel	15	33.3
Hooghalen	September 10	Flax	Elkana	109	68.8
Kooyenburg	September 10	Sugar beet	Florijn	106	76.4
Rolde	September 10	"	Florijn	77	87.0

Oospore formation in susceptible and partially resistant potato varieties

Oospores were detected in leaf tissue of all potato cultivars tested (Table 4-4). The highest numbers of oospores were observed in cultivars Bintje and Pimpernel, the lowest in Bildtstar, Nicola and Spunta. No significant difference in oospore production between the two experiments ($P = 0.281$) was observed and no significant cultivar x experiment interaction occurred ($P = 0.501$). Differences in oospore production between cultivars proved to be highly significant ($P < 0.001$). For example, cv. Pimpernel consistently produced large numbers of oospores (average oospore index 9.45) while cv. Bildtstar showed small numbers being produced (average oospore index 0.75). Except for cultivar Bintje, there seems to be a non-linear relationship between foliar resistance as presented by a 1 to 9 resistance rating (in which 1=very susceptible and 9=highly resistant) according to the Dutch National list of recommended potato cultivars (Ebskamp *et al.*, 1998) and oospore production. A polynomial model of the third order gave the best fit with significant associations between oospore production and the regression coefficients ($P < 0.004$). The fitted curve has the following equation:

oospore index = 29.3 - 15.9 x resistance_rating + 2.7 x resistance_rating^2 – 0.1 x resistance_rating^3. Only 59% of the variability of oospore production could be attributed to the foliar resistance rating ($R^2_{adjusted}$ = 0.593). The data for Bintje clearly represent outlying values for oospore production (Table 4-4).

Oospore survival under field conditions

Oospores remained infectious in the clay and sandy soil for up to 34 and 48 months after the start of the experiment respectively (Figure 4-3). The infection pattern, as assessed by means of the spore-baiting assay, was irregular and did not indicate a season-based pattern of inducible oospore germination. The sandy soil led to a higher incidence of infection compared to the clay soil. The infectivity of oospore-contaminated clay soil decreased faster than sandy soil with infectivity of clay and sandy soil showing highest infectivity after 7 and 28 months exposure to the prevailing field conditions, respectively.

Infectivity of oospore contaminated soils

The first blight lesions were detected by the floating leaflets bioassay at 84 to 96 hours after flooding the oospore-contaminated soil (Figure 4-4). In total, 21 lesions appeared on leaflets for up to 240 hours. The results obtained by the sequential testing of an oospore contaminated sandy soil by means of the floating leaflet bioassay are presented in Table 4-5. Soil sample A was tested 8 times within a period of 7 months. Infection became visible in bioassays 1, 2 and 4 but not in assays 3 and 5 to 8. High numbers of infections were found to be present during the second bioassay of soil samples B and C. When tested for the third time, both soil samples showed a dramatic drop in soil infectivity. None of the three soil portions was exhausted after one single spore-baiting experiment. Soil sample A remained infectious after 3 periods with frosts between 28 October 1994 and 17 January 1995.

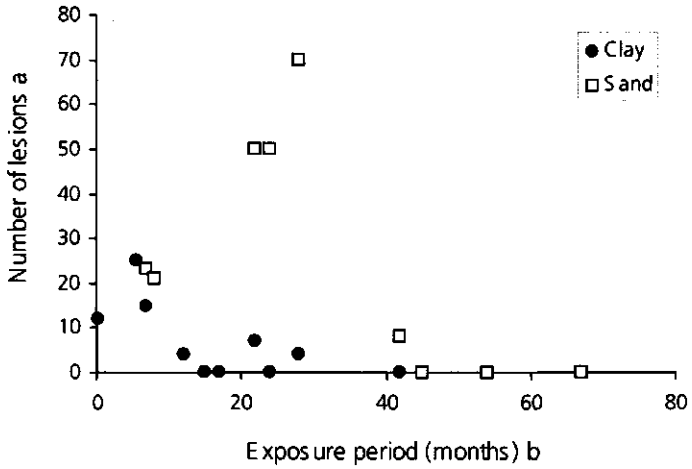

Figure 4-3. Infectivity of clay and sand soils contaminated with oospores of *Phytophthora infestans* over 6 years.

[a] Each data point presents the number of single lesions observed in a single spore-baiting, floating leaflet bioassay.

[b] eriod of exposure to local dutch climate conditions after start of the survival experiment in November 1992.

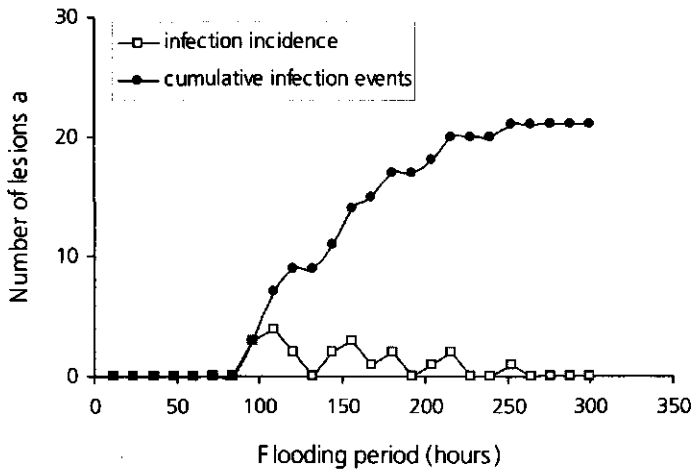

Figure 4-4. Cumulative mean number of infections by *Phytophthora infestans* on leaflets of cv. Bintje floating on a flooded oospore contaminated sandy soil.

[a] Number of lesions in floating leaflet bioassay. Each value represents the average of 10 bioassays.

Discussion

Viable oospores of *P. infestans* are being formed in field crops in the Netherlands. Both potato and tomato leaves serve as good hosts for the formation of oospores. However, no oospores were found in potato stems, which indicate that at the end of the crop development, oospores are mainly formed in leaf tissues.

Oospores were readily found when A1:A2 mating type ratios were in the range of 1:3 under field conditions. In commercial potato crops, A1 mating type percentages differed according to year and region from 99% to 30% (Drenth *et al.*, 1994; Turkensteen, unpublished). In a recent study, Cohen *et al.* (1997) showed that oospore formation is not significantly inhibited by skew mating type ratios. In their study, mixed A1/A2 sporangial inocula were used to infect leaflets of potato and tomato under controlled conditions. Therefore also at skew mating type ratios, oospores can be readily formed.

Table 4-5. Soil infectivity data obtained by a sequential testing procedure of a sandy soil artificially contaminated with oospores of *Phytophthora infestans* by means of a spore-baiting, floating leaflet bioassay.

	Lesion incidence in bioassay		
Bio assay (date)	**Soil sample A**	**Soil sample B**	**Soil sample C**
1 (94-10-28)	15*	15*	15*
2 (94-11-25)	8		
3 (94-12-22)	0		
4 (95-01-17)	2	38	
5 (95-02-07)	0	3	
6 (95-03-07)	0	0	
7 (95-04-07)	0	0	41
8 (95-05-15)	0	0	10

* All three-soil samples were tested together in bioassay 1. In total, 45 lesions were detected in this first bioassay.

Based on the hypothesis that more lesions per leaflet will increase the probability of A1 and A2 strains being present on a single leaflet, thus facilitating oospore formation, one might expect to find a positive correlation between the number of lesions and incidence of oospores leaflets. The proportion of blighted leaflets of clone Lan 22-21 containing oospores actually increased from 26% to 47% for leaflets with two and four lesions respectively, but the contingency test statistic provided no evidence for the presence of a trend, based on the four distinguished lesion incidence classes. The relationship presented in this paper is based on assumptions that are not usually met under field conditions. It is not realistic to assume that each individual

fungal strain in the local 'isolate-pool' has an equal chance of establishing a new lesion on a random host plant, thus totally ignoring the spatial distribution of both isolates and hosts. Strong founder effects will strongly increase the possibility of a common ancestry for lesions on a single leaflet. The presented formula might perhaps prove more useful as guidance for breeding efforts to minimise the probability of oospore formation in infected potato crops. Reducing the number of lesions formed, i.e. selecting for an infection resistance component of race-nonspecific resistance will reduce the chance of oospore formation, even under situations in which A1:A2 ratios are close to 1:1.

The level of race-nonspecific resistance present in the cultivars tested could explain a considerable part (59%) of the observed variation in oospore production. Elguezabal (1993) provided more evidence for the presence of a positive association between race-nonspecific resistance and oospore production. In his study, Mexican potato cultivars and breeding lines were included. Resistant cultivars like Norteña and Tollocan showed a remarkable and significantly higher level of oospore production compared with the susceptible cultivar Alpha. In a recent study Hermansen *et al.* (2000) reported on the presence of oospores in leaves of potato cultivars with intermediate levels of late blight resistance from three locations in the southern part of Norway. These observations are supported by another recent report on oospore production in partially resistant potato cultivars (Hanson & Shattock, 1998). When whole plants and leaf discs of 10 cultivars were inoculated, numbers of oospores were highest in cultivars with medium levels of race-nonspecific resistance. No oospores were observed in highly susceptible cultivars Home Guard and Bintje after inoculation of field and tunnel-grown whole plants.

As postulated by Hanson & Shattock (1998), factors like methodology and choice of parental isolates and cultivars might strongly influence oospore production. This is supported by our observations that cvs. Bintje and Bildtstar, with comparable levels of race-nonspecific resistance, showed large differences in oospore formation in inoculated leaf discs.

The differences found in oospore production in response to levels of host resistance (Drenth *et al.*, 1995; Hanson & Shattock, 1998; this paper) suggest that oospore formation is also influenced by factors other than the level of partial resistance to which 59% of the variability was attributed. Oospore formation might be influenced by other factors as well e.g. sterol contents of the host. Oospore formation is promoted by sterols *in vitro* (Elliot, 1983) and considerable differences in sterol production between potato cultivars have been reported (Langcake, 1974). This factor might possibly explain very high numbers of oospores found in cvs Bintje and Pimpernel with low and high race-nonspecific resistance respectively. The practical significance of Bintje as a potentially dangerous cultivar in terms of oospore production cannot be neglected. Bintje is still widely grown in the Netherlands (90.000 ha or approx. 50 % of the acreage). Based on the oospore formation experiment and the 1998 survey in Drenthe, we conclude that the replacement of this susceptible cultivar by more resistant ones will

not automatically lead to a reduction in oospore formation in field crops. We would recommend that future potato breeding programmes include an assessment of oospore production in progenitors and advanced breeding materials.

Oospores from cross F80029 x F88133 exposed to local weather conditions survived up to 48 months. Oospore survival time may vary; Pittis & Shattock (1994) reported much shorter survival periods, but longer periods may also be possible as longevity might depend on the parents of the cross and the conditions in the soil. For example, in our work after four periods of 25 days of flooding soil samples no more oospores appeared to be viable. In a trial at our laboratory, all oospores germinated within two months when stored at 15 °C in water in a beaker glass (unpublished results). It appears that the water content of soils may have a major impact on longevity. No season-based germination pattern could be discovered. Though germination peaks were recorded, the general conclusion may be that oospore germination in soil appears to be a rather erratic process. At present, all prerequisites are present for oospores to play an active role in the epidemiology of *P. infestans* in the Netherlands. Both mating types are commonly found in field crops, and as a consequence, oospore formation in field crops has become a reality. As oospores of *P. infestans* can survive up to four years, they may form a source of initial inoculum for most common crop rotation schemes.

Acknowledgements

We thank Dr. Andre Drenth for providing isolates and the oospore contaminated soils. We thank Dr. Richard Shattock, Dr. Hans van der Beek, Dr. Nyckle Fokkema and Prof. Rolf Hoekstra for their valuable comments. The Dutch Potato Association supported this work.

5

Formation, production and viability of oospores of *Phytophthora infestans* from potato and *Solanum demissum* in the Toluca Valley, central Mexica

W. G. Flier, N. J. Grünwald, W. E. Fry and L. J. Turkensteen

Mycological Research (2001): in press

Summary

Aspects of the ecology of oospores of *Phytophthora infestans* were studied in the highlands of central Mexico. From an investigation of a random sample of strains, it was found that isolates differed in their average capability to form oospores when engaged in compatible pairings. Most crosses produced large numbers of oospores but a few yielded none and some yielded only a few oospores. The results reveal that oospore production and fecundity is dependent on both isolates and the combining ability of a specific combination of parental strains. On average, 14 percent of the oospores produced were viable as determined by the plasmolysis method. Viability ranged from a low 1% in one cross to a high of 29% in another cross. Oospores were found in 10 – 20% of naturally infected *Solanum demissum* leaves from two different collections, and leaflets with two lesions per leaflet produced more oospores than did leaflets with three – five lesions per leaflet. There was no consistent trend for preferential mating between isolates from the same location or host.

Introduction

Phytophthora infestans (Mont.) de Bary, the causal organism of late blight in potatoes, is a heterothallic oomycete with two compatibility groups, referred to as mating types A1 and A2 (Shaw, 1996). Oospores are produced when two compatible fungal strains of opposite mating types interact (Galindo & Gallegly, 1960). In central Mexico, the presumed centre of origin of *P. infestans*, both mating types are present in approx. equal frequencies (Goodwin *et al.*, 1992a; Grünwald *et al*, submitted), and oospores are commonly found in potato crops (Niederhauser, 1956; Gallegly & Galindo, 1958). It is generally believed that each compatible cross between strains will yield oospores (Hohl & Iselin, 1984), although the ability of paired isolates to form oospores in both artificial media (Judelson *et al.*, 1995; Judelson, 1996a,b; Lee *et al.*, 1999) and host tissues (Mosa *et al.*, 1991) seems to vary among combinations of parental strains. The viability of oospores produced can vary considerably between crosses (Pittis & Shattock, 1994). There are several reports that describe the relative compatibility of Mexican strains of *P. infestans* (Shattock *et al.*, 1985; Shattock *et al.*, 1986; Spielman *et al.*, 1989; Spielman *et al.*, 1990; Al-Kherb *et al.*, 1995; Judelson, 1997). In addition, there are strong indications that there is diversity among isolates in their preference to serve as male or female parents (Judelson, 1997). However, most reports are based on experiments with a limited number of isolates. To date, only fragmentary data exist on the variation in mating ability, oospore production and viability present in *P. infestans* populations. A better understanding of the relative mating compatibility of isolates and the viability of the oospores produced is important in order to predict the relative importance of oospores in late blight epidemics in areas where A1 and A2 strains were only recently introduced.

The population of *P. infestans* in the Toluca Valley in central Mexico is considered to be the most diverse in the world (Tooley, *et al.*, 1985; Fry & Spielman, 1991; Goodwin *et al.*, 1992) and offers the unique opportunity to improve our

knowledge about the effect of a genetically diverse population on mating behaviour and oospore viability. To date, no information is available on presence and implications of isolation barriers on the production and viability of oospores of *P. infestans*. The presence of pre- or post-reproduction isolation could provide some support for the hypothesis of differentiation and eventually sympatric speciation (Kondrashov & Kondrashov, 1999) in local populations of *P. infestans* in central Mexico. Dieckmann & Moebeli (1999) recently presented theoretical evidence that assortative mating often leads to reproductive isolation between ecologically diverging sub-populations which in turn can lead to sympatric speciation. It has been stated that the speciation process can be accelerated by either resource use through host adaptation (strong selection for virulence genes in the pathogen to neutralise R-genes in *S. demissum*) or random genetic drift (Berlocher, 1998). It is likely that *P. infestans* populations in populations of *S. demissum* undergo severe bottlenecks at the end of each growing season, so it is to be expected that random genetic drift plays an important role in shaping populations of *P. infestans* with a very restricted size. The presence of several wild and cultivated host-species of *P. infestans* in central Mexico provides an excellent opportunity to test the hypothesis of sympatric speciation driven by ecological preferences in natural systems. Ordoñez *et al.* (2000) recently provided evidence for the presence of sympatric speciation in *P. infestans* populations. They reported on an Ecuadorian *Phytophthora* A2 population closely resembling *P. infestans* which appears to be strictly isolated from potato isolates by host-plant specificity.

Our objective was to study the ecology of oospores of *Phytophthora infestans* in the highlands of central Mexico. We evaluated oospore production, viability and mating ability in isolates collected from the native host-plant *Solanum demissum* and commercial potato crops in the Toluca Valley. In particular, we were interested in quantifying variation in mating ability, oospore production and oospore viability (using various viability tests) of *P. infestans* using *in-vitro* crosses. Isolates were collected in the Toluca Valley and along the slopes of the Volcano 'Nevado de Toluca' in central Mexico in 1997. Secondly, we assessed whether oospore formation occurs on wild *Solanum* species. A final goal was to test whether pre-reproduction or post-reproduction isolating mechanisms exist among isolates from different local populations of *P. infestans* in Toluca. Therefore, we compared oospore production and viability of isolates from three different populations of *S. demissum*.

Materials and methods

Source of isolates

All isolates except for the two reference strains (collected in 1996) were collected in the Toluca Valley in 1997. The selected strains represent three distinct geographical and host origins: (1) commercial potato production fields in the valley of Toluca, (2) small farmers' fields with local potato varieties of *Solanum tuberosum* (papa "criolla") on the

slopes of the volcano and (3) populations of wild *Solanum demissum* Lindl. occurring in pine forests on the Nevado de Toluca near the community of Loma Alta.

In the case of the valley and criolla survey, 20 potato fields were visited from which 212 isolates (ca. 10 from each field) were randomly sampled. For *S. demissum*, 66 isolates were obtained from three patches near Loma Alta. Reference strains Pic96001 and Pic96002 (Table 5-1), which had been isolated from local commercial potato crops, were kindly provided by Telesforo Zavala (INIFAP-Mexican National Potato Program). The isolates have been added to the PICTIPAPA-CEEM culture collection in Toluca, Mexico and are also maintained at Plant Research International, Wageningen, the Netherlands. Isolates are available for research purposes on request.

Isolation and culture

P. infestans strains were isolated from infected leaflets showing single lesions. Pieces of infected tissue adjacent to the sporulating region of the lesion were placed under potato tuber slices and incubated at 17-19 °C for 5 days until sporulation appeared. An inoculation needle was used to transfer mycelium from the tuber slice to Rye A agar (Caten & Jinks, 1968) supplemented with ampicillin (200 mg l^{-1}), Benlate (50% WP, 100 mg l^{-1}), PCNB (75% WP, 67 mg l^{-1}), polymixin B (50 mg l^{-1}) and rifampicin (20 mg l^{-1}) (Forbes, 1997). Petri plates (9 cm diameter) containing the Rye A selective medium were incubated at ambient temperature for 1-2 weeks. Subsequently, small pieces of selective medium containing actively growing *P. infestans* hyphae were transferred to Rye A agar plates. Isolates were maintained on Rye A agar at ambient temperature (20 ± 1 °C) with transfers every three to four months.

Mating type was determined by pairing Mexican isolates individually with tester strains Pic96001 (A1) and Pic96002 (A2) on Rye A agar according to standard procedures (Table 5-1) (Forbes, 1997).

Oospore production in 28 Mexican isolates

Variation in mating ability and oospore production for 28 Mexican isolates (Table 5-1) was assessed by pairing all 14 x 14, A1-A2 combinations. Crosses with parental isolates originating from different geographical locations were marked as inter-regional combinations while crosses in which both parental strains originated from the same geographical location were marked as intra-regional combinations. Parental isolates were transferred to Rye A agar plates and cultured for 10 days at ambient temperature (20 ± 1 °C). Agar discs (5 mm diameter) taken from margins of fast growing colonies of isolates of opposite mating types (one disc per isolate) were placed 30 mm apart in a Petri plate (6 cm diameter) containing 5 ml Rye A agar with 0.05 g l^{-1} ß-sitosterol to stimulate oospore production. Two plates were prepared for each parental combination. Plates were incubated for 15 days at ambient temperature in the dark. Presence of mate- and self-repulsion, mating region and oospore formation was determined for each plate. We define mate-repulsion as growth inhibition resulting in an un-colonised zone between parental colonies (Shaw, 1987; Shaw, 1991). When

these hyphal interactions are observed between neighbouring colonies of the same isolate, the phenomenon was referred to as self-repulsion. The presence of a mating region is marked by extensive stimulation of submerged hyphal growth at the interaction zone between the two parental strains, resulting in a distinct band visible by eye (Shaw, 1987).

Oospore production was estimated based on examination of 10 microscopic fields (0.2 mm^2 each) within the interaction zone of the two parental cultures (Smoot *et al.*, 1958). The zone in which oospores were formed was first located, and 10 microscopic fields were selected by random movement of the mechanical stage along this zone. The number of oospores in a cross-section from the agar surface to the bottom of the plate was quantified using an oospore production index (OPI). The OPI represents average oospore production in the mating region and is expressed in 4 classes: 0 = 0 oospores mm^{-2}, 1 = 1-50 oospores mm^{-2}, 2 = 51-250 oospores mm^{-2} 3 = 251-500 oospores mm^{-2}, and 4 = > 500 oospores mm^{-2}.

In a second experiment, oospore production and viability were studied in more detail to refine the results obtained in the main experiment and to compare three commonly used oospore viability tests. A subset of 8 isolates was randomly selected from the A1 and the A2 isolates used for the first experiment. Four agar discs (5 mm diameter), two of each parental isolate, were placed 40 mm apart on 10 ml Rye A agar amended with 0.05mgl^{-1} ß-sitosterol in 9 cm diam Petri dish. The agar discs from the same isolate were placed in the opposing corners so that the 4 discs formed a square. Three replicates were used.

Oospore production (OPI and oospore counts), viability and germination were determined. Mating regions of 21 days old cultures were excised using a scalpel, transferred to sterile 50 ml centrifuge tubes containing 9 ml sterile double distilled water. The agar was blended for 60 s at 20,000 rpm using an IKA T20 homogeniser with the S20 probe. The mean number of oospores was determined in three 50 µl aliquots, and the total number of oospores per mating event was calculated adjusting for the total volume of the homogenised sample.

A NovoZym 234 treatment was used to digest sporangia and mycelial fragments and to promote oospore activation and germination (Shaw, 1996). To monitor oospore viability prior to and after NovoZym 234 treatment, the plasmolysis and the tetrazolium test (Jiang & Erwin, 1990) were used. For the plasmolysis method, extracted oospores were suspended in 2 M NaCl solution for 3 hours. Plasmolysis was assessed by micoscopical observation of 150 oospores in three replicates. Viability is expressed as the percentage of oospores that were plasmolysed. For the tetrazolium test, oospore suspensions were dispersed in 0.1 M phosphate buffer containing 0.1% tetrazolium bromide (MTT). The suspensions were incubated for 2 days at 35 °C and stained oospores were subsequently examined microscopically for colour reactions according to Erwin & Ribeiro (1996). Rose oospores were considered to be dormant, while blue-pink oospores were assumed to be activated (ready to germinate). Unstained or black oospores were considered to be non-viable.

Table 5-1. Characteristics of the 32 Mexican *Phytophthora infestans* isolates used in the oospore production experiments.

Isolate	Host	Origin	Mating type	PEP	GPI	Metalaxyl resistance
Toluca Valley						
Pic97107	Potato c.v. Alpha	La Silva	A1	100/100	100/100	sensitive
Pic97111	Potato c.v. Alpha	La Silva	A2	100/100	86/100	sensitive
Pic97124	Potato c.v. Alpha	La Communidad	A2	100/100	100/122	sensitive
Pic97144	Potato c.v. Atlantic	Los Champignones	A1	92/100	122/122	intermediate
Pic97153	Potato c.v. Alpha	PICTIPAPA exp. fields	A2	100/100	100/100	intermediate
Pic97163	Potato c.v. Atlantic	El Cerrito	A1	92/100	86/100	sensitive
Pic97172	Potato c.v. Atlantic	El Cerrito	A2	78/96*	86/100	sensitive
Pic97175	Potato c.v. Atlantic	El Cerrito	A1	100/100	100/100	sensitive
Pic97196	Potato c.v. Alpha	Las Minas	A1	100/100	86/122	sensitive
Pic97236	Potato c.v. Atlantic	La Tolva	A2	100/100	86/100	sensitive
Nevado de Toluca						
Pic97301	Criolla (unknown)	Loma Alta	A1	100/100	86/86	sensitive
Pic97322	Criolla (unknown)	Loma Alta	A1	78/100*	100/122	sensitive
Pic97323	Criolla (unknown)	Loma Alta	A2	92/100	100/100	sensitive
Pic97333	Criolla (unknown)	Raices	A2	78/100*	86/100	sensitive
Pic97334	Criolla (unknown)	Raices	A1	92/100	100/111	sensitive
Pic97348	Criolla c.v. Rosita	Loma Alta	A1	100/100	100/100	sensitive
Pic97349	Criolla c.v. Rosita	Loma Alta	A2	100/100	92/100	sensitive
Pic97391	Criolla c.v. Marsiana	Loma Alta	A2	100/100	100/100	resistant
Pic97441	Criolla (unknown)	Buena Vista	A1	92/100	100/100	sensitive
Wild Solanum sp.						
Pic97701	*S. demissum*	Loma Alta, patch 1	A2	92/100	100/100	sensitive
Pic97709	*S. demissum*	Loma Alta, patch 1	A1	92/92	100/100	sensitive
Pic97711	*S. demissum*	Loma Alta, patch 1	A2	92/100	100/100	sensitive
Pic97728	*S. demissum*	Loma Alta, patch 2	A2	100/100	100/100	sensitive
Pic97731	*S. demissum*	Loma Alta, patch 2	A2	100/100	100/100	sensitive
Pic97735	*S. demissum*	Loma Alta, patch 2	A1	92/100	86/100	sensitive
Pic97743	*S. demissum*	Loma Alta, patch 3	A1	92/100	100/100	sensitive
Pic97746	*S. demissum*	Loma Alta, patch 3	A2	100/100	100/100	sensitive
Pic97750	*S. demissum*	Loma Alta, patch 3	A2	100/100	100/100	sensitive
Pic97754	*S. demissum*	Loma Alta, patch 3	A2	100/100	100/100	sensitive
Pic97757	*S. demissum*	Loma Alta, patch 3	A1	100/100	86/100	sensitive
Reference strains						
Pic96001	Potato c.v. Alpha	INIFAP	A1	100/100	100/122	sensitive
Pic96002	Potato c.v. Alpha	INIFAP	A2	100/100	100/100	sensitive

*) Tentatively assigned relative migration of a novel allele.

Germination of oospores was assessed using a plating technique described previously. A NovoZym 234 (Novo Biolabs) treatment was applied to lyse any mycelial fragments and sporangia in the suspension. A NovoZym 234 solution (50 mg NovoZym 234 per ml deionised water) was added to each of the crude oospore suspensions and incubated at 20 °C for 24 hours. After digestion, oospores were washed in three successive steps using 25 ml deionised water and re-suspended in 10 ml sterile deionised water. Oospore suspensions were then spread on sloppy water agar (5 g l^{-1}) and incubated at 20 °C under cool blue fluorescent light. Oospore germination was assessed after 14 days and expressed as percentage germination (Shaw, 1996).

Oospore production in wild Solanum species

Three populations of *S. demissum* were extensively monitored throughout the summer of 1997 for the presence of *P. infestans.* We defined a population here as a distinct group of *S. demissum* plants growing within a relatively small area (10-100 m^2) in a small valley on the slope of the Volcano Nevado de Toluca near Loma Alta. Population 1 consisted of approx. 500 plants of *S. demissum* growing in an open pine forest in the vicinity of an abandoned "Criolla" potato field. At the time of collection, 2-5% of the leaf area was infected by *P. infestans,* with most plants showing at least one sporulating lesion on lower leaves. Population 2 comprised approximately 250 plants and was located in the same valley separated from population 1 by at least 100 m of dense pine forest. Population 3 was located near a "Criolla" field under shrubs and bushes, at least 700 m distant from population 1 and 2 and consisted of approx. 200 *S. demissum* and 100 *S.* x *edinense* plants. Leaflets displaying multiple lesions were collected from two populations (1 and 2) of *S. demissum.* No leaflets with multiple lesions were found in population three during the 1997 field season.

Infected leaflets were examined for presence of oospores. Leaflets with multiple lesions were incubated for 6 days at ambient temperature on water agar (10 g l^{-1}) Petri dishes (9 cm diameter). Leaflets were then clarified in boiling ethanol (96% v/v) for 5 minutes, bleached in 1% NaHClO for at least 6 hours and mounted on microscope slides with glycerol. The entire clarified leaflets were examined for presence of oospores using a bright field microscope at a magnification of 10 x 10 and 10 x 40. The number of oospores per leaflet was counted.

Variation in fecundity of Phytophthora infestans isolates from Solanum demissum

Isolates of *P. infestans* collected in 1997 from three populations (as described above) of *S. demissum* were crossed to investigate the presence of reproductive isolation barriers by comparing intra- and inter-population variation in mating ability, oospore production and oospore viability. Isolates were collected on 11 September 1997. Crosses that involved isolates collected from different populations of *S. demissum* were marked as inter-population matings, while crosses involving isolates from the same location were marked as intra-population matings.

Five A1 isolates were mated with nine A2 isolates, including mating type tester strains Pic96001 and Pic96002. All crosses were performed according to the method described above using two replicates. Oospore density was determined by counting the number of oospores in ten randomly selected microscopic fields. Viability of oospores was assessed using the plasmolysis method.

Statistical analysis

All statistical analyses were performed using the Genstat 5 version 3.4.1 statistical package (Payne *et al.*, 1993). The presence of isolate specific effects and cross specific effects, analogous to the concept of general combining ability (GCA) and specific combining ability (SCA) in plants and livestock (Pooni *et al.*, 1984; Falconer & Mackay, 1996; Conner *et al.*, 1998) was investigated, using OPI as a estimation of oospore production. The OPI was $\log_e$-transformed since variance increased with means. Analysis of variance (ANOVA) was conducted to determine the relative importance of GCA and SCA on oospore production and viability. The experimental design and data analysis were adopted from Falconer & Mackay (1996). In the ANOVA the main effect of isolate is considered to be an estimate for GCA while the interaction effect of specific parental combinations resembles SCA. The significance of both factors GCA and SCA was tested using Fishers' F test. Associations between the presence of a mating region, mate-repulsion and oospore production in *in-vitro* crosses and independence of the frequency of oospore production in wild *S. demissum* leaflets were evaluated using contingency tables. A log-linear regression model was fitted to the counts. The test of independence was based on the chi-square approximation (Payne *et al.*, 1993). Spearman rank correlation coefficients were calculated for the various oospore production and viability parameters.

Results

Oospore production in 28 Mexican Phytophthora infestans isolates

In vitro pairings of A1 and A2 isolates from commercial potato fields, Criolla fields and populations of *S. demissum* led to production of oospores in all parental combinations tested, with the exception of pairings in which isolate Pic97301 (A1) was involved. Oospores were only produced in 4 out of 13 pairings when isolate Pic97301 was included as one of the parents (Table 5-2). In some mating combinations, only a few oospores were produced. No self-fertile isolates were observed.

In most parental combinations, a characteristic mating region was present at the interaction zone between two isolates. The presence of oospores, absence of sporangiophores and high density of both surface and sub-surface hyphae characterise this region. The presence of a mating region was found to be strongly associated with production of oospores ($P < 0.001$). Mate repulsion was observed in a few crosses, but appeared to have little effect on oospore production.

Table 5-2. Average oospore production index (OPI) values (0 = 0 oospores mm^{-2}, 1 = 1-50 oospores mm^{-2}, 2 = 51-250 oospores mm^{-2} 3 = 251-500 oospores mm^{-2}, 4 = > 500 oospores mm^{-2}) for *in vitro* matings between Mexican A1 and A2 isolates of *Phytophthora infestans*, at the interactionzone between the two isolates.

Isolates	***A1 isolates***														
A2 isolates	***Pic97107***	***Pic97144***	***Pic97163***	***Pic97175***	***Pic97196***	***Pic97301***	***Pic97322***	***Pic97334***	***Pic97348***	***Pic97441***	***Pic97709***	***Pic97735***	***Pic97743***	***Pic96001***	***Average***
Pic97111	1	3	4	4	2.5	0	3	3	3.5	4	4	3.5	3	4	3.0
Pic97124	1	3	4	3.5	2.5	0	2	3.5	3	3	4	3.5	4	4	2.9
Pic97153	1	4	4	3	4	0	3	4	4	4	4	3	4	3.5	3.3
Pic97172	3	4	3.5	4	3.5	0	1	3	4	3	4	4	4	4	3.2
Pic97236	2	4	3.5	3	1.5	1	4	3.5	3.5	3	-	2.5	4	2.5	2.9
Pic97323	1.5	4	4	3.5	2.5	0	2.5	4	4	3.5	3	3	4	3.5	3.1
Pic97333	1	3	3	3	2	2.5	2.5	2.5	4	3.5	3.5	3.5	4	4	3.0
Pic97349	1	3	2	3.5	3.5	1	4	3	4	2	2	3	4	3.5	2.8
Pic97391	0.5	3.5	4	4	2	0	4	3.5	4	3.5	3.5	2,5	4	2.5	3.0
Pic97701	1.5	4	4	2.5	2.5	0	2	4	3.5	4	4	4	4	3.5	3.1
Pic97711	1.5	3.5	2	4	2	0	1.5	2	3	1	2	4	4	4	2.5
Pic97728	3	3.5	2	3	3.5	0	3.5	1.5	1	4	4	2.5	2.5	4	2.7
Pic97754	0	3.5	4	3.5	2.5	2	2	4	3.5	2.5	4	4	4	4	3.1
Pic96002	3	3.5	4	3.5	2.5	-	3.5	4	4	4	4	4	4	4	3.4
Average	1.5	3.5	3.4	3.4	2.6	0.5	2.8	3.3	3.5	3.2	3.5	3.4	3.8	3.6	3.0

- = not determined due to contamination

The average value for OPI was 3.0 and variability in OPI was higher for A1 isolates (ranging from 0.5 to 3.8) than for A2 isolates (ranging from 2.5 to 3.4) (Table 5-2). The greater OPI diversity for A1 isolates is mainly caused by two isolates (Pic97107 and Pic97301), which in general produced very few oospores. Differences in oospore production between combinations of isolates (Table 5-2) were significantly dependent upon parental isolates (GCA) ($P < 0.001$) and specific combinations of isolates (SCA) ($P < 0.001$) based on ANOVA. The GCA : SCA variance ratio was calculated as 1.765, indicating that the effect of general combining ability on oospore production is almost twofold to that of specific combining ability.

Average oospore production was compared for crosses in which both parental isolates originated from the same geographical region (intra-regional combination) and crosses in which parental isolates were collected from different geographical regions (inter-regional combination) (Table 5-3). Isolates from *S. demissum* tend to be better parents when mated with each other since intra-regional combinations led to a significantly higher average OPI value ($P < 0.05$) compared to inter-regional combinations (Table 5-3). No preferential differences in OPI were found for isolates originating from the Toluca Valley or the slopes of the Nevado de Toluca.

The oospore production data from the viability experiment were in agreement with the results obtained in the first experiment. Among the ten matings between Mexican isolates *of P. infestans*, the average number of oospores produced varied between 1.6 x 10^3 to 1.5 x 10^5 oospores per Petri dish (Table 5-4). Three matings involving isolate Pic97107 yielded low numbers of oospores in comparison with other crosses in the same experiment (Table 5-4). Both GCA ($P < 0.001$) and SCA ($P = 0.019$) effects were found to influence the number of oospores produced in a mating event although the GCA : SCA ratio of 43.4 indicates that the effect of general combining ability on oospore production is more important than specific combining ability, based on our results using 10 crosses. A Spearman rank correlation was calculated to compare direct oospore counting and OPI values. Oospore production data generated by both methods were highly correlated ($r= 0.82$; $P<0.01$), (Table 5-5). Therefore, OPI values are a useful tool for assessing oospore production.

Variation in oospore viability and germination was evaluated in ten in-*vitro* matings using tetrazolium bromide staining, the plasmolysis method and oospore germination as different measurements for oospore vitality. Oospore viability prior to the NovoZym treatment ranged from 0.0 to 75.3% based on the tetrazolium test, and from 0.0 to 49.5% for the plasmolysis method (Table 5-4). Digestion of sporangia and mycelial fragments with NovoZym 234 reduced oospore viability for each of the seven matings that yielded viable oospores. After NovoZym treatment, viability varied between 0.0 and 67.6%, based on the estimates obtained with MTT staining (Table 5-4), as only MTT was used both before and after the NovoZym treatment.

The percentage of viable oospores, based on the MTT test criterion, was consistently higher in every cross than estimates obtained by the plasmolysis method. However, both methods gave highly correlated estimates for oospore viability ($r= 0.94$;

P<0.01) (Table 5-5). The few oospores obtained from the crosses Pic97107 x Pic97111, Pic107 x Pic97333 and Pic97107 x Pic97754 were found to be non-viable, both with MTT and the plasmolysis test. No germination was observed after NovoZym treatment of the oospores produced by these crosses. These results indicate that no viable oospores were produced in these three crosses. The NovoZym treatment induced oospores to shift from a dormant into an activated stage in all seven crosses that yielded viable oospores but did not significantly affect oospore viability since no drop in viability, based on MTT staining, was observed after the NovoZym treatment (Table 5-4).

The percentage germination in the seven oospore progenies that yielded viable oospores (based on MTT and the plasmolysis criterion) varied between 0.0 and 11.0%. A positive relation was found between oospore viability (as measured by MTT, plasmolysis and germination) and the number of oospores produced in a mating (Table 5-5); rank correlation coefficients varied between 0.65 and 0.87, and all associations were found to be significant (P<0.01).

Table 5-3. Average oospore production index (OPI) values for inter- and intra-population crosses for the three regional populations of *Phytophthora infestans* tested. Statistical significance using the Fisher's protected least significant difference (LSD) means comparison method.

Origin	Type of mating		$LSD_{(0.95)}$	$F_{probability}$
	Inter-population crosses (n)	Intra-population crosses (n)		
Toluca Valley	3.00 (170)	3.04 (25)	0.37	0.814
Slopes of Volcano	3.01 (175)	2.90 (20)	0.40	0.577
Populations of *S. demissum*	2.96 (183)	3.58 (12)	0.51	0.016

Oospore production in Solanum demissum

Sampling of leaflets with multiple lesions revealed that oospore formation is occurring in *S. demissum*. Oospores of *P. infestans* were observed in clarified leaflets that were examined for presence of oospores using a bright field microscope. Oospores were detected in leaflets showing multiple lesions from both population one and two. Oospore distribution in blighted leaflets appeared to be under-dispersed when whole leaflets were examined, their presence often concentrated in a few hot-spots of oospore production, which we refer to as clusters. Oospores were detected in leaflets with 2 to 5 late blight lesions per leaflet (Fig. 5-1), and up to 4 distinct oospore clusters per leaflet were observed. No oospores were detected in leaflets with 6 to 12 *P. infestans* lesions. Oospores were found to be present in 5 out of 42 and 5 out of 26 leaflets from population 1 and 2 respectively (Figure 5-1). Presence of oospores in leaflets collected from population 1 and 2 did not differ significantly for the two populations assessed (P = 0.412). The number of oospores ranged from 10 to ~ 5000 oospores per cluster.

Table **5-4**. Oospore production and viability measurements in ten *in vitro* matings of *Phytophthora infestans* isolates of central Mexican origin.

Parental Isolates		Oospore production		MTT				Plasmolysis	Germination
A2 parent	A1 parent	Oospores mm^{-2}	Oospores $plate^{-1}$	% activated		% dormant		% viability	% germination
				NovoZym -	NovoZym +	NovoZym -	NovoZym +		
Pic97111	Pic97107	7.8	2164	0.0	0.0	0.0	0.0	0.0	0.0
Pic97111	Pic97348	650.5	101565	17.6	28.0	17.3	3.0	16.5	3.1
Pic97111	Pic97743	317.3	49950	5.4	14.6	5.9	0.5	4.7	0.0
Pic97333	Pic97107	22.3	1665	0.0	0.0	0.0	0.0	0.0	0.0
Pic97333	Pic97348	247.8	42180	10.5	19.8	9.2	1.7	10.0	1.7
Pic97333	Pic97743	120.3	9435	2.3	8.3	5.6	3.3	1.5	0.0
Pic97754	Pic97107	80.5	17760	0.0	0.0	0.0	0.0	0.0	0.0
Pic97754	Pic97348	483.0	29970	22.5	30.8	16.3	9.5	20.5	1.8
Pic97754	Pic97743	240.3	78255	9.3	19.0	9.6	1.7	12.3	7.4
Pic96002	Pic96001	550.2	148185	49.7	53.7	25.6	13.9	49.5	11.0
$LSD_{(0.95)}$		*125.1*	*21634*	*5.3*	*9.0*	*8.9*	*3.5*	*11.5*	*1.9*

Table 5-5. Spearman rank correlation coefficients of different measurements on oospore production and viability in ten matings between *Phytophthora infestans* isolates originating from central Mexico.

Components	Oospore production		Oospore viability						
	Oospores per mm²	Oospores per plate	MTT prior to NovoZym treatment			MTT after NovoZym treatment			Plasmolysis
			% dormant	% activated	% viable	% dormant	% activated	% viable	% viability in 2 M NaCl
Oospores.plate	0.82								
% dormant MTT NovoZym -	0.86	0.78							
% activated MTT NovoZym -	0.83	0.80	0.93						
% viable MTT NovoZym -	0.83	0.78	0.97	0.98					
% dormant MTT NovoZym +	0.65	0.52	0.87	0.82	0.87				
% activated MTT NovoZym +	0.86	0.83	0.93	0.95	0.96	0.76			
% viable MTT NovoZym +	0.85	0.81	0.94	0.97	0.97	0.82	0.99		
% viable Plasmolysis	0.87	0.83	0.91	0.94	0.94	0.79	0.93	0.94	
% germination	0.65	0.81	0.78	0.79	0.78	0.64	0.64	0.85	0.81

All correlation coefficients were shown to be significantly different from zero ($P < 0.01$).

Table 5-6. Oospore production (mm^{-2}) and viability of oospores, based on the plasmolysis test (%, in brackets), after *in vitro* pairing of eight A1 and five A2 Mexican isolates of *Phytophthora infestans*.

	A2 Isolates								
A1 isolates	**Pic97701**	**Pic97711**	**Pic97728**	**Pic97731**	**Pic97746**	**Pic97750**	**Pic97754**	**Pic96002**	**Average**
Pic97709	430	488	350	154	546	214	543	-	389
	(9.0)	(1.0)	(25.7)	(3.7)	(12.3)	(8.0)	(6.3)		(9.4)
Pic97735	863	743	620	244	257	254	298	327	451
	(19.7)	(3.7)	(29.3)	(22.0)	(22.0)	(7.0)	(11.3)	(13.7)	(16.1)
Pic97743	716	312	277	129	500	1091	260	216	437
	(25.3)	(4.7)	(-)	(26.0)	(16.7)	(10.0)	(22.7)	(3.7)	(15.6)
Pic97757	491	457	283	352	1018	915	425	492	554
	(11.3)	(2.7)	(11.0)	(11.0)	(18.3)	(18.3)	(8.7)	(8.7)	(11.3)
Pic96001	248	164	320	289	404	367	432	416	330
	(29.3)	(11.0)	(19.0)	(19.0)	(19.7)	(20.0)	(18.7)	(13.0)	(17.6)
Average	549	433	370	233	545	568	391	362	432
	(18.9)	(4.6)	(21.3)	(14.6)	(17.8)	(12.7)	(13.5)	(9.8)	(14.1)

Least significant difference ($LSD_{(P=0.95)}$) for oospore production = 240; Least significant difference ($LSD_{(P=0.95)}$) for viability = 4.45

Table 5-7. Average oospore production (oospores mm^{-2}) and oospore viability (number of parental combinationsin brackets) in inter-patch and intra-patch matings between isolates of *Phytophthora infestans* obtained from *Solanum demissum*. Statistical significance was evaluated using Fisher's least significant difference method (LSD). The F-tests apply to inter- versus intra-population oospore production for each origin. Data shown after back-transformation.

Origin	Oospore production				Oospore viability			
	Inter-patch (n)	Intra-patch (n)	$LSD_{(P=0.95)}$	$F_{probability}$	Inter-patch (n)	Intra-patch (n)	$LSD_{(P=0.95)}$	$F_{probability}$
S. demissum Pop. 1	429 (26)	459 (2)	254	0.819	14.57 (27)	5.00 (2)	6.52	0.004
S. demissum Pop. 2	431 (26)	432 (2)	254	0.955	13.43 (27)	25.67 (2)	6.36	<0.001
S. demissum Pop. 3	384 (22)	757 (6)	144	<0.001	13.75 (27)	15.78 (6)	4.21	0.332

Variation in fecundity of Phytophthora infestans isolates from Solanum demissum

The number of oospores produced by pairing four A1 and eight A2 isolates originating from three populations of *S. demissum,* varied between 154 and 1091 oospores mm^{-2} (Table 5-6). Both GCA and SCA contributed to the observed variation in oospore production ($P < 0.001$). Preferential mating of isolates originating from the same population in terms of numbers of oospores produced was observed in only one out of three populations (Table 5-7). Isolates sampled from population 3 showed a two-fold increase in oospore production ($P < 0.001$) when mated with strains originating from that same population (Table 5-7) compared to matings with isolates from other populations. Average oospore production in population 3 was 384 and 757 oospores mm^{-2} for inter-population and intra-population matings respectively.

Considerable differences in oospore viability based on the plasmolysis test were present between both inter- and intra-population crosses. On average, 14.1% of the extracted oospores showed plasmolysis. Viability (based on the plasmolysis test) ranged from 1.0 to 29.3% with crosses Pic97709 x Pic97711 and Pic97735 x Pic97728, respectively (Table 5-6). Isolates originating from population 1 produced significantly more viable oospores ($P = 0.004$) when crossed with isolates from either population 2 or 3. On average 14.57 versus 5.00% viable oospores for inter-population and intra-population matings were produced, respectively (Table 7). Isolates originating from population 2 produced more viable oospores when involved in intra-population crosses ($P < 0.001$). On average, 3.43 versus 25.67% viable oospores were produced in inter-population and intra-population matings, respectively (Table 5-6). No differences in oospore viability in isolates originating from population 3 were observed between Intra- and inter-population matings (Table 5-6). No consistent trend indicating the presence of preferential mating or post-reproductive isolation barriers was found.

Discussion

All 28 isolates of *P. infestans* collected in central Mexico in 1997 were capable of mating with one or more isolates of the opposite mating type. Isolates differed in their average capability to form oospores when engaged in compatible matings, and certain specific parental combinations produced more oospores than other parental combinations. Therefore, the variation present in oospore production could be explained by both general combining ability, i.e. isolate-specific effects and specific combining ability, i.e. combination-specific effects analogous to the concept of GCA and SCA in plants and livestock (Falconer & Mackay, 1996). Pittis & Shattock (1994) found a similar pattern of oospore production and viability for *P. infestans* isolates originating from the United Kingdom.

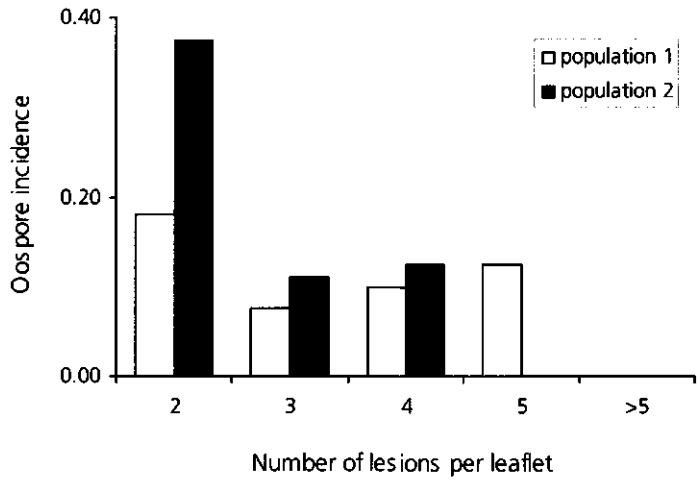

Figure 5-1. Incidence of oospores of *Phytophthora infestans* in infected leaflets of different *Solanum demissum* populations, showing 2,3,4,5 or more than 5 lesions per infected leaflet.

All parental combinations tested formed oospores with the exception of strains Pic97301 and Pic97107, which formed oospores with 4 out of 14 and 13 out of 14 compatible isolates respectively. Matings with isolate Pic97301 showed a clear mating region in all cases but this did not always result in oospore formation. The numbers of *in vitro* oospores produced in our experiments were in concordance with earlier reports (Smoot *et al.*, 1958; Pittis & Shattock, 1994) while low oospore viability in specific crosses has been reported by several authors including Shaw (1991), Shattock *et al.*, (1986) and Judelson *et al.*, (1995a,b). Our results provide an indication that oospore production and viability are related, as matings which yielded low numbers of oospores showed strongly reduced levels of oospore viability. These observations provide some evidence for the presence of sexual incompatibility or lethal factors in *P. infestans* strains leading to abortion and non-viable oospores (Erwin & Ribeiro, 1996). This is the first report of the presence of sexually or genetically induced incompatibility leading to diversity in mating success in crosses between A1 and A2 *P. infestans* strains in its centre of diversity in the Toluca Valley. Our results support the results obtained by Goodwin *et al.* (1992) who reported the presence of incompatible A1 and A2 *P. infestans* genotypes in the Los Mochis region in northern Mexico.

Differences in oospore production were observed between pairings of isolates collected from potato and *S. demissum*. Isolates originating from the wild host *S. demissum* tend to produce more oospores in crosses with compatible strains collected from the same host species than in crosses with isolates collected from potato. The observed differences in oospore production and viability among isolates from different *S. demissum* populations were not consistent.

We conclude that no indications were found suggesting the presence of pre-reproduction or post-reproduction isolating mechanisms among isolates from different local populations of *P. infestans* based on *in vitro* formation of oospores. The conclusion that no isolating mechanisms leading to accelerated sympatric speciation are present in *P. infestans* populations in the Toluca Valley is supported by recent allozyme analyses showing no significant sub-structuring among isolates from commercial potato fields, Criolla fields or native *Solanum* species (Grünwald *et al.*, 2000b). However, recent work based on RFLP and AFLP fingerprinting of isolates from the three different areas indicate restricted gene flow among populations from potato and *S. demissum* (Flier *et al.*, in prep.). In a recent study, Ordoñez *et al.* (2000) reported on an Ecuadorian *Phytophthora* A2 population closely resembling *P. infestans* which appears to be strictly isolated from potato isolates by host-plant specificity.

We observed formation of oospores in blighted leaflets of *S. demissum* in nature. Oospores were commonly detected in leaflets of *S. demissum* showing multiple lesions. The absence of oospores in leaflets with large numbers of lesions might be explained by the rapid decay of such leaflets, the time from infection to extended necrosis and decay being insufficient for oospore formation.

Support for the existence of a sexually reproducing population of *P. infestans* in central Mexico is traditionally based on the presence of the two known compatibility groups (Gallegly & Galindo, 1958; Smoot *et al.*, 1958) and oospores found in potato crops (Gallegly & Galindo, 1958). Despite earlier attempts (Gallegly & Galindo, 1958; Rivera-Peña, 1990c) to detect oospores in alternate hosts, the present study provides the first evidence for the occurrence of oospores in wild *Solanum* species from the Toluca Valley. Our results show that oospore formation in *S. demissum* is frequent in leaflets with 2 to 5 lesions. We did not determine how frequent leaflets with multiple lesions are in nature.

We did not find evidence for the presence of mating preference (as a form of assortative mating) in *P. infestans* populations from the Toluca Valley. Although inherent incompatibility between pairings of A1 and A2 strains of *P. infestans* was observed, no reproductive barriers based on geographical sub-structuring or host-plant specificity for *S. demissum* were detected. However, Ordoñez *et al.* (2000), provided evidence supporting the hypothesis that sympatric speciation, the origin of two or more new species from a single local ancestral population without geographical isolation (Kondrashov & Kondrashov, 1999), is possible in populations of *P. infestans.* In order to test the possibility of sympatric speciation within *P. infestans* in central Mexico, more detailed experiments investigating the presence of population sub-structuring due to host-plant specificity, including the role of self-fertilisation, oospore viability, *in planta* oospore formation and more alternative host-plant species, are needed.

Acknowledgements

The assistance of Alexei Smirnov and Edith Garay-Serrano is acknowledged. We thank Rolf Hoekstra, David Shaw and Anne Sturbaum for stimulating discussions and useful comments on the manuscript. Thanks to all at PICTIPAPA, ICAMEX and INIFAP for facilitating our research in Mexico. This work was conducted as part of module three of the International Co-operative Program for Potato Late Blight (PICTIPAPA) and was supported by grants from CEEM, the Dutch Potato Industry (HPA) and PICTIPAPA.

Section III

6

Differential interaction of *Phytophthora infestans* on tubers of potato cultivars with different levels of blight resistance

W. G. Flier, L. J. Turkensteen,T. B. M. van den Bosch, P. F. G. Vereijken and A. Mulder

Plant Pathology (2001): in press

Summary

Differential interactions in tuber blight attack between potato cultivars and *Phytophthora infestans* isolates were studied using whole tuber and tuber slice assays. Tuber blight incidence and severity was studied in a whole tuber assay while necrosis and mycelium coverage was evaluated in a tuber slice assay. The overall defence reaction of the potato cultivars tested varied considerably. Cultivars like Kartel and Producent showed resistant reactions while Bintje and (to a lesser extent) Astarte reacted more susceptible after inoculation with aggressive strains of *P. infestans*. A highly significant cultivar by year interaction was observed when tuber blight incidence was evaluated in two successive years. Differential responses occur as revealed by changing ranked order of cultivars after exposure to aggressive isolates of *P. infestans*. The results show that cultivar by isolate interactions existed for all components of tuber blight resistance studied. The quantitative nature of the observed resistance responses suggests the presence of Quantitative Trait Loci governing resistance to tuber blight. The consequences of differential interactions in relation to the stability of tuber resistance are discussed.

Introduction

Late blight of potatoes caused by the oomycete *Phytophthora infestans* (Mont.) de Bary is considered to be among the most important pathogens of potato crops worldwide. Yield losses caused by late blight can be attributed to reduced yields due to premature killing of vines and the infection of potato tubers; the latter leading to tuber rot in the field and in storage, which may be extensive. Worldwide crop losses and pesticide applications have been estimated to cost nearly 3 billion US Dollar annually (Mackay, 1996).

In Western Europe and North America, where *P. infestans* is being controlled by numerous applications of both protective and curative fungicides, commercial potato growers have recently experienced unexpectedly high tuber blight incidences that in some cases have led to severe yield losses in the United States (Kadish & Cohen, 1992; Fry & Goodwin, 1997). The increased problems with tuber blight control coincide with the displacement of the US1 clonal lineage by several more aggressive genotypes of the pathogen (Lambert & Currier, 1997; Peters *et al.*, 1999) and the presence of a variable population of *P. infestans* in Western Europe (Spielman *et al.*, 1991). Drenth *et al.* (1994) provided strong evidence for the presence of sexual reproduction of *P. infestans* in the Netherlands.

In reaction to the growing public demand for more sustainable forms of agriculture with less adverse effects on health and the environment, potato breeders have been increasing their efforts to breed potato cultivars with enhanced levels of late blight resistance. Potato cultivars with moderate to high levels of rate-reducing resistance have been shown to provide acceptable levels of disease control together with a substantially decreased requirement for fungicide applications (Fry, 1975). Traditionally, most emphasis is given to late blight resistance in the foliage. Until recently, most potato breeders (Umaerus & Umaerus, 1994) have not given high priority

to tuber blight resistance. It is common belief that high levels of foliar resistance will lead to reduced levels of inoculum to which the tubers will be exposed, and therefore minimise the risk of tuber blight (Toxopeus, 1958; Wastie, 1991).

It has been argued that aggressive, variable populations of the pathogen may affect the stability of field resistance (Nelson, 1979; Latin *et al.*, 1981) due to accelerated adaptation of aggressive forms of the pathogen with higher levels of parasitic fitness (Caten, 1974; Nelson, 1979). In addition, the sexual stage of the pathogen is supposed to have a considerable impact on the stability of field resistance. Besides an overall and equally decrease in average resistance scores of all cultivars due to the increased aggressiveness of *P. infestans,* differential responses might change the ranked order of cultivars. The latter effect could serve as an operational definition of instability of field resistance and is synonymous with the definition of erosion of field resistance according to Nelson (1979). The stability of tuber blight resistance might be affected in a similar way. Flier *et al.* (1998) studied the variation in aggressiveness to potato tubers present in populations of *P. infestans* in the Netherlands. Wide variation in aggressiveness to tubers was present in the three regional populations of the pathogen monitored.

To date, little is known about the way newly established strains, which are highly aggressive to the foliage, interact with tuber resistance. Bjor & Mulelid (1991) were the first to investigate the possibility of erosion of resistance to tuber blight. In their study, they presented evidence that adaptation of specific isolates to tubers of potato cultivars might lead to erosion of tuber blight resistance.

Late blight resistance in tubers can be attributed to three major components (Pathak & Clarke, 1987). The first defence barrier consists of several layers of phellem cells, known as the periderm. When intact, the periderm provides an absolute barrier to infection. Tuber infection can only take place through buds, lenticels or through cracks and wounds of the periderm. The second defence barrier is located in the peripheral layers of the cortex. The outer cortex cell layers may retard the growth of late blight lesions and can, in extreme cases, completely block hyphal growth. The third defence barrier is located in the storage tissues of the tuber, known as the medulla. This resistance component is marked by reduced hyphal growth and sporulation of *P. infestans.*

To obtain more detailed data on the stability of the various defence mechanisms we studied the pathogenicity of several aggressive isolates of *P. infestans* on potato tuber tissues of a range of potato cultivars that reportedly varied in tuber resistance. Whole tuber and tuber slice inoculation experiments were performed which enabled us to estimate defence reactions in the periderm, cortex and the medulla. Knowledge of the relative importance of the various defence barriers and the correlation's between them will lead to a better understanding of blight resistance in tubers, which will facilitate future breeding programmes. In addition, the order of magnitude of differential interactions between cultivars and isolates was determined in order study the impact of different aggressive isolates on the stability of tuber blight resistance.

Materials and methods

Cultivars

Nine potato cultivars were selected for their foliar and tuber blight resistance ratings according to the 73rd Dutch list of varieties of field crops (Table 6-1), including existing information on the presence of R-genes (Ebskamp *et al.*, 1998). All tubers for the inoculation experiments were grown from certified seed in a single experimental field of the Dutch Plant Protection Service at Assen. Standard crop management procedures for ware potatoes were followed during the growing season.

Isolates

Information on virulence factors, mating type and data concerning the collection of the isolates used is presented in Table 6-2. Five isolates, two representing the old population and three representing the new population of *P. infestans* in the Netherlands, were selected (Table 6-2). Isolate VK 6C is the representative of the old, clonal population and has been commonly used for tuber blight resistance testing including the evaluation for the Dutch list of cultivars (Flier *et al.*, 1998). The race 0 isolate also belongs to the US1 lineage and was selected in order to detect the expression of R-genes in tuber slice inoculations. Isolates F95573, I655-2A and I428-2 belong to the new, sexually reproducing population of *P. infestans* in the Netherlands and showed high levels of aggressiveness to tubers in previous preliminary experiments (unpublished data).

Culturing and inoculum preparation

Isolates taken from liquid nitrogen storage were first inoculated on tuber slices of the general susceptible potato cv. Bintje and incubated in the dark at 15 °C for 5 to 7 days. When sporulating mycelium was present, small tufts of mycelium were placed in a drop of water on the abaxial epidermis of leaflets of cultivar Bintje placed in 9 cm Petri dishes containing 10 ml 2% water agar. The inoculated leaflets were incubated for seven days in a climate chamber at 15 °C with a 16 hours light period (Philips fluorescence tubes type 33, intensity of 12 W m^{-2}).

Sporangial inoculum was prepared by dipping the leaves in 20 ml of tap water. The crude sporangium suspension was washed several times and spores were collected on a 15 µm nylon filter cloth. After re-suspension, the concentration was adjusted to 2.0×10^4 sporangia ml^{-1} using a Coulter Counter Z1 (Coulter Electronics Inc.) and kept for maximal 30 minutes at 15 °C until inoculation.

Table 6-1. List of potato cultivars used for tuber blight inoculation experiments.

Cultivar	Foliar blight rating[1]	Tuber blight rating[1]	Presence of R-genes[2]
Astarte	6.5	6	-?
Bintje	3	3	-
Elkana	5	8	+
Elles	7	7	-?
Florijn	5.5	5.5	+
Karnico	8.5	6	+
Kartel	8	6.5	+
Producent	6.5	8	+
Seresta	7	8	+

1) Resistance scores according to the 73rd Dutch List of Varieties of Field Crops (Ebskamp *et al.*, 1998), on a 1 to 9 scale where a low rating indicates high susceptibility.
2) Based on unpublished data and various literature sources.

Table 6-2. Information on the isolates of *Phytophthora infestans* used in the tuber blight inoculation experiments.

Isolate	Race	Mating type	Year of collection	Source
F95573[a]	1.3.4.7.10.11	A1	1995	Cull pile, Flevoland
I428-2[b]	1.2.3.4.5.6.7.8.9.10.11	A2	1992	Allotment garden at Ede
I655-2A[b]	1.2.3.4.5.6.7.8.9.10.11	A1	1992	Allotment garden at Ede
VK 6C[c]	1.4	A1	1958	Commercial potato crop, Drenthe
US1 race 0[d]	0	A1	<1970	Commercial potato crop, USA

[a] Isolate kindly provided by M. Zwankhuizen, Department of Phytopathology, Wageningen Agricultural University, the Netherlands.
[b] Isolates from the Plant Research International *Phytophthora infestans* collection.
[c] Reference isolate used for tuber blight resistance assessment in the Netherlands.
[d] Isolate kindly provided by W. E. Fry, Department of Plant Pathology, Cornell University, Ithaca, USA.

Whole tuber assay

Whole tuber inoculation experiments were performed in 1997 and 1998. Tubers of the selected potato cultivars grown on a sandy soil were harvested at the end of September by hand in order to minimise wounding and were immediately transferred to the laboratory and washed to remove adhering soil. Undamaged tubers, about 80 tubers per crate, were placed in plastic seed tuber crates with rose ends facing upward.

Three replicates per cultivar-isolate combination (one crate per replicate) were used whenever possible. In cases where there was a shortage of tubers, two crates

were used. Tubers received 25 ml of inoculum per crate sprayed with a low-pressure nozzle at a pressure of 300 kPa. Inoculated tubers were incubated in the dark at 15 °C for 14 days. During the first 24 hours RH was 98-100% RH to provide favourable conditions for tuber infection. After the first day, RH was kept at 80%. Individual tubers were visually examined for the presence of tuber blight symptoms and the percentage of infected tubers per crate was calculated as an indicator for the peridermal resistance component. The invasive ability of the isolates in relation to the cortical resistance component of the tubers was evaluated by means of an invasive ability index (IAI) (Flier *et al.*, 1998). Ten randomly selected diseased tubers per crate were cut longitudinally and disease severity was scored for each individual tuber using the following scale: 0 = no symptoms, 1 = < 2.5% of cut area with symptoms, 2 = 2.5- 10% of cut area with symptoms, 3 = 10-25% of cut area with symptoms, 4 = 25-50% of cut area with symptoms and 5 = > 50% of cut area with symptoms.

Tuber slice assay

The tuber slice assay was adopted from Bhatia & Young (1985) and Dorrance & Inglis (1998). Undamaged tubers of similar size were taken from 5 °C storage, eight weeks after harvest, surface sterilised in 5% sodium hypochlorite for 5 minutes, rinsed in tap water and wiped dry with Kleenex tissue paper. Two one-cm thick slices were cut from the central part of a single tuber and placed in 9 cm Petri dishes; one slice in each Petri dish. Each slice taken from a single tuber was randomly assigned to one of the two replicates that consisted of 10 slices each.

A single 10 µl droplet of inoculum was applied in the middle on the upper side of each individual slice. The inoculated tuber slices were incubated for 8 days in closed Petri dishes in an incubator at 15 °C and 85% RH in the dark. Individual slices were evaluated for the presence of necrotic tissue on the tuber slice surface as an indicator for the area of invaded tissue. In addition, the coverage with mycelium was estimated using a linear scale of 0 = no mycelium, to 9 = completely covered with mycelium. We assume that both necrotic tissue and mycelium coverage are features that are related to medullar resistance in potato tubers.

Statistical analysis

All statistical analyses were performed using GENSTAT statistical package, version 5.3.2 (Payne *et* al., 1993). The infection response in the whole tuber assay, which is presented by the percentage blighted tubers, was considered to represent a continuous proportion in the interval [0,1]. This response was analysed using a logistic regression model in which the relation between the probability of an infected tuber (p) and the explanatory factors was described as logit (p): Logit (p) = log (p/(1-p)) = constant + year + cultivar + isolate + all two-factor interaction terms.

In addition, it was assumed that the variance of the observed number of blighted tubers (Y) could appropriately be described by a variance proportional with

binomial variance var(Y) = φ np(1-p) where φ is the dispersion parameter and n the number of tubers assessed per replicate (McCullagh & Nelder, 1989).

Numerical estimation problems using the logit transformation arose when no blighted tubers were found to be present for all replications within a treatment. In such cases we therefore substituted a small positive value (0.2), which corresponds with the logit value of a mean number of 2 infected tubers in a sample of 800 inoculated tubers, to avoid unreliable parameter estimates for the corresponding means on the logit scale and unreliable standard errors.

The quasi-likelihood procedure in the GENSTAT statistical package was used to estimate model parameter values. Cultivar-by-isolate interaction on the logit scale was explored using Generalised Additive Main effects and Multiplicative Interaction effects (GAMMI) models (Van Eeuwijk, 1995, 1996). Analysis of the tuber infection data by means of GAMMI modelling was chosen because of the presence of several significant effects of two-factor interactions thus preventing the use of more easy interpretable analysis methods like interaction clustering. Biplots (Gower & Hand, 1996) were constructed to visualise cultivar by isolate interactions and facilitate the biological interpretation of the results.

IAI scores were analysed using Restricted Maximum Likelihood (REML) analysis. The main effects of isolate, cultivar, year and the interaction terms cultivar-by-isolate and cultivar-by-year were treated as fixed effects in the linear model. The relative contributions of fixed effects were evaluated using the Wald test of F-tests for the variance ratios in case of ordinary linear regression (Payne *et al.*, 1993).

Data from the tuber slice experiment were transformed (^{10}Log for mycelium coverage and square root for the necrotic tissue score) prior to analysis. Differences between treatments were studied by calculating a compatibility index (CI = mean percentage necrotic tissue x mean mycelium coverage). Differences in the fixed model terms cultivars, isolates and cultivars-by-isolates and interaction effects were evaluated using the Wald test.

Cultivar-by-isolate interaction for IAI scores and tuber slice data was structured by interaction clustering using the CINTERACTION procedure in GENSTAT (Corsten & Denis, 1990). Homogeneous groups were visualised by dendrograms derived from interaction clustering.

Results

Whole tuber assay

The eight potato cultivars tested showed remarkable differences in their defence response to the four *P. infestans* isolates in the whole tuber assay. Considerable differences in tuber blight incidence and severity were present between the two years of evaluation. Average values for invasive ability of the isolates and the general level of tuber blight resistance in the potato cultivars assessed for the two years are presented

in Table 6-3. Cultivar Bintje was marked by a high incidence of tuber infection and a deep penetration of the four isolates tested. The other cultivars were moderately to highly resistant to tuber infection and varied considerably with respect to their ability to retard the penetration of lesions of *P. infestans* in the outer cortical region (Table 6-3). Isolate F95573 caused the highest percent blighted tubers whereas isolate I655-2A was marked by a low percentage blighted tubers in the whole tuber assay (Table 6-3). Differences in mean IAI between the four isolates were relatively small, with isolates F95573 and I428-2 showing a higher invasive ability than I655-2A or VK 6C. In general, higher tuber rot incidences were assessed in the 1998 experiment.

Logistic regression analysis of the tuber blight incidences in the whole tuber inoculation experiments of 1997 and 1998 revealed highly significant interaction effects of year-by-isolate, year-by-cultivar and cultivar-by-isolate interactions ($P< 0.001$) (Table 6-3). Tuber blight incidence was therefore analysed for each separate year of testing. Logistic regression analysis performed on the 1997 data showed a highly significant ($P< 0.001$) cultivar-by-isolate effect. This cultivar-by-isolate interaction effect was also significant in the 1998 experiment ($P= 0.017$).

The two axes GAMMI model satisfactorily describes the differential interaction for tuber blight incidence between cultivars and isolates in both 1997 and 1998. The biplot of the 1997 data (Figure 6-1) reveals that the cultivars Producent and Florijn and the isolates F95573 and I655-2A are responsible for the largest contribution to the cultivar-by-isolate differential interaction. For 1998, several clusters of potato cultivars can be distinguished, based on the corresponding biplot (Figure 6-2). The cultivar-clusters: {Producent, Elles}, {Seresta, Astarte}, {Kartel, Florijn} and {Bintje, Elkana} showed different resistance responses after inoculation with the *P. infestans* isolates concerned. The highest contribution to the differential interaction can be attributed to the cultivars Seresta, Elles and Florijn and the isolates VK 6C and I655-2A.

Restricted Maximal Likelihood (REML) analysis revealed a highly significant cultivar-by-isolate interaction of IAI scores of the combined 1997 and 1998 experiments ($P< 0.001$). No significant year by isolate or year by cultivar interaction effect was found. Interaction clustering was applied to structure groups of isolates and cultivars with similar IAI and cortical resistance patterns. Five and four significantly different groups ($P=0.05$) are distinguished for cultivars and isolates, respectively (Figure 6-3). Cultivars Astarte, Bintje, Producent and Seresta showed a very similar behaviour in their cortical resistance responses and where therefore assigned to a single cluster. Four other clusters for cortical resistance response could be identified: {Elkana}, {Kartel}, {Elles} and {Florijn}. All four isolates showed a unique IAI pattern when tested with eight potato cultivars (Figure 6-3).

Table 6-3. Whole tuber response of eight potato cultivars to infection by four isolates of *Phytophthora infestans*.

Year	Cultivar	Isolate							
		F95573		I428-2		I655-2A		VK 6C	
		Blighted tubers (%)	IAI[1]	Blighted tubers (%)	IAI[2]	Blighted tubers (%)	IAI[2]	Blighted tubers (%)	IAI[2]
1997	Astarte	7.1	1.0	2.0	1.5	0.0	0	0.4	0.5
	Bintje	17.2	3.0	10.5	2.0	8.3	1.5	1.9	2.3
	Elkana	4.8	1.2	5.0	1.0	10.9	2.3	0.0	0
	Elles	6.7	2.0	1.4	0.3	0.5	1.0	0.9	1.0
	Florijn	0.0	0	0.5	1.3	1.3	0.7	1.7	1.2
	Kartel	0.0	0	0.0	0	1.7	0.7	0.0	0
	Producent	1.2	0.7	11.5	1.0	0.0	0	0.0	0
	Seresta	2.2	1.2	2.1	1.3	0.8	0.7	0.4	0.3
	Average	*4.9*	*1.1*	*4.1*	*1.1*	*2.9*	*0.9*	*0.7*	*0.7*
1998	Astarte	7.1	1.9	6.3	1.7	0.6	1.0	0.4	0.7
	Bintje	53.4	3.0	40.9	3.5	22.7	2.5	55.3	2.9
	Elkana	7.1	1.6	3.0	1.6	2.6	0.5	0.0	0
	Elles	6.6	2.2	2.6	2.2	0.0	0	2.7	3.1
	Florijn	0.0	0	1.3	0.5	3.5	0.7	0.9	2
	Kartel	0.0	0	0.0	0	0.4	0.3	0.0	0
	Producent	2.1	2.3	0.5	1.0	0.0	0	0.4	0.7
	Seresta	8.3	1.8	5.2	2.0	0.0	0	0.0	0
	Average	*10.6*	*1.6*	*7.5*	*1.6*	*3.7*	*0.6*	*7.5*	*1.2*

[1]Invasive ability index (IAI) scores (Flier *et al.*, 1998).

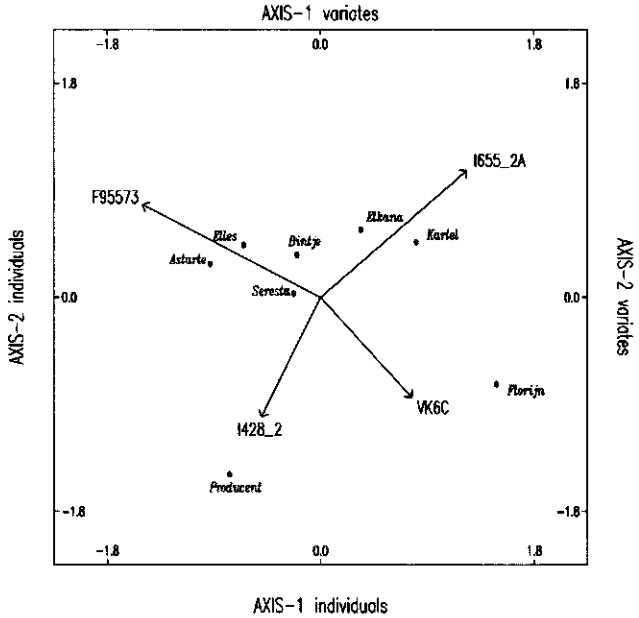

Figure 6-1. Biplot of the interaction in the tuber blight data in 1997 following from GAMMI-2 model with logit link and over-dispersed Binomial distribution. Cultivars are represented by dots ; isolates by lines.

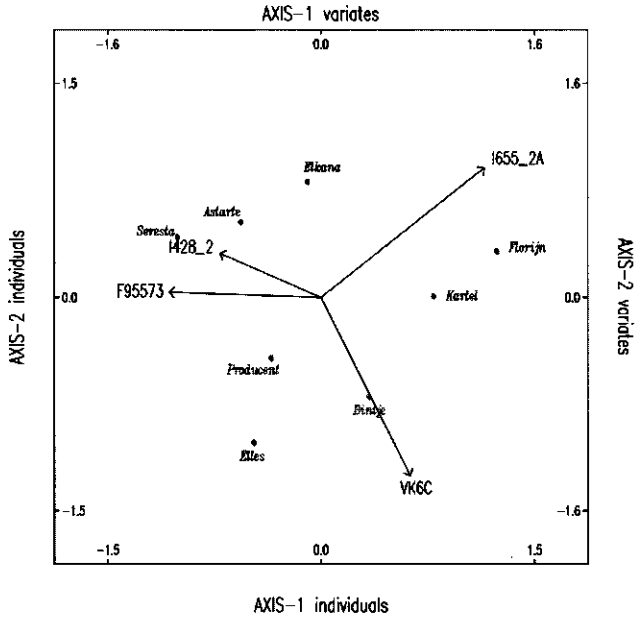

Figure 6-2. Biplot of the interaction in the tuber blight data in 1998 following from GAMMI-2 model with logit link and over-dispersed Binomial distribution. Cultivars are represented by dots ; isolates by lines.

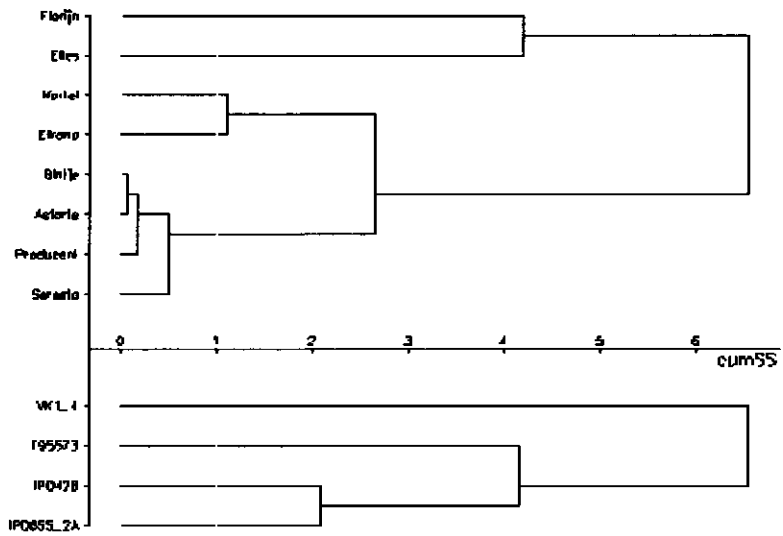

Figure 6-3. Interaction clustering by CINTERACTION (Corsten & Denis, 1990) of internal tuber rot symptoms as measured by the invasive ability index after inoculation of whole tubers of 8 potato cultivars with 4 isolates of *Phytophthora infestans*. The vertical line indicates a significance level at $P = 0.05$.

Tuber slice assay

All isolates induced necrosis on the upper surface of inoculated tuber slices of the nine potato cultivars tested (Table 6-4). For most cultivar-isolate combinations, mycelium and sporangia were readily formed.

Isolate race 0 failed to produce aerial mycelium on Astarte, whereas isolate I655-2A did not produce mycelium on Kartel and Astarte (Table 6-4). Cultivar-by-isolate differential interaction effects contributed significantly ($P<0.001$) to the assessed differences in the percentage necrotic tissue, mycelium coverage and the composite compatibility index.

Interaction clustering was performed in order to structure groups of isolates and cultivars with similar interaction patterns. Four cultivar-clusters; {Kartel, Florijn}, {Bintje, Elles, Producent}, {Elkana} and {Astarte, Karnico, Seresta} and four isolate-clusters; {race 0, I428-2}, {I655-2A}, {VK 6C} and {F95573} could be assigned based on the percentage necrotic tissue on the surface of inoculated tuber slices ($P=0.05$). For mycelium coverage, interaction clustering yielded an entirely different pattern. No less than six cultivar-clusters accounted for the cultivar by isolate interaction ($P=0.05$); {Astarte}, {Elkana}, {Bintje, Producent}, {Elles, Karnico, Seresta}, {Florijn} and {Kartel}. Four isolate-clusters could be assigned; {I428-2}, {F95573}, {VK 6C} and {I655-2A, race 0}.

Table 6-4. Tuber slice defence response of nine potato cultivars to infection and invasion by five isolates of *Phytophthora infestans*.

Cultivar	Isolate									
	F95573		I428-2		I655-2A		VK 6C		Race 0	
	% Necrosis	Index[1]	% Necrosis	Index[1]	% Necrosis	Index[1]	% Necrosis	Index[1]	% Necrosis	Index[1]
Astarte	66.8	4.3	36.5	2.6	5.8	0.0	15.6	1.0	6.1	0.0
Bintje	49.3	3.3	47.0	4.6	54.5	4.9	43.3	6.1	19.0	3.6
Elkana	82.8	5.0	46.5	5.7	20.5	0.1	67.5	3.7	23.0	0.3
Elles	82.3	4.3	84.3	3.7	76.8	5.3	62.3	3.8	61.0	3.9
Florijn	83.4	3.0	76.5	1.7	67.0	0.5	86.2	2.1	59.4	0.5
Karnico	72.8	5.5	48.0	2.9	50.0	3.8	43.3	4.9	34.5	4.1
Kartel	26.0	0.7	22.9	0.9	13.7	0.0	26.9	0.4	14.1	0.2
Producent	55.5	4.4	60.0	4.4	46.3	4.0	37.3	5.4	36.0	3.3
Seresta	58.8	0.2	35.0	0.0	20.4	0.4	16.8	0.3	13.9	0.2

Fishers LSD after back-transformation : % necrosis = 15.44, Index = 0.94

[1] Coverage with mycelium using a scale of 0 = no mycelium, to 9 = completely covered with mycelium.

Associations between components of tuber blight resistance

Associations between the assessed components of tuber blight resistance (percentage blighted tubers, cortical resistance, percentage necrotic tissue and mycelium coverage) and the overall levels of tuber and foliar resistance according to the Dutch list of varieties of field crops were evaluated by calculating Spearman Rank Correlation coefficients (Table 6-5). The percentage-infected tubers in the tuber assay (If_t) were highly correlated (r=0.88) with the invasive ability index (IAI_t). Both If_t and IAI_t were loosely, although significantly (r=0.36 and r=0.33 respectively) (P=0.05), correlated with mycelium density in the tuber slice assay (My_s). Surprisingly, no association was found between both If_t and IAI_t and the tuber blight resistance score in the National List (Tr_{nl}). A significant (P=0.05) negative association (r = -0.35) was found present between If_t and the foliar blight resistance score in the National List (Fr_{nl}).

The percentage necrotic tissue (Nc_s) and mycelium density (My_s), both assessed in the tuber slice assay, were positively correlated (P<0.01). No association was found between Tr_{nl} and both components assessed in the tuber slice assay (Table 6-5).

An overall measure of agreement for the assessed components of tuber blight resistance in the whole tuber assay and the tuber slice experiment was evaluated by means of the Kendall coefficient of concordance. The resulting concordance coefficient $W_{[If_t\ ,\ IAI_t\ ,\ Nc_s\ ,\ My_s]}$ was estimated at 0.56, indicating excellent agreement between the components assessed (P < 0.001). Agreement between the various measurements dropped sharply when foliar and tuber blight resistance scores were included in the analysis ($W_{[If_t\ ,\ IAI_t\ ,\ Nc_s\ ,\ My_s\ Tr_{nl}\ ,Fr_{nl}]}$ = 0.21; P = 0.121), indicating that little congruency exits between the components assessed in the bioassays and the resistance scores as presented in the Dutch National List.

Table 6-5. Spearman Rank Correlations of the various components of tuber blight resistance assessed in whole tuber and tuber slice experiments and foliar and tuber blight resistance rating according to the national list.

Component[1]					
	If_t	**IAI_t**	**Nc_s**	**My_s**	**Tr_{nl}**
If_t	1				
IAI_t	0.88**	1			
Nc_s	0.16	0.26	1		
My_s	0.36*	0.33*	0.46**	1	
Tr_{nl}	-0.27	-0.32	-0.13	-0.09	1
Fr_{nl}	-0.35*	-0.25	-0.20	-0.24	0.21

*) Denotes a significant correlation at P = 0.05 with 34 df

**) Denotes a significant correlation at P < 0.01 with 34 df.

[1]) If_t = percentage infected tubers in the tuber assay, Si_t = invasive ability index in the tuber assay, Nc_s = percentage necrotic tissue in tuber slice assay, My_s = mycelium density in tuber slice assay, Tr_{nl} = tuber blight resistance score in national list, Fr_{nl} = Foliar blight resistance score in national list.

Discussion

The periderm, cortical and medulla defence responses of tubers of the potato cultivars tested varied depending on the *P. infestans* isolate used in both the whole tuber and the tuber slice inoculation experiments. With cortical and medulla defence responses, large differences in susceptibility were observed among the cultivars tested, even for potato cultivars with reputed high levels of field resistance to tuber blight. The periderm resistance responses for cultivars were not the same in 1997 and 1998, as can be deduced from a highly significant cultivar by year interaction term. This phenomenon has been observed in earlier studies (Dorrance & Inglis, 1998; Pietkiewicz & Jellis, 1976; Stewart *et al.*, 1983, 1996).

Differences in tuber infections can be attributed to differences in periderm development (Lapwood, 1977), lenticel resistance (Zan, 1962; Lacey, 1967) and tuber maturity (Bjor, 1987). As the defence reaction of the tuber surface usually increases with maturity (Lapwood, 1977), experimental results using field or whole tuber inoculations will likely be influenced by the physiological condition of the tubers tested (Toxopeus, 1958).

The defence reaction located in the outer cortex and the medulla is thought to be less dependent upon the physiological age of the tuber during the first weeks in storage. However, it has been reported that the levels of both cortical (Pathak & Clarke, 1987) and medulla resistance (Bhatia & Young, 1985) appear to diminish towards the end of the storage period. Differences in maturity per cultivar may be held responsible for the observed cultivar by year interactions in the whole tuber experiments. Since all inoculations in this study were performed directly after harvest of the tubers, it is not likely that differences in cortical and medulla resistance responses are attributable to the effect of physiological ageing. In general, laboratory assays for testing tuber blight resistance are necessarily based on a compromise between the harvest date and the various ripening times of the cultivars assessed.

Resistance in the periderm and outer cortical region might be induced or attenuated by the action of R-genes (Toxopeus, 1958; Lapwood & McKee, 1961). Some reports suggest that potato cultivars with resistance gene R1 confer a strong hypersensitivity reaction in the cortical region after inoculation with incompatible isolates (Lapwood & McKee, 1961; Toxopeus, 1961). Other R-genes do not seem to inhibit the growth of *P. infestans* after tuber infection. Pathak & Clarke (1987) studied this phenomenon in more detail and concluded that many more cells are involved in the hypersensitive-like defence reaction in the cortical region, compared to the hypersensitive response in the foliage. In addition, inhibitors of respiratory metabolism do not seem to inhibit the defence reaction in the cortical region (Mucharromah & Kuc, 1995). As compared to the hypersensitivity observed in the foliage, this component of tuber resistance therefore appears to be based on a passive defence mechanism rather than an active hypersensitivity response.

Based on our observations, we conclude that it is very unlikely that the existing gene-for-gene pathosystem in potato (Black *et al.*, 1953; Malcolmson & Black, 1966) is

to be held responsible for the observed differential interaction in tuber infection. Both I428-2 and I655-2A are very complex races, which are compatible to all eleven identified R-genes in potato, yet their interaction patterns observed are very different for most components of tuber blight resistance assessed. Based on our knowledge on the presence of R-genes in the cultivars tested, incompatible interactions were to be expected for the cultivars Kartel and Producent in combination with isolate VK 6C and F95573. The very resistant response of Kartel to infection with complex *P. infestans* races suggests that this cultivar combines high levels of peridermal and cortical resistance. However, it cannot be excluded that the tuber resistance of Kartel is based on an unclassified R-gene.

All isolates were capable of infecting medulla tissue after drop-inoculations on tuber slices. The Race 0 strain used did not significantly cause less necrotic tissue compared with the complex strain I655-2A or strain VK 6C. However, both Race 0 and I655-2A seem to have a strongly reduced level of compatibility when inoculated on tuber slices of Astarte. Interaction clustering based on the percentage necrosis led to a clustering of isolates and cultivars with similar interaction patterns that was strikingly different from the grouping that was obtained when analysing mycelium coverage.

There appears to be two different genetic systems governing resistance against late blight in potato. Observed cultivar- and isolate-cluster discrepancies for the three components of tuber blight resistance suggest that the three components might be under different genetic control although associations between the defence components in the host were found to be present, suggesting that these components may not be completely independent. The presence of novel, putative R-genes or resistance genes with a more quantitative effect, which are only expressed in tuber tissues, can provide an explanation for the phenomenon of lesion arrest in the cortical region and reduced mycelium production in the medulla. The defence responses in the tuber could be under the control of putative Quantitative Trait Loci (QTL's).

The significance of cultivar-by-isolate interaction, which is presumably not based on the classical gene-for-gene system in potato, has several important implications for both plant breeding and integrated control of late blight epidemics. The use of a single *P. infestans* isolate in the selection process may lead to the selection of potato cultivars, which may not express the same level of tuber blight when exposed to field populations of *P. infestans*. The unpredictability of tuber blight resistance in the field, based on single-isolate evaluations, may interfere with the need for the selection of stable and durable sources of tuber blight resistance. In the United States and Canada, recently emerged clonal lineages of *P. infestans* have been shown to be more pathogenic to potato tubers compared to the US1 genotype (Lambert & Currier, 1997; Peters *et al.*, 1999). Flier *et al.* (1998) showed that variation for both tuber infection and invasive growth is present within local field populations of *P. infestans* in the Netherlands. Tuber blight resistance ratings as presented in various recommended lists of potato cultivars should therefore be used with caution. The selection for stable tuber blight resistance

should involve both inoculation studies and field evaluations with one or more isolates that ideally should be compatible to all "vertical" tuber resistance genes.

Combining stable tuber blight resistance with high levels of resistance in the foliage could be used to reduce the risk of tuber infection. After infection, partially resistant potato cultivars tend to produce inoculum during a longer period as compared to more susceptible cultivars, therefore extending the period during which tuber infections may occur (Toxopeus, 1958; Wastie, 1991). Tuber blight resistance can be used to minimise the risk of tuber infections during the growing season and at harvest, thus preventing yield loss. The use of tuber blight resistant potato cultivars will therefore also reduce the level of infected seed tubers at harvest. As a consequence, less blighted seed tubers will overwinter in storage, which will lead to a decrease of initial disease pressure in the following spring.

It has been argued that aggressive *P. infestans* strains which cause extensive rot in susceptible potato seed tubers may not survive the winter period, thus leading to a negative selection pressure upon aggressive strains of the pathogen (Shattock, 1976). The occurrence of differential interactions in tuber blight attack suggests that *P. infestans* strains that are compatible to the potato cultivars concerned might be selected during the infection and overwintering period, leading to increased survival rates of those adapted strains. Such a directional selection for adapted pathogen genotypes will decrease the general level of tuber blight resistance.

Our results provide strong indications that the stability of tuber blight resistance in potato is to be reconsidered. The presence of specificity between components of tuber blight resistance and pathogenicity of strains of the present *P. infestans* population has an impact on the stability of tuber blight resistance. The presence of differential interactions between host and pathogen for tuber infection and colonisation suggest that tuber blight resistance may itself is subject to erosion of resistance (*sensu* Nelson, 1979). The use of a limited set of potato cultivars and *P. infestans* strains in this study combined with the presence of a highly variable, sexually reproducing population of *P. infestans* in North West Europe makes it difficult to predict to which extend erosion of tuber blight resistance is to be expected under field conditions. As stated by Latin *et al.* (1981), widespread use of potato cultivars with high levels of blight resistance is likely to cause selection in the pathogen population for increased levels of pathogenicity to tubers. Accumulation of pathogenicity factors in *P. infestans* could therefore eventually lead to pathogen strains that are able to partially or completely overcome this type of host resistance. It is however unlikely that a complete breakdown of tuber blight resistance will be observed for all cultivars under field conditions, as many different potato cultivars, each with a specific combination of genes involved in tuber blight resistance, are grown on a regional scale. Cultivar diversity might therefore play an important role in integrated pest management, preventing adaptation of *P. infestans* to tuber blight resistance and therefore delay erosion of resistance.

Acknowledgements

We thank Margriet Boerma, Marieke Förch, Henri van Raay and Jans Roosjen for technical assistance. The useful suggestions and comments on the manuscript given by Dr. Hans van der Beek, Dr. Gert Kema, Dr. Piet Boonekanp Prof. Rolf Hoekstra (WU) and Prof. William E. Fry (Cornell University) are highly appreciated. The Dutch Potato Association and the Dutch Ministry of Agriculture, Fisheries and Nature Management supported this work.

7

Stability of partial resistance in potato cultivars exposed to aggressive strains of *Phytopthora infestans*

W. G. Flier, L. J. Turkensteen and T. B. M. van den Bosch

Submitted to Plant Pathology

Summary

Potato cultivars were evaluated for their resistance responses to aggressive strains of *Phytophthora infestans* in field and laboratory experiments. Results from two successive years revealed significant differences in expression of foliar and tuber blight resistance. Analysis of variance revealed differential cultivar-by-isolate interactions for both foliar and tuber blight. Differential responses occur as revealed by changing rank order. In general, severity of late blight epidemics as observed in the haulm did not correlate well with foliar blight resistance ratings as presented in the National List of Recommended Potato Varieties. No significant correlation was found between tuber blight incidence under field conditions and the tuber blight rating in the National List. Also there was no relation between the field and laboratory tuber blight resistance assessments. A significant relation between late blight infection in the foliage and tuber blight incidence was demonstrated. The presence of differential interaction, independent of R-gene based resistance indicates the presence of adaptation of *P. infestans* to partial resistance and consequently the existence of adverse effects on the stability and durability of partial resistance to potato late blight.

Introduction

The oomycete *Phytophthora infestans* (Mont.) de Bary, the cause of late blight in potato, is considered to be one of the most important pathogens of potatoes worldwide. The pathogen affects leaves, stems and tubers, leading to serious yield losses and high costs for chemical control.

To date the vast majority of potato cultivars commonly grown in Western Europe (Colon *et al.*, 1995) and North America (Platt & Tai, 1998) are susceptible to late blight. The use of numerous applications of both protective and curative fungicides is common practise in order to control potato late blight, which is both expensive and has several adverse effects on the environment. Host resistance could enable a significant reduction of fungicide use while maintaining the present yield and quality standards (Inglis *et al.*, 1996). Numerous attempts to achieve durable resistance in potato by the incorporation of R-genes from *S. demissum* and *S. stoloniferum* (Black *et al.*, 1953; Malcolmson & Black, 1966) proved to be unsuccessful due to rapid adaptation of the pathogen as reviewed by Van der Plank (1971) and Turkensteen (1993).

With the growing public demand for crop protection methods with no adverse effects on health and the environment, breeding for durable resistance against late blight has become a focus for most modern potato breeding programmes (Colon *et al.*, 1995; Inglis *et al.*, 1996; Peters *et al.*, 1999). Coincidental with these efforts has been the displacement of the US1 clonal lineage by several more aggressive genotypes of the pathogen in North America (Lambert & Currier, 1997) and the presence of a diverse population of *P. infestans* in Western Europe (Spielman *et al.*, 1991). Evidence is accumulating that *P. infestans* is sexually reproducing in many countries in Western Europe (Spielman *et al.*, 1991; Drenth *et al.*, 1994; Sujkowski *et al.*, 1994; Andersson *et al.*, 1998; Turkensteen *et al.*, 2000). Sexual reproduction and the presence of functional

oospores result into high levels of variation in the pathogen and might lead to increased and more rapid adaptation to systemic fungicides and host resistance.

The newly established population in Western Europe is marked by increased levels of aggressiveness. Flier *et al.* (1998) studied the variation in aggressiveness to potato tubers in local populations of *P. infestans* in the Netherlands. It was concluded that high levels of aggressiveness to tubers are present in three local populations of the pathogen, and that large variation for aggressiveness is being maintained in these populations. Similar results were obtained when the variation in aggressiveness to the foliage was evaluated (Flier & Turkensteen, 1999).

In recent years, commercial potato growers repeatedly claimed severe late blight outbreaks in cultivars with high levels of partial resistance and high tuber blight incidences leading to serious yield losses (Kadish & Cohen, 1992; Inglis *et al.*, 1996; Platt & Tai, 1998). Adequate information on the level of tuber blight resistance is especially important for farmers applying organic or integrated cropping systems. In a recent study, Varis *et al.*, (1996) reported that late blight was a serious problem in cultivar Bintje in both integrated and organic systems in Finland. Total yields were 10% and 36%, respectively, lower compared to the conventional cropping system. Similar late blight problems have been reported in organic potato production in the Netherlands (Lammerts-van Bueren, *pers. comm.*). Resistant potato cultivars like Escort and Santé, which are widely grown in organic farming in the Netherlands show considerable levels of tuber blight leading to serious yield losses in recent years. The observation of increased yield losses due to tuber blight raised doubts about the stability of partial resistance.

The presence of an aggressive, variable population of the pathogen may affect the durability of partial resistance (Nelson, 1979; Latin *et al.*, 1981) due to accelerated adaptation of aggressive forms of the pathogen with higher levels of parasitic fitness (Nelson, 1979). Besides an overall and equally decrease in average resistance scores of all cultivars due to the increased aggressiveness of *P. infestans,* differential responses is marked by an unequally effectiveness of rate reducing resistance against pathogen genotypes (Leonard & Moll, 1979). The latter effect could serve as an operational definition of instability of field resistance and is synonymous with the definition of erosion of field resistance according to Nelson (1979). In practise, instability of resistance will change the ranked order of cultivars. In a recent study, Flier *et al.* (2001a) explored the presence and relative importance of cultivar-by-isolate interaction in tuber blight incidence and severity. Specificity in cultivar-by-isolate interactions was clearly demonstrated. Additional studies are needed to extend our knowledge about host x pathogen differential interaction in the potato–late blight pathosystem to partial resistance in the foliage and about the specificity of this interaction under field conditions.

The aim of the present study was (1) to evaluate the level of partial resistance of some potato cultivars with respect to the foliage and tubers and (2) to evaluate the stability of foliar and tuber blight resistance for partially resistant potato cultivars to

selected strains of a sexually reproducing *P. infestans* population. Consequently, we discuss the role of specificity in the interaction between partially resistant potato cultivars and aggressive strains of *P. infestans.* The nature of the host-pathogen interactions was tested under field and climate chamber conditions and interrelationships were studied between components of partial resistance.

Materials and methods

Isolate selection, culturing and inoculum preparation

Information on virulence factors, mating type and data concerning the collection of the isolates is presented in Table 7-1. Five isolates, all belonging to the new population of *P. infestans* in the Netherlands, were selected (Table 7-1). IPO82001 serves as a reference strain since it has been used for resistance testing by breeders and to obtain foliar resistance ratings for the national list of recommended potato varieties (Ebskamp *et al.*, 1999). It is marked by an intermediate level of aggressiveness to the foliage (Flier & Turkensteen, 1999) and a low level of tuber pathogenicity (Flier *et al.*, 1998). Isolate IPO98014 is at present among the most aggressive strains in the collection and was obtained from a blighted potato stem found on a potato dump in early spring. Isolates F95573, IPO655-2A and IPO428-2 showed high levels of aggressiveness to tubers in previous whole tuber inoculation experiments (Flier *et al.*, 2001a). Isolates taken from liquid nitrogen storage were first inoculated on tuber slices of the general susceptible potato cv. Bintje and incubated in the dark at 15 °C for 5 to 7 days. When sporulating mycelium was present, small tufts of mycelium were placed in a drop of water on the lower epidermis of leaflets of cultivar Bintje placed in 9 cm Petri dishes containing 10 ml 2% water agar. The inoculated leaflets were incubated for seven days in a climate chamber at 15 °C with a 16 hours light period (Philips fluorescence tubes type 33, intensity of 12 W m^{-2}). For large-scale inoculum production, greenhouse grown leaves were cut, placed on wetted filter paper in plastic trays and inoculated. Trays were wrapped in transparent polythene bags and incubated for 5 to 7 days as described previously. Inoculum for field infection was prepared by dipping sporulating leaflets in a 10 l bucket filled with tap-water, filtering the crude suspension through a cheese cloth and adjusting the sporangia concentration to 1.0 x 10^4 sporangia ml^{-1}.

Table 7-1. Isolates of *Phytophthora infestans* used in the inoculation experiments, specific virulence spectrum, mating type, year and origin of collection.

Isolate	Race	Mating type	Year of collection	Source
F95573[a]	1.3.4.7.10.11	A1	1995	Cull pile, Southern Flevoland
IPO428-2[b]	1.2.3.4.5.6.7.8.9.10.11	A2	1992	Allotment garden at Ede
IPO655-2A[b]	1.2.3.4.5.6.7.8.9.10.11	A1	1992	Allotment garden at Ede
IPO82001[c]	1.2.3.4.5.6.7.10.11	A2	1982	Commercial potato crop
IPO98014[b]	1.2.3.4.7.11	A1	1998	Commercial starch potato crop

[a] Isolate kindly provided by M. Zwankhuizen, Department of Phytopathology, Wageningen University, the Netherlands.

[b] Isolates from the Plant Research International *Phytophthora infestans* collection.

[c] Reference isolate used for foliar blight resistance assessment in the Netherlands.

Table 7-2. Potato cultivars used in this study, the presence of identified R-genes and known late blight resistance ratings according to the National List of Recommended potato Varieties.

Cultivar	Purpose	Known resistance	R-genes[3]	Late blight resistance rating[1]	
				Foliar blight	Tuber blight
Astarte	*Starch potato*	*High partial resistance*	*R3*	7	6
Bintje	Ware potato	Susceptible	R0	3	3
Eigenheimer	Ware potato	Susceptible	R0	5	3
Hertha	Ware potato	Partial & R-gene resistance	R1R3R10	6	7
Karnico	Starch potato	Partial & R-gene resistance	n.a.	7.5	6.5
Kartel	Starch potato	Partial & R-gene resistance	n.a.	8[2]	6.5[2]
Pimpernel	Ware potato	High partial resistance	R0	8	8
Producent	Starch potato	Partial & R-gene resistance	R10	7	8
Santé	Ware potato	Partial	R1R10	4	8
Sirtema	Ware potato	Susceptible	R0	4	6

1) Rating according to the National list of recommended potato varieties 1999

2) Ratings according to the National list of recommended potato varieties 1988

3) Based on unpublished data

Response to aggressive isolates

Over a two-year period, eight potato cultivars (Astarte, Bintje, Eigenheimer, Hertha, Kartel, Pimpernel, Producent and Sirtema) with varying levels of resistance to late blight according to the National List of Recommended potato Varieties (Table 7-2) were evaluated under field conditions for their response to two aggressive *P. infestans* isolates F95573 and IPO98014. Field evaluations were performed on a sandy loam soil at the experimental farm at Lienden, the Netherlands.

Certified seed was hand-planted during mid-April using a randomised complete block design with three replicates of 4 hill plots of 6 m each. Plants were spaced 0.7 m between rows and 0.3 m between hills in a row. Plots had 5 m bare fallow spacing on all sites to reduce interplot-interference and to provide adequate access for sprinkler irrigation and weed control. Silage maize was grown as a buffering crop surrounding the experimental field. All plots received fertilizer according to common practise for ware potato production. A weekly fungicide application (Mancozeb, 2 kg a.i. ha^{-1}) was given to test plots until the third week of July in order to prevent early-uncontrolled late blight development. Plots were inoculated during the first week of August with a sporangial suspension (1.0×10^4 ml^{-1}) of either strain at a rate of approx. 10 ml per plant. An overhead irrigation system (5 mm h^{-1}) was used for 2 hours per day for 5 weeks to accelerate late blight epidemics in the foliage and enhance infection of the tubers. Plots were visually evaluated for late blight development twice a week. Late blight was assessed as percent infected haulm area following James (1971).

Vines were killed after the first week of September and tubers were dug and harvested by hand and incubated at room temperature for two weeks. Only tubers from hill 2 and 3 were used for tuber blight assessments. The number of blighted tubers (FPTUB) per plot was assessed visually and percent blighted tubers calculated.

Stability of partial resistance

The stability of field resistance of three (Bintje, Santé and Pimpernel; 1998) and four (Bintje, Karnico, Santé and Pimpernel; 1999) potato cultivars with varying levels of rate reducing resistance to late blight (Table 7-2) were evaluated for two years under field conditions for their response to two highly aggressive *P. infestans* strains (IPO655-2A and IPO98014) and one moderately aggressive isolate IPO82001. Field evaluations were performed on heavy clay at the IPO experimental farm in Wageningen, the Netherlands. The experiment consisted of three randomised blocks in both years of evaluation. Plot size was five hills, each 4 m long with 0.7 m between rows and 0.3 m spacing between plants within a row. Each plot was isolated by 5 m of bare fallow. Silage maize was grown as a buffer crop surrounding the experiment and a 5 m buffer between the blocks.

Plots were inoculated in mid July in both years with a sporangial suspension (1.0×10^4 ml^{-1}). Sprinkler irrigation (5mm h^{-1}) was applied approximately three times a week for 2 hours during the first three weeks after inoculation. Disease assessments were made every three days for 5 weeks post inoculation using the methods described above.

Tuber infections were evaluated by digging up tubers from hill two, three and four at 5 weeks post inoculation. Tubers were incubated for two weeks at ambient temperature and subsequently visually examined for the presence of blighted tubers. The percent blighted tubers (FPTUB) were calculated for each plot.

Whole tuber inoculations

Whole tuber inoculation experiments were performed using tubers from additional plots for experiment I (8 cultivars in 1998 and 1999) and experiment II (3 and 4 cultivars in 1998 and 1999, respectively). Whole tuber inoculations were performed during the end of August using tubers from additional plots. For this purpose, tubers were dug by hand to minimise wounding, immediately transferred to the laboratory and washed to remove adhering soil. Undamaged tubers, about 35 tubers per crate, were placed in plastic seed tuber crates with rose ends facing upward. Tubers were inoculated using strains IPO82001, IPO655-2A and IPO98014 and subsequently incubated according to the methods described by Flier *et al.*, (2001a). Three replicates were used.

After two weeks in storage, tubers were visually examined for the presence of tuber blight symptoms. The percentage of infected tubers per crate (WPTUB) was calculated. In addition, tubers were cut in two (longitudinal axis), and tuber rot severity was evaluated by means of an invasive ability index (IAI) (Flier *et al.*, 1998, 2001a).

Compatibility tests

In two separate experiments in 1999, detached leaflets of the potato cultivars used in the field experiments were inoculated with five *P. infestans* isolates to determine the compatibility of the isolates used in the field experiments. Seven days before field inoculation, fully developed lateral leaflets were collected from the Lienden and Wageningen sites.

Ten leaflets were inoculated for each cultivar-isolate combination. Individual leaflets were placed in 9 cm Petri dishes filled with 10 ml 2% water agar and inoculated by placing one 10 µl droplet of sporangial inoculum ($1,0 \times 10^4$ sporangia ml^{-1}) on the abaxial side of each leaf. Inoculum was prepared from infected leaflets of cv. Bintje according to Flier & Turkensteen (1999). Petri plates with the inoculated leaves were wrapped in transparent polythene bags and incubated in a climate chamber for one week at 15 °C with a light intensity of 12 Wm^{-2}, 16 hours light per day. In both experiments, lesions were measured three times, usually between day three and five, using an electronic calliper. Length and width of each lesion was measured and the average diameter, lesion area and lesion growth rate (LGR) were calculated.

We defined incompatibility here as the mechanism leading to the predominance of unsuccessful infections where the pathogen had been arrested by a hypersensitive reaction by the plant. Therefore, cultivar x isolate interaction leading to an average LGR of 0.0 was regarded as incompatible.

In the first experiment, sporulation (SPOR) was visually assessed using a 0-4 rating scale (0= no sporulation to 4= heavily sporulating). For experiment two, five expanding lesions were randomly selected and sporangia were collected by gently dipping the infected leaflet in 10 ml Isoton II solution (Beckman Coulter BV, Mijdrecht, the Netherlands). Sporangia were counted using a Coulter Counter Z10 (Beckman

Coulter BV). Sporangia density and average sporangia production per cm^2 lesion area were calculated.

Data analysis

All statistical tests were performed using the statistical software Genstat version 5.2.1 (Payne *et al.*, 1993). Foliar resistance levels of potato cultivars in Wageningen and Lienden were compared in each year by analysis of variance on the standardised area under the disease progress curve values (stAUDPC) (Campbell & Madden, 1990). Tuber blight data were analysed using ANOVA and residual maximum likelihood (REML) procedures. The relation between foliar attack (with stAUDPC as a condensate measure) and tuber blight incidence was explored using polynomial linear regression analysis. Spearman rank correlations between the ANOVA estimates for stAUDPC, FPTUB, WPTUB IAI LGR and SPOR were calculated to determine their interrelationship. The foliar and tuber blight ratings according to the Dutch National List of Recommended Varieties were transformed by Y= 10–(rating) in order to be able to calculate positive correlations between listed ratings and the various disease parameters assessed.

Results

Response to aggressive isolates

Resistance response of eight potato cvs to infection in the haulm and tubers by two aggressive isolates, F95573 and IPO98014, during 1998 and 1999 was estimated in terms of standardised AUDPC values (stAUDPC) and percent blighted tubers (Table 7-3). Analysis of variance performed on stAUDPC show significant effects ($P < 0.001$) due to cultivars and year of evaluation. Significant contributions of cultivar-by-isolate (C x I; $P = 0.042$) and cultivar-by-year (C x Y; $P < 0.001$) interactions were also detected. Cultivar-by-isolate differential interactions contributed for 0.5% of the total variation accounted for by the model. Cultivar Kartel was the most resistant to both *P. infestans* strains (Table 7-3). All other cvs showed rather susceptible reactions under field conditions when exposed to these isolates. Moreover, the resistant standard cv. Pimpernel in three out of four tests had higher stAUDPC values as the susceptible standard cv. Bintje (Table 7-3). Late blight epidemics were more severe in 1999 as compared to the 1998 season (average stAUDPC values of 24.3 and 12.1 respectively). Both isolates evoked similar levels of disease in the foliage; average stAUDPC values of 18.2 and 18.3, respectively.

Tubers exposed to inoculum produced in the haulm of the eight potato cultivars resulted in significant tuber infection after two weeks of incubation following the harvest of the tubers (Table 7-3). The main factors cultivar, isolate and year showed highly significant effects ($P < 0.001$). The contribution of C x I, C x Y and isolate-by-year (I x Y) interactions were also significant (ranging from $P = 0.013$ to $P < 0.001$). The C x I interaction accounted for 9.5% of all the variation that could be attributed to the

analysis of variance model. The starch potato cvs Kartel and Producent showed the lowest levels of tuber blight attack (0.83 and 0.81% blighted tubers, respectively) (Table 7-3). Bintje and Eigenheimer were the cultivars with the most susceptible tubers (16.9 and 14.2%, respectively). Isolate IPO98014 was significantly more aggressive on tubers than isolate F95573 (Table 7-3). Higher levels of tuber blight attack were observed in 1998 as compared to 1999 (with 10.7 and 4.1% blighted tubers, respectively).

Stability of partial resistance

Stability of partial resistance was evaluated by estimating resistance response of 3 and 4 potato cvs. to infection in the haulm and tubers by 3 isolates during 1998 and 1999 in terms of stAUDPC and percent blighted tubers (Table 7-4). Vines were totally killed by late blight within three weeks post inoculation in both years of evaluation. The effects for cultivar, isolate and year were significant ($P < 0.001$). The probability levels of C x I and C x Y interactions were $P = 0.018$ and $P < 0.001$, respectively and 5.6% of the variation was explained by cultivar-by-isolate interaction. Bintje was the most susceptible cultivar with an average stAUDPC of 52.1 while cvs Santé and Pimpernel showed intermediate levels of resistance in the foliage with stAUDPC values of 21.9 and 24.9, respectively (Table 7-4). Cultivar Karnico was the most resistant cultivar with an average stAUDPC value of 1.9 (1999 data only). Isolates IPO655-2A and IPO98014 showed similar levels of aggressiveness while IPO82001 caused less severe late blight epidemics in the foliage.

Like in Lienden, average late blight epidemics in Wageningen were more severe in 1999 as compared to 1998. Remarkable differences in stability of foliar resistance were observed for the four cultivars tested in 1999. Both cvs Karnico and Santé showed a fairly stable reaction pattern when exposed to three *P. infestans* strains, while cvs Bintje and Pimpernel showed a surprisingly high level of susceptibility when exposed to the highly aggressive strains IPO655-2A and IPO98014 (Table 7-4).

Tuber blight attack at Wageningen showed less pronounced differences between cultivars and isolates as compared to the Lienden site. Analysis of variance revealed significant differences among cultivars ($P = 0.026$) and year of evaluation ($P = 0.002$). Although 27.0% of the total explained variation was accounted for by cultivar-by-isolate interaction, no significant effect was demonstrated. Cvs Bintje and Pimpernel showed a similar susceptibility of tubers to late blight attack (8.2 and 9.3% blighted tubers, respectively) (Table 7-4). Cv. Santé showed the most resistant reaction (3.9 % on average) closely followed by cv. Karnico with 4.9% blighted tubers (1999 data only for cv. Karnico). All three isolates showed similar levels of aggressiveness to tubers.

In concordance with the results obtained at the Lienden site, average tuber blight incidence at Wageningen was higher in 1998 as compared to 1999. An increase in the average percentage of blighted tubers as observed in 1998 tended to be associated with slower late blight epidemics in the foliage (as measured by stAUDPC values) in both experiments.

Table 7-3. Resistance response of eight potato cultivars to infection in the haulm and the tuber by two aggressive isolates of *Phytophthora infestans* during two years of evaluation at Lienden.

	Foliar blight (stAUDPC)				Tuber blight (% blighted tubers)			
Isolate	**F95573**		**IPO98014**		**F95573**		**IPO98014**	
Cultivar	**1998**	**1999**	**1998**	**1999**	**1998**	**1999**	**1998**	**1999**
Astarte	14.6	27.1	14.2	27.7	12.1	2.7	23.1	5.2
Bintje	12.2	27.6	14.0	28.0	14.8	10.2	36.3	14.7
Eigenheimer	12.5	28.4	12.7	30.8	10.7	3.7	59.3	14.3
Hertha	12.6	28.1	17.5	26.7	9.8	1.9	8.2	2.3
Kartel	0.1	4.2	3.6	0.8	0.1	0.7	1.9	0.7
Pimpernel	14.9	29.9	12.1	31.9	4.5	0.5	19.8	2.0
Producent	13.5	25.8	13.5	22.9	0.2	1.2	0.6	1.3
Sirtema	12.3	26.6	13.1	22.7	3.8	1.5	5.1	2.0
$LSD_{cultivar}$ ($P = 0.05$)				3.4				3.8
$LSD_{isolate}$ ($P = 0.05$)				0.9				1.9
$LSD_{cultivar\text{-}isolate\text{-}year}$ ($P = 0.05$)				5.4				7.5

Table 7-4. Resistance response of four potato cultivars to infection in the haulm and the tuber by three isolates of *Phytophthora infestans* during two years of field evaluation at Wageningen.

	Foliar blight (stAUDPC)						Tuber blight (% blighted tubers)					
Isolate	IPO82001		IPO655-2A		IPO98014		IPO82001		IPO98014		IPO98014	
Cultivar	1998	1999	1998	1999	1998	1999	1998	1999	1998	1999	1998	1999
Bintje	35.7	40.5	46.3	70.9	45.5	73.6	10.5	5.9	14.5	2.5	14.3	1.6
Santé	24.8	21.1	22.6	22.1	21.1	20.0	3.3	1.4	7.0	3.3	5.4	3.1
Pimpernel	6.9	24.6	13.6	38.9	22.4	43.0	15.7	16.7	6.3	2.3	11.3	3.9
Karnico	nd[1]	1.9	nd	1.2	nd	2.6	nd	5.8	nd	6.2	Nd	2.6
$LSD_{cultivar}$ ($P = 0.05$)	7.3						4.6					
$LSD_{isolate}$ ($P = 0.05$)	5.5						3.5					
$LSD_{cultivar\text{-}isolate\text{-}year}$ ($P = 0.05$)	8.1						8.2					

1) = not determined

Whole tuber inoculations

Large differences among cultivars and isolates were found in whole tuber tests with tubers from location Lienden and Wageningen (Table 7-5 and 7-6, respectively). No absolute tuber resistance was observed in the whole tuber tests; inoculations always led to visually detectable tuber blight.

In 1998 percent blighted tubers varied between 5.6% for cv. Santé in combination with IPO655-2A and 35% for cv. Pimpernel inoculated with IPO98014 (Table 7-5) and ranged in 1999 from a low 26.2% (cv. Karnico inoculated with IPO655-2A) to a high of 90.3% (cv. Pimpernel inoculated with either IPO655-2A or IPO98014) (Table 7-5). Analysis of variance performed on percent blighted tubers (after angular transformation) for the 1998 and 1999 Lienden data showed significant effects due to cultivars ($P < 0.001$), isolates ($P < 0.001$) and years ($P = 0.013$). Significant interaction terms were found for C x I ($P < 0.001$) and C x Y ($P = 0.002$). A total of 89.2% of the observed variation could be attributed to the model and 14.2 % of the explained variance was due to the C x I differential interaction term.

Analysis of variance for the 1998 and 1999 IAI Lienden data revealed significant cultivar, isolate and year effects ($P < 0.001$) and a C x Y interaction effect ($P = 0.001$). No significant C x I interaction term was observed although 9.7% of the explained variance could be attributed to cultivar-by-isolate interaction. The model accounted for 68.3% of the observed variation in tuber blight severity. IAI varied from 1.7 (cv. Bintje in combination with IPO98014 and cv. Pimpernel with IPO655-2A) to 3.0 (cv. Santé inoculated with IPO98014) in 1998 and from 1.5 (cv. Pimpernel inoculated with IPO82001) to 2.6 (cvs Bintje and Pimpernel inoculated with IPO98014) in 1999 (Table 7-5).

The whole tuber inoculation tests with tubers from location Wageningen showed similar levels of variation for tuber attack (Table 7-6) as found using tubers from location Lienden. Analysis of variance on the % blighted tubers showed highly significant effects of cultivar, isolate, year and all interaction terms. The model accounted for 95.2% of the observed variation and 9.7% of the explained variance could be attributed to C x I differential interaction. Percent blighted tubers ranged between a low 4.5% in 1998 for cv. Pimpernel inoculated with IPO655-2A and a high 90.3% in 1999 for cv. Pimpernel inoculated with either IPO655-2A or IPO98014.

For IAI, only 52.8% of the observed variation could be attributed to the model used in the analysis of variance. Significant effects were detected for cultivars ($P = 0.032$), isolates ($P = 0.009$) and C x Y interaction ($P = 0.015$). No significant effect was detected for C x I interaction ($P = 0.231$) although 20.5% of the variance accounted for could be attributed to cultivar-by-isolate interaction. Tuber blight severity varied from an IAI of 1.5 for cv. Pimpernel in combination with IPO82001 in 1999 to 3.0 for cv. Santé inoculated with IPO98014 in 1998.

Table 7-5. Percent blighted tubers and tuber blight severity (as presented as a 1-4 index value) of eight potato cultivars evaluated in whole tuber inoculation experiments with four aggressive strains of *Phytophthora infestans* in two successive years at Lienden.

	Blighted tubers (%)								Tuber blight severity (IAI)							
Isolate	**F95573**		**IPO428-2**		**IPO655-2A**		**IPO98014**		**F95573**		**IPO428-2**		**IPO655-2A**		**IPO98014**	
Cultivar	**1998**	**1999**	**1998**	**1999**	**1998**	**1999**	**1998**	**1999**	**1998**	**1999**	**1998**	**1999**	**1998**	**1999**	**1998**	**1999**
Astarte	39.3	51.6	17.5	35.2	6.2	7.7	23.2	40.1	1.8	2.3	2.2	2.2	1.2	2.6	1.6	2.1
Bintje	70.6	82.4	74.8	74.6	22.0	6.8	57.7	67.7	2.4	3.4	2.9	2.9	2.1	2.5	2.1	3.1
Eigenheimer	89.6	82.7	78.0	80.4	13.6	12.2	75.4	74.3	2.2	3.0	1.9	3.0	1.3	2.8	1.6	2.8
Hertha	89.8	64.5	44.2	54.0	7.2	1.0	67.7	71.2	2.1	3.4	2.0	2.9	1.0	2.5	1.8	3.0
Kartel	1.9	0.8	0.9	0.0	0.0	2.1	6.4	3.6	1.3	1.7	1.2	0.0	0.0	0.0	2.1	1.0
Pimpernel	34.5	75.7	35.2	66.1	0.0	0.0	50.1	72.5	1.7	1.8	2.3	1.8	0.0	1.3	1.9	2.2
Producent	21.1	50.5	4.4	7.1	0.0	0.0	3.6	11.9	2.3	1.7	1.2	2.3	0.0	1.1	1.7	2.4
Sirtema	53.2	54.7	57.0	63.8	4.6	1.1	40.7	25.3	1.8	3.2	2.6	3.3	1.6	2.6	2.0	2.8
$LSD_{cultivar}$ ($P = 0.05$)								7.4								0.4
$LSD_{isolate}$ ($P = 0.05$)								5.2								0.3
$LSD_{cultivar\text{-}isolate\text{-}year}$ ($P = 0.05$)								20.9								1.1

Table 7-6. Percent blighted tubers and tuber blight severity (as presented as a 1-4 index value) of four potato cultivars evaluated in whole tuber inoculation experiments with two aggressive strains and a reference isolate of *Phytophthora infestans* in two successive years at Wageningen.

		Blight tubers (%)						Tuber rot (index)					
	Isolate	IPO82001		IPO655-2A		IPO98014		IPO82001		IPO655-2A		IPO98014	
Cultivar		1998	1999	1998	1999	1998	1999	1998	1999	1998	1999	1998	1999
Bintje		16.1	67.2	8.6	45.2	23.4	80.3	2.8	2.0	1.7	2.2	1.7	2.6
Santé		7.7	84.6	5.6	71.0	18.3	79.7	2.8	1.6	1.8	1.2	3.0	2.0
Pimpernel		13.8	78.3	4.5	90.3	35.0	90.3	1.9	1.5	1.7	1.8	2.4	2.6
Karnico		nd	66.7	nd	26.2	nd	73.5	nd	2.2	nd	2.1	nd	2.4
LSD ($P = 0.05$)		9.6						0.6					

Compatibility test

Infection experiments using detached leaflets collected from field grown plants at the locations Lienden and Wageningen showed highly significant ($P < 0.001$) effects of cultivar, isolate and C x I differential interaction when both LGR and SPOR were analysed using analysis of variance. The model accounted for most of the variation observed (ranging from 79.6 to 85.9%) and the differential interaction between cultivars and isolates accounted for 11.0 to 13.6% of the variation that could be attributed to the model used. Incompatible combinations that might be attributed to the action of *R*-genes were only detected in the case of isolate IPO82001 in combination with cvs Astarte and Kartel (Table 7-7).

LGR ranged from a low 0.0 mm d^{-1} for cvs Astarte and Kartel in combination with IPO82001 to a high 7.9 mm d^{-1} for cv. Hertha in combination with IPO655-2A (Table 7-7). Sporangia production, as measured by a sporulation index, varied from 0.0 (cvs Kartel, Hertha and Astarte in combination with IPO82001 and Kartel inoculated with IPO428-2) to 4.0 (cvs Eigenheimer, Bintje and Santé inoculated with IPO98014) (Table 7-7).

Analysis of variance for the second compatibility experiment showed significant contributions to observed variation in LGR by cultivars and isolates ($P < 0.001$) as well as the differential interaction between cultivars and isolates ($P = 0.023$). The percent variance accounted for by the model was 64.8%, 8.4% of the explained variance could be attributed to C x I interaction. LGR varied between 2.2 mm d^{-1} for the combination of cv. Pimpernel and IPO82001 to 4.7 mm d^{-1} for cv. Bintje in combination with IPO655-2A and IPO98014 (Figure 7-1).

Analysis of variance using the $\log_e$-transformed data for sporulation density revealed significant effects of cultivars ($P = 0.016$) and C x I differential interaction ($P = 0.007$). The amount of variation accounted for was 57.2% and 43.1% of this variation could be attributed to cultivar-by-isolate interaction. Spore production was also variable, ranging from 2.9 x 10^4 sporangia cm^{-2} for cv. Santé and IPO98014 to 10.8 x 10^4 sporangia cm^{-2} for cv. Santé and IPO655-2A (Figure 7-1).

When exposed to *P. infestans* strains, cultivars show a different expression of their components of partial, rate-reducing resistance. Cv. Pimpernel and Bintje show remarkable differences in LGR when exposed to the isolates IPO82001, IPO655-2A and IPO98014, while SPOR seems more or less fixed at a certain level. Cv. Santé shows a striking increase (ranging from 2.9 to 10.8 x 10^4 sporangia cm^{-2}) in SPOR, while maintaining a stable LGR of approx. 3.5 mm d^{-1} for all three isolates tested. Cv. Karnico appears to express the most stable expression for both components tested with relatively small differences in LGR (ranging from 2.7 to 3.3 mm d^{-1}) and SPOR (ranging from 3.7 to 5.8 x 10^4 sporangia cm^{-2}).

Table 7-7. Lesion growth rates (mm d^{-1}) and sporulation density (presented as a 0-4 index value) of four aggressive strains and a reference isolate of *Phytophthora infestans* assessed using inoculated detached leaflets collected from eight and four potato cultivars respectively during the 1999 field season.

		LGR (mm d^{-1})					SPOR (index)				
	Isolate	**F95573**	**IPO428-2**	**IPO655-2A**	**IPO82001**	**IPO98014**	**F95573**	**IPO428-2**	**IPO655-2A**	**IPO82001**	**IPO98014**
Location	**Cultivar**										
Lienden	Astarte	4.9	4.8	4.8	0.0	5.0	2.0	3.0	3.2	0.0	3.8
	Bintje	5.4	5.9	6.6	0.8	5.6	2.4	3.0	3.0	0.6	3.8
	Eigenheimer	5.9	4.6	6.2	1.9	6.9	2.6	3.2	3.4	0.6	4.0
	Hertha	6.2	6.4	7.9	2.1	6.2	3.0	1.8	3.0	0.0	2.8
	Kartel	0.7	0.3	1.0	0.0	1.5	0.2	0.0	0.4	0.0	0.8
	Pimpernel	4.4	5.7	4.8	1.1	5.3	3.0	3.0	3.0	1.2	3.2
	Producent	4.7	2.1	2.7	1.2	2.7	3.0	3.6	3.2	1.2	3.8
	Sirtema	6.2	5.9	6.5	2.0	6.2	2.2	1.2	1.4	1.0	2.2
Wageningen	Bintje	6.3	6.1	5.9	2.4	6.7	3.2	3.8	3.8	1.6	4.0
	Karnico	5.1	2.7	5.3	0.8	5.8	3.0	2.0	3.0	0.8	3.0
	Pimpernel	5.3	5.8	5.4	2.6	5.6	1.4	2.4	2.8	1.2	3.0
	Santé	6.0	2.8	6.1	1.0	6.7	3.0	2.0	3.4	1.2	4.0

Lienden LGR-$LSD_{ras\text{-}isolaat}$=1.6; SPOR- $LSD_{ras\text{-}isolaat}$ =0.7

Wageningen LGR-$LSD_{ras\text{-}isolaat}$=1.1; SPOR- $LSD_{ras\text{-}isolaat}$ =0.6

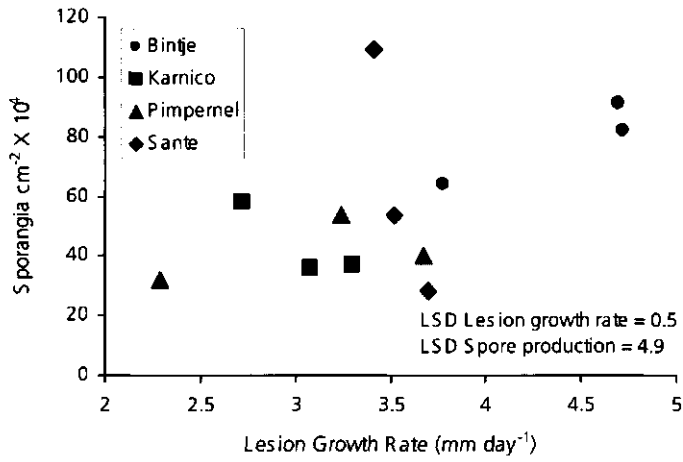

Figure 7-1. Relationship between sporulation capacity (sporangia cm^{-2} x 10^4) and the lesion growth rate in mm d^{-1} for four potato cultivars inoculated with three isolates (IPO655-2A, IPO98014 and the reference strain IPO82001) of *P. infestans* in a detached leaflet test. Each dot represents a cultivar by isolate combination.

Correlations

The relationships between the various measures for foliar and tuber blight resistance were explored by means of Spearman rank correlations. The foliar resistance ratings in the Dutch National list of Recommended Potato Varieties did not correlate with the actual stAUDPC values from the Lienden and Wageningen sites. The Spearman rank correlation coefficient was 0.138, not significantly different from zero (P = 0.67). The foliar disease ratings were found correlated with both LGR (r = 0.569, P = 0.05) and SPOR (r = 0.625, P = 0.03). LGR nor SPOR significantly correlated with observed foliar blight resistance as measured by stAUDPC values (r = 0.529 and 0.372, respectively).

The tuber resistance ratings were found poorly correlated with tuber blight incidence under field conditions (r = 0.593, P = 0.04). No correlations between the tuber resistance rating and percent blighted tubers or IAI in the whole tuber assay were detected. The tuber blight incidence observed under field conditions was associated with the percent blighted tubers obtained by the whole tuber test (r = 0.648, P = 0.02).

A strong relationship between the severity of haulm blight and tuber blight incidence was found for both years of evaluation at the Lienden site when average stAUDPC values were plotted against average percent tuber blight attack for all 16 cultivar-isolate combinations for each year of evaluation (Figure 7-2). A quadratic non-linear equation $Y = 0.0106\ x^{2.2841}$ fitted the 1998 data with a R^2 of 0.94. The exponential equation $Y = 0.6019\ e^{0.1642x}$ fitted the relationship between haulm blight and tuber blight fairly well (R^2 = 0.75) (Figure 7-2). No clear relationship between stAUDPC and

percent blighted tubers could be established for the Wageningen data. No significant correlation coefficients were demonstrated between foliar and tuber blight other than a correlation coefficient ($r = 0.705$; $P = 0.01$) between the foliar resistance rating in the national list and tuber blight severity as measured by IAI in the whole tuber experiments.

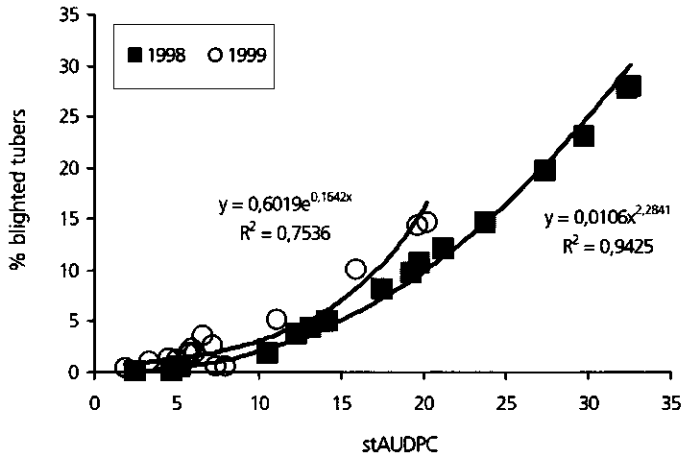

Figure 7-2. Relationship between percent blighted tubers as assessed in field evaluations at Lienden in 1998 and 1999 and the standardized Area Under the Disease Progress Curve as recorded during the epidemic in the haulm for 8 potato cultivars with varying levels of partial resistance in the foliage and tubers and two aggressive strains of *Phytophthora infestans*. Each marker represents a cultivar by isolate combination.

Discussion

Cultivars used in this study differed considerably for partial late blight resistance in the haulm as well as tubers. The level of tuber infection was not found closely related to foliar blight resistance, which is in agreement with earlier studies (Wastie, 1991) although other reports have claimed a good correlation between foliar disease and results based on tuber inoculations (Stewart *et al.*, 1996; Platt & Tai, 1998). Whole tuber inoculation assays led on average to higher incidences of tuber infection as compared to the field experiments. The whole tuber test rules out escape mechanisms might explain the relatively high levels of tuber attack observed. Stewart *et al.* (1996) stated that late blight resistance in foliage and tuber are determined by the same genes or by different linked genes, but the lack of association between levels of foliar and tuber blight observed under field conditions might be explained by the influence of environmental variation and temporally or spatially escape mechanisms of tubers (Bain & Möller, 1999).

Our results provide strong indications for the presence of specificity between *P. infestans* isolates and potato cultivars with partial resistance. The presence of differential interaction between potato cultivars and *P. infestans* strains was confirmed at nearly all phases of host-pathogen interaction. Our observations are in agreement with Caten (1974) who stated that differential interactions between pathogen strains and non-immune host varieties are a common feature of host-pathogen systems. Support for this view was presented by Leonards-Schippers *et al.* (1994) who identified at least one 'isolate-specific' QTL for quantitative late blight resistance in diploid potato lines by means of interval mapping. Doubt is generated on the existence of forms of resistance totally independent of the invading strain. It is very unlikely that R-genes are to be held responsible for the observed specificity since the compatibility studies using detached leaves did not indicate the presence of incompatibility between cultivars and the isolates used in the field evaluations. In addition, R-genes do not seem to inhibit the growth of *P. infestans* after tuber infection, although the R1 resistance gene has been reported to confer a strong hypersensitivity reaction in the cortical region after inoculation with incompatible isolates (Lapwood & McKee, 1961; Toxopeus, 1961). Recent work (Flier *et al.*, 2001a) indicate that a different class of resistance genes with a quantitative effect might be involved in the resistance reaction in the outer cortex region of potato tubers.

Evidence for the presence of specificity in potato tuber blight has been reported by de Bruyn, (1947), Caten (1974), Bjor and Mulelid (1991) and Peters *et al.*, (1999). However, reports on specificity in foliar late blight epidemics of partially resistant potatoes are scarce (Latin *et al.*, 1981; James & Fry, 1983) and their results have been questioned (Kulkarni & Chopra, 1982). In laboratory studies, James and Fry (1983) were able to increase the level of foliar pathogenicity of isolates slightly by repeated sequential cycles of culturing on two cultivars. However, they did not find evidence for differential adaptation since the gain in aggressiveness was not limited to the cultivar in which the cycling had occurred. They concluded that specificity, measured as cultivar-by-isolate interaction, was very limited and of no practical importance. Others have pointed out that the cultivar-by-isolate interaction described by Latin *et al.* (1981) could be confounded with environmental interaction (Kulkarni & Chopra, 1982; Fry & Spielman, 1991). More recently, Inglis *et al.* (1996) compared cultivar rankings in response to foliar infection with new *P. infestans* strains with rankings obtained with isolates that had been predominant in the United States before 1990. Cultivar rankings were found nearly identical to the previously obtained data. These data provide additional support for the view that erosion of partial resistance is of little importance.

All published reports on the role of specificity for stability of partial resistance to potato late blight are based on studies concerning either the asexually reproducing US1 clonal lineage which appeared to be pan-globally distributed until recently (Spielman *et al.*, 1991), or new immigrant strains of the pathogen. No data exists on the contribution of cultivar-by-isolate interaction considering highly variable, sexually reproducing *P. infestans* populations that have been reported for Central Mexico (Goodwin *et al.*,

1992a) and Western Europe (Drenth *et al.*, 1994). Specificity and adaptation to late blight resistance is more likely to occur when genetic variation is being maintained at a very high level. Consequently, adaptation of strains to partial resistance to late blight are far more likely to occur in areas with sexually reproducing *P. infestans* populations as compared to clonally propagating populations.

The order of magnitude of cultivar-by-isolate specificity relative to the differences in cultivar resistance and aggressiveness of isolates determines the potential ability of the pathogen population to adapt to partial resistance and therefore the stability of such resistance (Caten, 1974), It has been stated that only a small portion of the observed variation in experiments can be attributed to cultivar-by-isolate interaction when analysis of variance is used to evaluate experimental data (Parlevliet & Zadoks, 1977; Carson, 1987). Carson (1987) evaluated six genetic models of host-pathogen interaction in pathosystem featuring partial resistance. All models that allowed for substantial cultivar-by-isolate interactions resulted in a small cultivar-by-isolate interaction estimate in the analysis of variance of disease reaction. The percent variance accounted for by C x I ranged from 1.6 to 6.5% for the Log_e transformed multiplicative and interactive multiplicative models respectively (Carson, 1987). As a consequence, the relative importance of cultivar-by-isolate interaction will be underestimated when analysis of variance is applied to experimental data and the detection of significant cultivar-by-isolate interaction in field or greenhouse experiments requires fairly small estimates for experimental error. Approximately 10% of the total variation in our experiments could be explained by cultivar-by-isolate interactions, which is in the expected order of magnitude as compared to the model-based studies of Parlevliet and Zadoks (1977) and Carson (1987).

The presence of specificity implies that screening for partial resistance and predicting the stability of partially resistant potato cultivars using only one isolate may lead to the selection of breeding lines which will not express stable forms of partial resistance. We believe that proper screening for partial resistance should involve two stages. First, the breeding lines should be exposed to a well defined virulent (R-gene compatible) and aggressive *P. infestans* strain. Potato clones with an outstanding performance in this first test, which should take place for at least two seasons, should then be exposed to a highly variable population of *P. infestans*. This could be either a natural existing population or a mix of many genotypes with a broad genetic base. Only clones that express stable resistance to the variable pathogen population should be selected and compatible isolated should be collected from those clones for additional stability tests. This approach might reduce the risk of selection for non-stable forms of resistance but can however not completely exclude the possibility that erosion will take place after introduction of the new cultivar. As a consequence, continuation of late blight testing for the national list using the not very aggressive strains IPO82001and VK 6C can no longer be recommended. Instead, an isolate showing a high level of aggressiveness to the foliage and tubers combined with a fairly complex virulence spectrum should be used, for example isolate IPO98014, F95573 or IPO428-2.

From our results we conclude that specificity plays a significant role in interactions between *P. infestans* strains and potato cultivars. We have demonstrated that erosion of partially resistant potato cultivars occurs under field conditions as shown in the case of the partial resistant cv. Pimpernel and isolate IPO98014. The presence of specificity in interactions between *P. infestans* and partially resistant potato cultivars support the concept of "erosion of resistance" as alluded by Niederhauser (1962) and Nelson (1979) and will affect the stability and the durability of partial resistance against late blight in potatoes. This is in agreement with substantial anecdotic evidence for erosion of partial resistance in potato in the Netherlands in recent years. This stresses the need of a better insight in the adaptive ability of the pathogen in order to be able to predict the relative durability of partially resistant potato cultivars when exposed to variable *P. infestans* populations.

Acknowledgements

We thank Maarten Holdinga and the personnel at the experimental farm for their great help with the field experiments and the tuber blight assessments. This work was supported by grants from the Ministry of Agriculture, Fisheries and Nature Management of the Netherlands.

Section IV

8

Influence of host and management on the population structure of *Phytophthora infestans* within the Toluca Valley of central Mexico

N. J. Grünwald, W. G. Flier, A. K. Sturbaum, E. Garay-Serrano,
T. B. M. van den Bosch, C. D. Smart, J. M. Matuszak, H. Lozoya-Saldana,
L. J. Turkensteen and W. E. Fry

Potato Research (1991) **41**: 345-354

Summary

The relationships among populations of *P. infestans* from wild *Solanum* species, from locally grown ("criolla") varieties in low input production systems, and from modern cultivars in high-input agriculture in the central highlands of Mexico were studied. This region contains the most diverse populations of *P. infestans* in the world and probably represents the centre of origin of this species. Isolates were sampled from the three different host-pathosystems in 1988 and 1989 (n = 179) and in 1997 and 1998 (n = 401). Analyses of genotypic diversity (distinguishing aspects of richness and evenness, as well as diversity) were based on mating type and two allozyme loci. Two groups of isolates from diverse host/management systems but monomorphic for these markers were further investigated via RFLP fingerprinting, and most individuals (65% and 85%) in the two subsets were demonstrated to be unique. Additionally, the frequency of metalaxyl resistance was assessed as a selectable marker. The population structure in 1988-89 was similar to that in 1997-98, except that the frequency of metalaxyl resistance was 13% in 1997-98 and 60% in 1988-89. The populations from the three different associations were only slightly different from each other, with greatest diversity found in the population from commercial cultivars. The diversity data and the chronology of disease occurrence during the season are consistent with the hypothesis that populations of *P. infestans* on wild *Solanum* populations are now derived from populations on cultivated potatoes in the central highlands of Mexico near Toluca.

Introduction

Much evidence has accumulated that the central highlands of Mexico, including the Toluca Valley, are the centre of origin of *Phytophthora infestans* (Mont.) de Bary, the causal organism of potato late blight. Genetic diversity for virulence (Mills & Niederhauser, 1953; Niederhauser *et al.*, 1954; Tooley *et al.*, 1986; Turkensteen, 1993), allozyme (Tooley *et al.*, 1985; Goodwin *et al.*, 1992a) and RFLP loci (Goodwin *et al.*, 1992b) is higher in central Mexico than elsewhere, and mating types can be found in approximately a 1:1 ratio (Goodwin *et al.*, 1992a). Despite this general knowledge, the population structure of *P. infestans* within the Toluca Valley is not well understood.

The Toluca Valley is located at 2,600 m above sea level and several wild *Solanum* species can be found there. To the SW, the valley is delimited by the volcano Nevado de Toluca (Figure 1), reaching an altitude of 4,660 m. Potatoes are grown at altitudes up to 3,500 m, while wild species of *Solanum* have been found at the edges of pine and *Abies* forests at 3,800 m (Hawkes, 1990). Several *Solanum* species are endemic to the Toluca Valley and can be infected by *P. infestans*. *S. demissum* Lindt. is by far the most abundant species of *Solanum*, followed by *S. verrucosum* Schlechtd., *S. iopetalum* (Bitt.) Hawkes, *S. brachycarpum* Correll, *S.* x *edinense* Berth. subsp. *salamanii* (Hawkes) Hawkes, and *S. stoloniferum* Schlechtd. et Bché. (Rivera-Peña & Molina-Galan, 1989). All of these species can be infected by *P. infestans*, and an extensive survey between 1982 and 1986 by Rivera-Peña established that about 10% of these plants were actually infected (Rivera-Peña, 1990b,c,d). Levels of rate-reducing resistance and the composition of R-genes vary among specific genotypes of *S. demissum* (Malcolmson

& Black, 1966; Rivera-Peña, 1990b; Lozoya-Saldaña *et al.*, 1997), and these characteristics are also expected to vary among genotypes for the other wild *Solanum* species.

Solanum species occur in three distinctly different types of associations in the central highlands. The first association is in patches of wild *Solanum* species (WSS) in areas of subsistence farming or uncultivated areas. The second association is on low-input, rural farms (RURAL). These farms produce mostly "criolla" potato (*S. tuberosum*) cultivars for additional income or subsistence. Individual fields are on average about 0.2 to 3 ha in size and the potato varieties range from moderately resistant to highly susceptible to late blight. Some of these varieties harbour R-genes conferring resistance to *P. infestans* (Grünwald & Fernandez, *unpubl.*). Applications of agro-chemicals vary from field to field and can be quite sporadic, ranging anywhere from an application of fungicide 1-2 times per season to once a week depending on the income level and attitude of the farmer. In this study, the first and second associations occurred on the slopes of the volcano Nevado de Toluca (Figure 8-1). The third association consists of large, commercial seed-tuber production farms that grow cultivars susceptible to potato late blight (cv. Alpha has no R-genes, and cv. Atlantic has R1). The farming practices of these operations are intensive, applying pesticides 2 to 3 times per week and fields are large, ranging anywhere from 2-10 ha. In our study, these farms occurred in the valley SE of the city of Toluca (VALLEY).

The two different production systems and variation in the wild *Solanum* species might be expected to exert detectable selection on populations of *P. infestans* in this region. Host resistance and fungicide usage are probable forces of selection. Most known R-genes originated from *S. demissum* (Niederhauser, 1991), but levels of rate-reducing resistance and R-gene composition vary among plants (Black & Gallegly, 1957; Malcolmson & Black, 1966; Rivera-Peña, 1990d) and presumably among populations. Consequently, one might expect greater genetic diversity in populations of *P. infestans* growing on *S. demissum* than in populations from cultivated potatoes. Fungicide usage is most intense in the central valley and no fungicides are used on wild species of *Solanum*. Again, higher selection pressures would be expected in environments with more intense fungicide use, leading to less genetic diversity in populations from the central valley than from wild species of *Solanum*. In general, we might expect to find greatest genetic diversity in *P. infestans* populations growing on wild potato species.

Analysis of genotypic diversity in plant pathology typically relies on calculation of indices of genotypic diversity and does not distinguish richness and evenness as components of diversity (Borchart *et al.*, 1998a,b; Caffier *et al.*, 1999; Chen & McDonald, 1996; Chen *et al.*, 1994; Goodwin *et al.*, 1992; Goodwin *et al.*, 1994; Goodwin *et al.*, 1993; McDonald *et al.*, 1996; Sujkowski *et al.*, 1994). However, genotypic diversity depends both on genotype richness, namely the number of genotypes observed in a sample, and evenness (reflecting whether any genotypes dominate the sample) (Ludwig & Reynolds, 1988; Magurran, 1988; Pielou, 1975). Others have noted that richness and evenness are confounded in diversity analyses (Müller *et al.*, 1996; Groth & Roelfs, 1987). For certain inferences it is important to

distinguish richness and evenness. A decision to use a fungicide, which could select strongly for resistance, would be better informed by knowledge of both richness and evenness rather than by knowledge of diversity alone. Two populations with the same number of genotypes might have high diversity when one resistant clone dominates (low evenness) compared to a population where all susceptible and resistant clones occur at equally low frequencies (high evenness).

Our overall objective is to understand the population biology of *Phytophthora infestans* in the Toluca Valley area. Generalities learned in this area may have application to recently sexual populations of *P. infestans* in different global locations. Our focus in the present study was to determine the influence of different host and management systems on the population structure of *P. infestans*. We studied populations of *P. infestans* derived from three host-pathosystems, namely (i) patches of wild *Solanum* species (WSS), (ii) *S. tuberosum* ("criolla") varieties in rural, low-input potato fields (RURAL) and (iii) *S. tuberosum* cultivars in central valley pesticide-intensive potato fields (VALLEY). Our hypothesis was that the WSS population is genetically more diverse than the other two populations. Our approach was to determine mating type and allozyme patterns at the *Glucose-6-phosphate isomerase (Gpi)* and *Peptidase (Pep)* loci (as neutral markers) as well as metalaxyl sensitivity (as a selectable marker) of each individual pathogen isolate. There were two collections; one from 1988/1989 and the other from 1997/98. This enabled a comparison of results contrasting neutral vs. non-neutral markers in two collections separated by nearly a decade. Some isolates from diverse host/management systems had the same mating type and allozyme genotype. Diversity within the two most frequent of these monomorphic groups of isolates was determined by nuclear DNA fingerprinting using probe RG57 (Goodwin *et al.*, 1992b).

Materials and methods

Sampling strategy

An operational definition of population was used (Milgroom, 1995). Populations of *P. infestans* were defined by host/management system: (1) wild *Solanum* species (WSS) populations sampled from *S. demissum* (200-2000 plants per population) and *S.* x *edinense* (usually individual plants within "Criolla" fields), (2) small, rural fields of low-income farmers growing a mixture of land-race type potatoes colloquially called "criollas" and commercial varieties (mostly Alpha and Rosita) (RURAL), and (3) large agricultural fields in the central valley, where the cultivars Alpha and Atlantic are grown on a large scale (VALLEY). Separate fields sampled within each of these systems are considered to harbour sub-populations. In the 1997/1998 collection, the VALLEY populations were geographically distant from the RURAL and WSS populations. The WSS and RURAL populations occur in close proximity on the slopes of the volcano Nevado de Toluca (Figure 8-1). In the 1988/89 collections, the WSS population was

primarily from the valley floor in close proximity to commercial fields, but also from the slopes of the volcano.

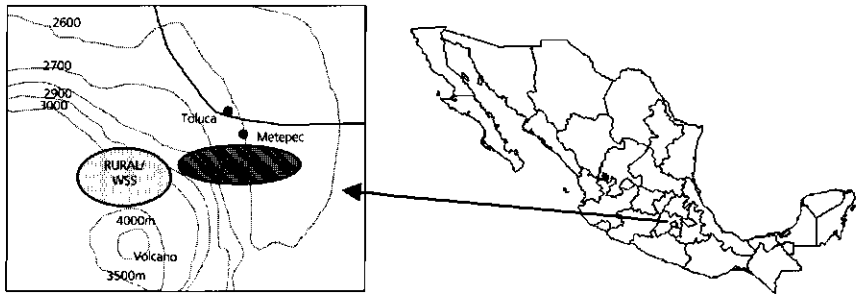

Figure 8-1. Sites of collection of *Phytophthora infestans* isolates in Mexico in 1997-1998. The insert shows a topographic map of the Toluca Valley located in the State of Mexico. Isolates were obtained in 1997 and 1998 (n = 401) from three different pathogen-plant associations. They were: i) populations of *P. infestans* growing on populations of wild *Solanum* species on the slopes of the volcano Nevado de Toluca (3,000-3,500 m beyond sea level) where no fungicides are applied and all plants are expected to carry R-genes (WSS); ii) on potatoes in rural, low-input potato fields on the slopes of the volcano Toluca (3,000-3,500 m above sea level) where fungicide is applied sporadically and R-genes occur (RURAL); and iii) on potatoes in pesticide-intensive potato fields where fungicides are applied every 2-3 days and only susceptible cultivars with no R-genes are grown (VALLEY).

1988 and 1989 samples

Isolates collected in 1988-89 were sampled and isolated as described previously (Matuszak *et al.*, 1994). All isolates that could be clearly identified as coming from one of the three populations (WSS, VALLEY and RURAL) were selected, resulting in a total of 179 isolates (Table 8-1). Most of the isolates from wild *Solanum* spp were collected in the Valley and not on the slopes of the volcano. Although not recorded in that collection, these isolates probably came from one of three species of *Solanum* (*S. demissum*, *S. stoloniferum* and *S.* × *edinense*) because these are the only species that are somewhat common. (In the 1997/98 collections, no other wild species were detected in the valley.) Mating types, and allozyme patterns at the *Pep* and *Gpi* loci for these isolates are reported here, while results for metalaxyl resistance were reported previously (Matuszak *et al.*, 1994).

1997 and 1998 samples

Three hundred and seventy one isolates were collected in 1997 and 30 isolates in 1998 (Table 8-2). The sampling scheme in 1997 consisted of obtaining 10-15 isolates

each from at least 10 fields in the valley (VALLEY), from at least 10 fields on the slopes of the volcano (RURAL) and from 10 patches of wild Solanum species (WSS). Each isolate originated from a randomly selected, single-leaf lesion of *Solanum tuberosum*, *S. demissum*, or *S.* x *edinense*. Additional isolates were sampled in 1998 from RURAL and mostly WSS populations to ensure a sufficiently large sample size. WSS isolates were more difficult to sample as they occurred sporadically and only late in the season.

Isolation and storage of isolates

Isolates collected in 1997 and 1998 were obtained by placing a portion of a leaf lesion under a slice of potato tuber (ca 0.5 cm thick) in a Petri dish. Tuber slices were obtained from surface sterilized, healthy tubers of cultivar Alpha. Isolation from mycelial tufts on top of the tuber slice was performed 4-5 days later by transfer onto selective or regular Rye-A agar (Caten & Jinks, 1968). Selective Rye A agar was regular Rye-A agar containing the following antibiotics and fungicides: 20 mg l^{-1} rifampicin, 50 mg l^{-1} polymyxin-B sulfate, 200 mg l^{-1} ampicillin, 67 mg l^{-1} PCNB (pentachloronitrobenzene), and 100 mg l^{-1} benomyl.

All isolates collected in 1997 and 1998 were subsequently maintained on Rye A agar at 18° C with transfers every 2-3 months. Replicate cultures of most isolates are stored temporarily by cryogenic storage in liquid nitrogen (Tooley, 1988) and under mineral oil at 18 °C at the PICTIPAPA culture collection in Toluca, Mexico (N. J. Grünwald) and permanently by cryogenic storage in liquid nitrogen (50) at Plant Research International, Wageningen, the Netherlands (W. G. Flier).

Neutral markers

Mating type and allozyme genotypes were assessed as putative neutral markers. Mating type was determined by pairing each isolate with known A1 (PIC96001) and A2 (PIC96002) tester strains on Rye-A agar. Allozyme genotypes for *Gpi* (EC 5.3.1.9) and *Pep* (EC 3.4.3.1) were determined using potato starch gels as described by Spielman *et al.* (1990) for the 1988 and 1989 samplings and cellulose acetate membranes as described by Goodwin *et al.* (1995a) for the 1997 and 1998 samplings. Further analysis was performed on two subsets of isolates; each being monomorphic for mating type, and *Gpi* and *Pep* allozyme patterns (A2, *100/100*, *100/100* or A2, *86/100*, *100/100*). The moderately repetitive clone RG-57 (Goodwin *et al.*, 1992b) was used for Southern blot analysis using the Renaissance non-radioactive detection kit as described by the manufacturer (New England Nuclear, Boston, MA).

Table 8-1. Sampling information for isolates collected in 1988 and 1989 (n = 179). n = number of isolates collected.

Population	Sub-population	Isolate numbers	Location type	Location name[1]	Date collected	Number of isolates	Host
1	1	ME885007-ME885041	VALLEY	Calimaya	13-Jul-88	13	Potato
1	2	ME885406-ME885428	VALLEY	Atizapán	11-Jul-88	14	Rosita
1	3	ME886000-ME886024	VALLEY	Metepec	22-Aug-89	22	Rosita
1	4	ME886025-ME886048	VALLEY	Metepec	22-Aug-89	19	Alpha
1	5	ME886052-ME886060	VALLEY	Metepec	27-Aug-89	9	Atzimba
1	6	ME886063-ME886073	VALLEY	Metepec	27-Aug-89	9	Lopez
1	7	ME885708-ME885717	VALLEY	S.E. Tenango	28-Jul-88	7	-
1	8	ME885718-ME885727	VALLEY	N.E. Calimaya	28-Jul-88	9	-
1	9	ME885729-ME885733	VALLEY	E. Calimaya	28-Jul-88	4	-
1	10	ME885736-ME885740	VALLEY	Conalep	28-Jul-88	4	-
1	11	ME885743-ME885754	VALLEY	Lerma	28-Jul-88	8	-
1	12	ME885755-ME885763	VALLEY	San Felipe del Progreso	28-Jul-88	6	-
1	13	ME885773-ME885788	VALLEY	Santiago Tianguistenco	29-Jul-88	14	-
2	1	ME885659-ME885660	RURAL	El Mesón	26-Jul-88	2	-
2	2	ME885664-ME885665	RURAL	La Puerta	26-Jul-88	2	-
2	3	ME885670-ME885674	RURAL	Raices	26-Jul-88	3	-
2	4	ME885679-ME885694	RURAL	Ojo de Agua	26-Jul-88	7	-
2	5	ME885695-ME885699	RURAL	San Juan Huerta	26-Jul-88	2	-
3	1	ME885790-ME885791	WSS	El Mesón	26-Jul-88	1	WSS
3	2	ME885792-ME885795	WSS	San Felipe del Progreso	28-Jul-88	4	WSS
3	3	ME885796-ME885805	WSS	East of S. Felipe P.	28-Jul-88	8	WSS
3	4	ME885808-ME885810	WSS	Atizapán	29-Jul-88	2	WSS
3	5	ME885811-ME885816	WSS	S.N. Coatepec	29-Jul-88	2	WSS
3	6	ME885818-ME885819	WSS	Metepec	18-Aug-88	1	WSS
3	7	ME885820-ME885931	WSS	Atizapán	19-Aug-88	7	WSS

[1] - = no information available on coordinates, altitude and fungicide use.

Table 8-2. Sampling information for isolates collected 1997 and 1998 (n = 405). n = number of isolates collected.

Population	Sub-pop	Isolate numbers	Location type	Location name	Location coordinates	Date collected	Alt.(m)	Number of isolates	Fungicides	Host
1	10	PIC97001-PIC97023	VALLEY	Metepec	N19°14'35.5" S99°35'23.3"	3-Jul-97 8-Jul-97	2599	7	None	cv. Alpha
1	11	PIC97146-PIC97159	VALLEY	Metepec	N19°14'35.5" S99°35'23.3"	18-Jul-97	2599	13	Chloroth.	cv. Alpha
1	12	PIC97031-PIC97048	VALLEY	Metepec	N19°14'35.5" S99°35'23.3"	19-Jul-97	2599	13	None	cv. Alpha
1	13	PIC97651-PIC97665	VALLEY	Metepec	N19°14'35.5" S99°35'23.3"	1-Sep-97	2599	12	None	cv. Alpha
1	14	PIC97601-PIC97641	VALLEY	Metepec	N19°14'35.5" S99°35'23.3"	1-Sep-97	2599	34	Chloroth.	cv. Alpha
1	1	PIC97101-PIC97115	VALLEY	La Silva	N19°10'52.0" S99°35'01.8"	18-Jul-97	2707	11	Acrobat	cv. Alpha
1	2	PIC97116-PIC97130	VALLEY	La Comunidad	N19°11'12.8" S99°34'52.9"	18-Jul-97	2615	15	Manzate	cv. Alpha
1	3	PIC97131-PIC97144	VALLEY	Los Champiñones	N19°11'48.4" S99°33'29.3"	18-Jul-97	2590	14	Yes	cv. Atlantic
1	4	PIC97161-PIC97175	VALLEY	El Cerrito	N19°11'45.4" S99°36'48.5"	23-Jul-97	2600	12	Yes	cv. Atlantic
1	5	PIC97176-PIC97190	VALLEY	La Loma	N19°11'21.6" S99°36'04.5"	23-Jul-97	2650	13	Yes	cv. Atlantic
1	6	PIC97191-PIC97204	VALLEY	Las Minas	N19°14'27.2" S99°36'43.3"	23-Jul-97	2680	12	Yes	cv. Alpha
1	7	PIC97206-PIC97211	VALLEY	El Corral	N19°14'11.7" S99°34'11.4"	23-Jul-97	2650	6	Yes	cv. Alpha

Table 8-2. Continued.

Population	Sub-pop	Isolate numbers	Location type	Location name	Location coordinates	Date collected	Alt.(m)	Number of isolates	Fungicides	Host
1	8	PIC97221-PIC97235	VALLEY	El Refugio	N19°13'16.7" S99°41'52.1"	23-Jul-97	2821	9	Yes	cv. Atlantic
1	9	PIC97236-PIC97249	VALLEY	La Tolva	N19°12'35.5" S99°42'19.4"	23-Jul-97	2985	7	Yes	cv. Atlantic
2	1	PIC97301-PIC97310	RURAL	Loma Alta	N19°10'13.4" S99°48'17.7"	30-Jul-97	3447	7	Yes	"Criolla"
2	2	PIC97316-PIC97329	RURAL	Loma Alta	N19°10'08.4" S99°48'10.5"	30-Jul-97	3385	13	Yes	"Criolla"
2	3	PIC97331-PIC97344	RURAL	Raices	N19°09'54.8" S99°48'18.6"	30-Jul-97	3520	11	Yes	"Criolla"
2	4	PIC97348-PIC97359	RURAL	Loma Alta	N19°10'18.5" S99°48'31.0"	30-Jul-97	3578	9	Yes	"Criolla"
2	5	PIC97361-PIC97381	RURAL	Loma Alta	N19°10'45.5" S99°48'48.7"	30-Jul-97	3466	17	Yes	cv. Alpha
2	6	PIC97382-PIC97390	RURAL	Loma Alta	N19°10'40.6" S99°48'15.2"	30-Jul-97	3334	8	Yes	"Criolla"
2	7	PIC97391-PIC97403	RURAL	Loma Alta	N19°11'13.8" S99°48'31.0"	30-Jul-97	3323	9	Yes	cv. Marsiana
2	8	PIC97406-PIC97420	RURAL	Loma Alta	N19°11'13.0" S99°48'24.9"	30-Jul-97	3320	8	Yes	cv. Marsiana
2	9	PIC97421-PIC97432	RURAL	La Puerta	N19°11'31.0" S99°48'29.4"	30-Jul-97	3300	9	Yes	"Criolla"
2	10	PIC97438-PIC97448	RURAL	Buena Vista	N19°12'09.0" S99°49'39.9"	30-Jul-97	3150	6	Yes	"Criolla"

Table 8-2. Continued.

Population	Sub-pop	Isolate numbers	Location type	Location name	Location coordinates	Date collected	Alt.(m)	Number of isolates	Fungicides	Host
2	11	PIC98301-PIC98322	RURAL	Loma Alta	N19°11'13.0" S99°48'24.9"	2-Jul-98	3320	18	Yes	cv. Marsiana
3	1	PIC97701-PIC97721	WSS	Patch 1	N19°11'16.9" S99°48'31.9"	26-Sep-97	3225	20	None	S. demissum
3	2	PIC97722-PIC97736	WSS	Patch 3	N19°11'13.6" S99°48'29.4"	26-Sep-97	3240	12	None	*S. demissum*
3	3	PIC97737-PIC97742	WSS	Patch 3	N19°11'13.6" S99°48'29.4"	26-Sep-97	3240	6	None	S.x edinense
3	4	PIC97769-PIC97778	WSS	Patch 3	N19°11'13.6" S99°48'29.4"	4-Oct-97	3240	7	None	*S. demissum*
3	5	PIC97763-PIC97768	WSS	Patch 3	N19°11'13.6" S99°48'29.4"	4-Oct-97	3240	6	None	S.x edinense
3	6	PIC97743-PIC9757	WSS	Patch 4	N19°14'35.5" S99°35'23.3"	26-Sep-97	3200	15	None	*S. demissum*
3	7	PIC97779-PIC97789	WSS	Patch 7	-[1]	4-Oct-97	-	8	None	*S. demissum*
3	8	PIC97791-PIC97797	WSS	Patch 9	-	4-Oct-97	-	7	None	*S. demissum*
3	9	PIC98360-PIC98369	WSS	Patch 2	N19°11'16.9" S99°48'31.9"	14-Sep-98	3240	11	None	*S. demissum*
3	10	PIC98370-PIC98393	WSS	Patch 7	-	14-Sep-98	-	20	None	*S. demissum*

[1] - = information not available

Sensitivity to metalaxyl

Response to metalaxyl for the 1997 and 1998 samplings was determined by growing isolates *in vitro* on metalaxyl amended Rye A agar. Radial growth of each isolate on Rye A agar amended with 5 and in a separate test on Rye A agar with 100 µg ml^{-1} reagent grade metalaxyl (Novartis, Greensboro, NC) was compared to radial growth of the same isolate in the absence of metalaxyl. Each litre of medium was treated with 1ml of dimethylsulfoxide (DMSO) containing 0, 5 or 100 µg metalaxyl. Agar plugs (5mm diameter) were transferred to the centre of a Petri dish and incubated at 20°C in the dark for 8 days. Mean radial growth was measured in two directions and averaged for each of 3 replications in the non-amended treatment and 2 replications in the metalaxyl-amended treatments. Radial growth was corrected for plug diameter. To compare distribution of metalaxyl resistance, all readings were separated into 5 classes for relative growth on 5 or 100 µg ml^{-1} metalaxyl (= growth relative to the DMSO amended control) defined as: 0 – 19%, 20 – 39%, 40 – 59%, 60 – 79%, and 80 – >100%. To determine the reproducibility of the method, a subset of 47 isolates was assessed some months after the first assessment and the results of the two independent assessments were compared.

Because the frequency of metalaxyl sensitivity in 1997/98 was different from that reported for the 1988/89 collection (see Results), further tests on different formulations of metalaxyl (or mefenoxam, the active isomer of metalaxyl) were conducted. The different formulations were reagent grade metalaxyl, Ridomil-2E (metalaxyl, 25.1%) and Ridomil Gold-4E (mefenoxam, 46.2%) (Novartis Agro, Mexico City, Mexico). The sensitivities of a subset of 47 isolates to each of the formulations were determined. Reagent grade metalaxyl was dissolved in DMSO as described above with the same concentration of DMSO used for the corresponding control. For metalaxyl and mefenoxam, the active ingredients were already dissolved in a solvent of unknown composition, so distilled water was used as control.

Data analysis

A multi-locus genotype was constructed for each isolate by combining data for mating type, *Gpi* and *Pep* loci at each location sampled (Goodwin *et al.*, 1994). The A2 mating type was considered to be homozygous and the A1 heterozygous (Fabritius & Judelson, 1997; Judelson, 1996a,b; Judelson *et al.*, 1995). A chi-square analysis for mating type frequencies was conducted to detect departure from a 1:1 ratio. Genetic differentiation among populations was estimated using Nei's coefficient of differentiation (G_{ST}) (Nei, 1973; Slatkin & Barton, 1989) using POPGENE (Yeh & Boyle, 1997; Yeh *et al.*, 1997) POPGENE is available on-line at no cost from the University of Alberta, Canada. Unbiased expected heterozygosities (Levene, 1949; Nei, 1978) were calculated using TFPGA (Tools for Population Genetic Analyses, version 1.3; Department of Biological Sciences, Northern Arizona University, Flagstaff, AZ). Exact tests for population differentiation were determined using a Monte Carlo approach (10 batches, 2000

permutations per batch and 1000 dememorization steps) (Raymond & Rousset, 1995) as described in the TFPGA manual. Cluster analysis of multi-locus genotypes was based on allele frequencies observed for each population. Trees were constructed using the unweighted pair-group method of averages (UPGMA) algorithm from a Rogers' modified genetic distance matrix (Wright, 1978) based on multi-locus genotype as defined above using TFPGA. Statistical support for phenogram branches was obtained using 1,000 bootstrapped samples using TFPGA.

Genotypic diversity depends both on genotype richness, defined as the number of genotypes (g) observed in a sample, and evenness, which is the distribution of genotypes within the sample and reflects whether or not one or a few genotypes dominate the sample (Grünwald & Fry, 2000; Ludwig & Reynolds, 1988). Genotypic diversity was calculated as Shannon-Wiener's index $H' = \{-\Sigma_i[n_i \times \ln(n_i)]\}$ (Shannon & Weaver, 1949). Significance of differences between genotypic diversities were calculated for H' using a t-test (Magurran, 1988). The evenness index E_5 was calculated as

$E_5 = \frac{(1/\hat{\lambda})-1}{e^{H'}-1}$ (Ludwig & Reynolds, 1988),

where $\hat{\lambda}$ corresponds to Simpson's index (Simpson, 1949):

$\hat{\lambda} = \sum_{i=1}^{g} \frac{n_i(n_i-1)}{n(n-1)}$.

Genotypic richness expresses the number of expected genotypes in a sample and was estimated using rarefaction curves. Rarefaction curves assume that the number of genotypes g expected in a sample of n individuals in a population sampled to a total of N individuals, with number of individuals per genotype (n_i) distributed among each genotype i is

$$E(g_n) = \sum_{i=1}^{g} \left\{1 - \left[\frac{\binom{N-n_i}{N}}{\binom{N}{n}}\right]\right\}$$

$E(g_i)$ computes the expected number of species in a random sample of size n as the sum of the probabilities that each species will be included in the sample (Ludwig & Reynolds, 1988). The algorithm, implemented in C, calculates $E(g_i)$ for each sample size n = 1 to N and was validated using published data sets (Grünwald & Fry, 2000; Krebs, 1989; Livingston, 1976; Ludwig & Reynolds, 1988; Magurran, 1988). The algorithm is available upon request from the first author.

Classes of metalaxyl resistance were compared using the nonparametric Wilcoxon-Mann-Whitney test for two groups and Kruskal-Wallis test for three groups, which test the null hypothesis that the distribution of a response variable is the same in multiple independently sampled populations (PROC NPAR1WAY WILCOXON in SAS) (Stokes *et al.*, 1995). Linear regressions restricting the intercept to be zero (PROC REG with RESTRICT INTERCEPT = 0 option) were used to evaluate different formulations of

metalaxyl. This approach redefines the coefficient of determination (R^2) and is preferable to using the NOINT option in PROC REG (Freunds & Littell, 1991).

Results

Total population

The characteristics of the overall population in each of the two sampling periods (1988 and 1989 vs. 1997 and 1998) were quite similar as determined by neutral markers. For both sampling periods, there was much diversity and each population contained many rare genotypes. The 179 isolates sampled in 1988-89 and 401 isolates sampled in 1997-98 could be classified into 32 and 48 different genotypes (50 including genotypes with a five-banded *Gpi* allozyme pattern), respectively, based on mating type and *Gpi* and *Pep* allozyme patterns (Table 8-3). There were unique genotypes in each collection. A total of 15 out of 32 genotypes and 21 out of 48 genotypes (excluding isolates with five-banded *Gpi* allozyme) were detected only once each in the two sampling periods, corresponding to 8.4% and 5.2% of isolates appearing only once per sample for the 88-89 and 97-98 samples, respectively.

The most common genotypes (based on mating type, *Gpi* and *Pep*) were common to both sampling periods. The "A2, *86/100, 100/100*" genotype was the most common genotype in the 88-89 sample with a frequency of 18.4% (Table 8-3). The "A2, *100/100, 100/100*" genotype was most common in the 97-98 sampling period and showed a frequency of 12.6% (Table 8-3). In both sampling periods the *100 Gpi* and *Pep* alleles were most frequent (Table 8-4). The *83* and *130 Gpi* alleles were rare in the 1997-98 sampling period and were not detected in the 1988-89 sampling. A five-banded genotype was detected only in the 1997-98 sampling period at a low frequency (Table 8-3). Frequency patterns for each mating type and allozyme allele for the two sampling periods were very similar. Mating type frequencies did not depart significantly from a 1:1 ratio ($P = 0.204$ for 1988-89; $P = 0.803$ for 1997-98).

Genomic fingerprint

Southern blot analysis of two subsets of isolates belonging to the two, most common multi-locus genotypes in the 97-98 sampling period (defined by mating type, *Gpi* and *Pep*), revealed much diversity within each multi-locus genotype. Among 26 individuals with the "A2, *100/100, 100/100*" multi-locus genotype, 17 (65%) had unique RFLP fingerprints, and among 27 individuals with the "A2, *86/100, 100/100*" multi-locus genotype, 23 (85%) had unique RFLP fingerprints.

Table 8-3. Frequencies of multi-locus genotypes based on mating type, *glucose-6-phosphate isomerase* (*Gpi*) allozyme, and *peptidase* (*Pep*) allozyme patterns of isolates of *P. infestans* sampled in 1988 and 1989 (n = 179) and 1997 and 1998 (n = 401) excluding genotypes with five-banded *Gpi* allozyme pattern).

				1988-89						1997-98[2]						
				VALLEY		RURAL		WSS		overall	VALLEY		RURAL		WSS		overall
Geno-type	Mating type[1]	Gpi[2]	Pep	n[3]	g_i[4]	n	g_i	n	g_i	g_i	n	g_i	n	g_i	n	g_i	g_i
1	A1	*100/100*	*100/100*	6	0.043	-[5]	-	4	0.160	0.056	15	0.085	18	0.158	3	0.027	0.089
2	A1	*100/100*	*78/100*	-	-	-	-	-	-	-	-	-	1	0.009	-	-	0.002
3	A1	*100/100*	*92/100*	7	0.051	-	-	1	0.040	0.045	7	0.040	1	0.009	5	0.045	0.032
4	A1	*100/100*	*92/92*	-	-	-	-	-	-	-	1	0.006	1	0.009	1	0.009	0.007
5	A1	*100/100*	*96/100*	-	-	-	-	-	-	-	-	-	-	-	1	0.009	0.002
6	A1	*100/111*	*100/100*	2	0.014	-	-	-	-	0.011	-	-	-	-	1	0.009	0.002
7	A1	*100/122*	*100/100*	13	0.094	3	0.188	3	0.120	0.106	20	0.113	4	0.035	11	0.100	0.086
8	A1	*100/122*	*78/100*	-	-	-	-	-	-	-	-	-	1	0.009	-	-	0.002
9	A1	*100/122*	*92/100*	4	0.029	-	-	-	-	0.022	2	0.011	1	0.009	2	0.018	0.012
10	A1	*100/122*	*92/92*	-	-	1	0.063	-	-	0.006	-	-	1	0.009	-	-	0.002
11	A1	*100/122*	*96/100*	-	-	-	-	-	-	-	1	0.006	-	-	-	-	0.002
12	A1	*100/122*	*96/96*	-	-	-	-	-	-	-	2	0.011	-	-	-	-	0.005
13	A1	*122/122*	*100/100*	1	0.007	-	-	-	-	0.006	4	0.023	1	0.009	6	0.055	0.027
14	A1	*122/122*	*92/100*	-	-	-	-	-	-	-	2	0.011	-	-	-	-	0.005
15	A1	*122/122*	*92/92*	1	0.007	-	-	-	-	0.006	-	-	1	0.009	-	-	0.002
16	A1	*122/122*	*96/100*	-	-	-	-	-	-	-	1	0.006	-	-	-	-	0.002
17	A1	*83/122*	*100/100*	-	-	-	-	-	-	-	2	0.011	-	-	-	-	0.005
18	A1	*86/100*	*100/100*	14	0.101	2	0.125	-	-	0.089	14	0.079	11	0.096	7	0.064	0.079
19	A1	*86/100*	*92/100*	1	0.007	-	-	1	0.040	0.011	4	0.023	5	0.044	-	-	0.022
20	A1	*86/100*	*92/92*	2	0.014	-	-	-	-	0.011	1	0.006	-	-	-	-	0.002

Table 8-3. Continued.

Genotype	Mating type[1]	Gpi[2]	Pep	1988-89							1997-98[2]						
				VALLEY		RURAL		WSS		overall	VALLEY		RURAL		WSS		overall
				n[3]	g_i[4]	n	g_i	n	g_i	g_i	n	g_i	n	g_i	n	g_i	g_i
21	A1	*86/100*	*96/100*	-	-	-	-	-	-	-	2	0.011	-	-	1	0.009	0.007
22	A1	*86/100*	*96/96*	-	-	-	-	-	-	-	1	0.006	-	-	-	-	0.002
23	A1	*86/122*	*100/100*	20	0.145	1	0.063	1	0.040	0.123	12	0.068	1	0.009	3	0.027	0.040
24	A1	*86/122*	*92/100*	2	0.014	-	-	-	-	0.011	2	0.011	2	0.018	-	-	0.010
25	A1	*86/130*	*100/100*	-	-	-	-	-	-	-	1	0.006	-	-	-	-	0.002
26	A1	*86/86*	*100/100*	6	0.043	-	-	1	0.040	0.039	3	0.017	5	0.044	3	0.027	0.027
27	A1	*86/86*	*92/100*	-	-	-	-	-	-	-	1	0.006	2	0.018	-	-	0.007
28	A1	*86/86*	*92/92*	1	0.007	-	-	-	-	0.006	-	-	-	-	-	-	-
29	A2	*100/100*	*100/100*	6	0.043	2	0.125	4	0.160	0.067	13	0.073	13	0.114	25	0.227	0.126
30	A2	*100/100*	*78/100*	-	-	-	-	1	0.040	0.006	-	-	1	0.009	-	-	0.002
31	A2	*100/100*	*92/100*	1	0.007	-	-	-	-	0.006	-	-	6	0.053	7	0.064	0.032
32	A2	*100/100*	*92/92*	1	0.007	-	-	-	-	0.006	-	-	1	0.009	-	-	0.002
33	A2	*100/100*	*96/100*	-	-	-	-	-	-	-	-	-	-	-	8	0.073	0.020
34	A2	*100/100*	*96/96*	1	0.007	-	-	-	-	0.006	-	-	-	-	-	-	-
35	A2	*100/111*	*100/100*	-	-	-	-	1	0.040	0.006	-	-	-	-	-	-	-
36	A2	*100/111*	*92/100*	1	0.007	-	-	-	-	0.006	-	-	-	-	-	-	-
37	A2	*100/122*	*100/100*	9	0.065	-	-	-	-	0.05	15	0.085	8	0.070	10	0.091	0.081
38	A2	*100/122*	*78/100*	-	-	-	-	-	-	-	-	-	1	0.009	-	-	0.002
39	A2	*100/122*	*92/100*	1	0.007	-	-	-	-	0.006	5	0.028	5	0.044	-	-	0.025
40	A2	*100/122*	*92/92*	-	-	-	-	-	-	-	1	0.006	-	-	-	-	0.002
41	A2	*111/122*	*100/100*	-	-	1	0.063	-	-	0.006	-	-	-	-	-	-	-

Table 8-3. Continued.

				1988-89							1997-98[2]						
				VALLEY		RURAL		WSS		overall	VALLEY		RURAL		WSS		overall
Geno-type	Mating type[1]	Gpi[2]	Pep	*n*[3]	g_i[4]	*n*	g_i	*n*	g_i	g_i	*n*	g_i	*n*	g_i	*n*	g_i	g_i
42	A2	*122/122*	*100/100*	4	0.029	-	-	-	-	0.022	1	0.006	2	0.018	-	-	0.007
43	A2	*83/100*	*100/100*	-	-	-	-	-	-	-	-	-	1	0.009	-	-	0.002
44	A2	*86/100*	*100/100*	25	0.181	3	0.188	5	0.200	0.184	28	0.158	9	0.079	2	0.018	0.096
45	A2	*86/100*	*78/100*	-	-	-	-	-	-	-	-	-	2	0.018	-	-	0.005
46	A2	*86/100*	*78/96*	-	-	-	-	-	-	-	1	0.006	-	-	-	-	0.002
47	A2	*86/100*	*92/100*	3	0.022	-	-	3	0.120	0.034	2	0.011	2	0.018	1	0.009	0.012
48	A2	*86/100*	*92/92*	-	-	-	-	-	-	-	2	0.011	-	-	1	0.009	0.007
49	A2	*86/111*	*100/100*	-	-	-	-	-	-	-	1	0.006	-	-	-	-	0.002
50	A2	*86/122*	*100/100*	3	0.022	3	0.188	-	-	0.034	6	0.034	4	0.035	11	0.100	0.052
51	A2	*86/122*	*92/100*	1	0.007	-	-	-	-	0.006	1	0.006	-	-	-	-	0.002
52	A2	*86/122*	*92/92*	1	0.007	-	-	-	-	0.006	-	-	-	-	-	-	-
53	A2	*86/86*	*100/100*	1	0.007	-	-	-	-	0.006	2	0.011	3	0.026	1	0.009	0.015
54	A2	*86/86*	*92/100*	-	-	-	-	-	-	-	1	0.006	-	-	-	-	0.002
55	A1	*100/111/122*[2]	*100/100*	-	-	-	-	-	-	-	-	-	1	-	-	-	0.002
56	A2	*100/111/122*[2]	*100/100*	-	-	-	-	-	-	-	1	-	-	-	2	-	0.007

[1] The self-fertile isolate was excluded from analysis.

[2] The three-banded *Gpi* genotype was included in the overall frequency analyses, but ignored in the frequency analysis per population (VALLEY, RURAL, and WSS) and in the genotypic analysis. In the genotypic analysis *P. infestans* is considered to be a diploid organism and *Gpi* a co-dominant marker.

[3] *n* = number of isolates collected per multi-locus genotype.

[4] g_i = frequency of *i*th genotype.

[5] - = genotype not detected in this sample.

Populations from host-management associations

In both sampling periods the greatest diversity (based on neutral markers) occurred in the populations from the VALLEY and RURAL populations. Mating type A1 was more dominant in the VALLEY population for both sampling periods, while A2 predominated in the RURAL and WSS populations (Table 8-4). Only the WSS population in the 1997-98 sampling period deviated significantly from a 1:1 ratio for mating type frequencies (Table 8-4). The VALLEY population showed the largest number of multi-locus genotypes *g* (Table 8-5). Multi-locus genotypic diversity *H'* decreased from VALLEY to WSS populations (Table 8-5), and was significantly lower in the WSS population than in the VALLEY population for both sampling periods (Table 8-6).

The evenness index E_5, reflecting whether or not one or a few genotypes dominate the population, was very similar for the three populations in the 97-98 sampling period where sample size was large enough to test for evenness (Table 8-5). Using the rarefaction method on a sample size of n = 110 isolates in a population, we would expect to find 29 (or 25 in the 1988-89 sampling) distinct genotypes in the VALLEY, and 30 and 21 in RURAL and WSS populations, respectively (Table 8-5; Figure 8-2). Using a sample size of n = 16 isolates, which is the smallest sample size present, the number of expected genotypes decreases again from VALLEY to RURAL and WSS populations (Table 8-5). Combining information on evenness and richness, it appears that for the 1997-98 sampling period, where sample size was large, genotypic diversity differs mostly due to changes in richness rather than evenness (Table 8-5).

The VALLEY, RURAL and WSS populations differed from each other in population structure. Unbiased expected heterozygosity was greatest in the VALLEY populations and least in the WSS populations (Table 8-5). The proportion of total genetic diversity that is due to differentiation (G_{ST}) among subpopulations within populations was low to moderate within all populations. In the 1988/89 collections it ranged from 0.071 for the VALLEY population to 0.363 for the WSS population (Table 8-5). In the 1997/1998 collections it ranged from 0.053 in the VALLEY population to 0.174 in the WSS population (Table 8-5). Overall differentiation between the VALLEY, RURAL and WSS populations was moderate with G_{ST} values of 0.173 for the 88-89 and 0.095 for the 97-98 sampling periods.

Based on Rogers' genetic distance the VALLEY and RURAL populations are more closely related to each other than to the WSS populations in both sampling periods, and the VALLEY-RURAL node appeared in 70 and 50% of 1,000 bootstrapped samples (Figure 8-3). Based on exact tests, differentiation among the three populations was significant ($P < 0.001$). The VALLEY population was significantly differentiated from the WSS population in both sampling periods, while RURAL and VALLEY and RURAL and WSS populations were only significantly different in the 97-98 sampling period (Table 8-6).

Table 8-4. Frequencies of mating type, *glucose-6-phosphate isomerase* (*Gpi*), and *peptidase* (*Pep*) alleles by population (i.e., sampling location and date) (n = 179 and 401, respectively). Four isolates with five-banded *Gpi* genotype (*100/111/122*) were excluded from this analysis.

			Mating type			*Gpi*						Pep			
Date	**Location**	***N*** [1]	**A1**	**A2**	***P-value*** [2]	***83***	***86***	***100***	***111***	***122***	***130***	***78***	***92***	***96***	***100***
1988-89	VALLEY	138	0.580	0.420	0.061	- [3]	0.319	0.431	0.011	0.239	-	0.000	0.120	0.007	0.873
	RURAL	16	0.438	0.563	0.617	-	0.281	0.406	0.031	0.281	-	0.000	0.063	0.000	0.938
	WSS	25	0.440	0.560	0.549	-	0.240	0.660	0.020	0.080	-	0.020	0.100	0.000	0.880
1997-98	VALLEY	177	0.554	0.446	0.153	0.006	0.260	0.489	0.003	0.240	0.003	0.003	0.105	0.031	0.862
	RURAL	114	0.491	0.509	0.851	0.004	0.246	0.592	0.000	0.158	0.000	0.026	0.140	0.000	0.833
	WSS	110	0.400	0.600	0.036	0.000	0.155	0.618	0.005	0.223	0.000	0.000	0.086	0.046	0.868

[1] n = number of isolates collected.

[2] Probability values for a chi-square analysis for mating type frequencies to detect departure from a 1:1 ratio expected under Hardy-Weinberg conditions.

[3] - = genotype not detected in this sampling.

Table 8-5. Sample size (*n*), number of observed multi-locus genotypes (*g*), multi-locus genotypic diversity (*H'*), evenness (E_5), richness (g_{16}, g_{110}), unbiased expected heterozygosity (*Het*), and differentiation within and among populations for multiple loci (G_{ST}) for two sampling periods (1988-89 and 1997-98) and three populations (VALLEY, RURAL and WSS). This analysis excludes the five-banded *Gpi* genotype observed in the 1997-98 sampling.

Date	Location	n[1]	g[2]	H'[3]	E_5[4]	g_{16}[5]	g_{110}[6]	Het[7]	G_{ST}[8]
1988-89	VALLEY	138	28	2.8	0.74	10	25	0.432	0.071
	RURAL	16	8	2.0	1.59	8	-[9]	0.391	0.123
	WSS	25	11	2.2	1.23	9	-	0.360	0.363
	Overall	179	32	2.8	0.72	10	26	0.420	0.173
1997-98	VALLEY	177	35	2.9	0.74	11	29	0.429	0.053
	RURAL	114	30	2.9	0.81	11	30	0.408	0.062
	WSS	110	21	2.6	0.78	9	21	0.369	0.174
	Overall	401	48	3.1	0.71	11	28	0.409	0.095

1 *n* = number of isolates collected per population.

2 *g* = number of multi-locus genotypes observed.

3 *H'* = Shannon-Wiener diversity index.

4 E_5 = index of evenness.

5 g_{16} = expected number of genotypes calculated for a sample size of *n* = 16 isolates per population estimated using the rarefaction method.

6 g_{110} = expected number of genotypes calculated for a sample size of *n* = 110 isolates per population estimated using the rarefaction method. This index was only calculated for populations with large enough sample-sizes.

7 *Het* = unbiased expected heterozygosity.

8 G_{ST} = genetic differentiation among populations estimated using Nei's coefficient of differentiation (G_{ST}).

9 - = can only be calculated on populations with a sample size *n* >110.

Table 8-6. Probability values for population differentiation are shown above and for genotypic diversity are shown below the diagonal for both sampling periods. Probability values for the Shannon-Wiener genotypic diversity index were calculated using t-tests based on Magurran (1988). Probability values for population differentiation over three loci (mating type, *Gpi*, and *Pep*) were calculated using exact tests based on Raymond and Rousset (1995).

Date		VALLEY	RURAL	WSS
1988-89	VALLEY	-	0.773	0.019
	RURAL	<0.001	-	0.366
	WSS	<0.001	0.004	-
1997-98	VALLEY	-	0.006	<0.001
	RURAL	0.924	-	<0.001
	WSS	0.002	0.002	-

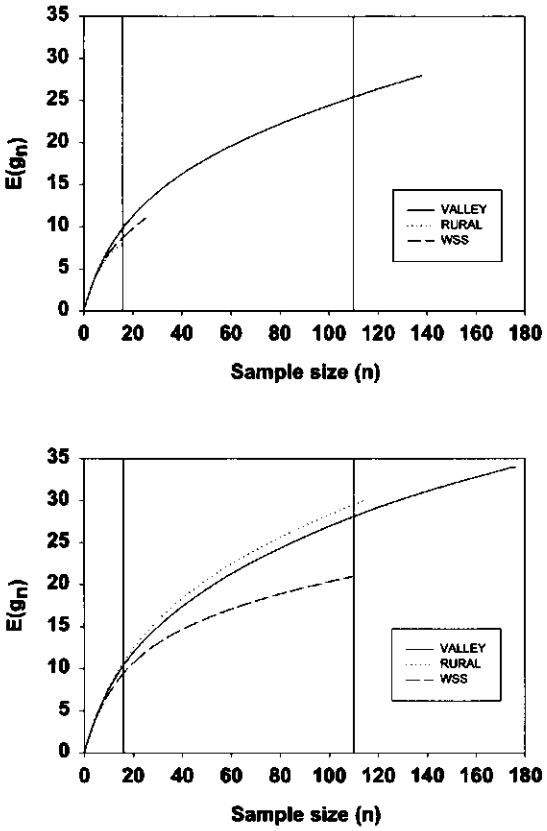

Figure 8-2. Expected number of multi-locus genotypes estimated using rarefaction curves for three populations of *P. infestans* within the Toluca Valley sampled in **A,** 1988-1989 and **B,** 1997-1998. The reference lines are drawn at n = 16 and n = 110 corresponding to information presented in Table 5. See figure 1 for description of populations.

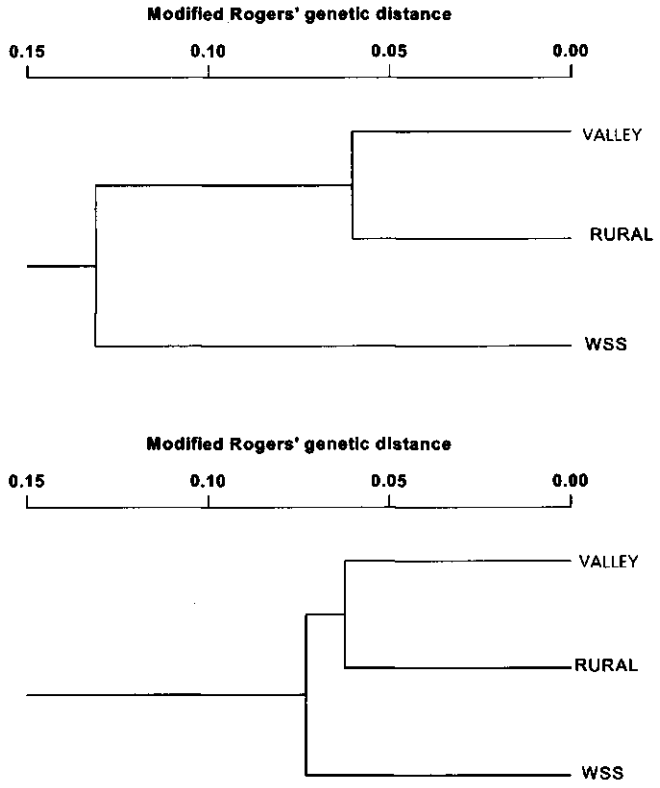

Figure 8-3. UPGMA cluster analysis of three populations of *Phytophthora infestans* within the Toluca Valley based on Rogers' genetic distance of *Pep* and *Gpi* allozyme loci sampled in **A,** 1988-1989 and **B,** 1997-1998. Statistical support for phenogram branches was obtained using 1,000 bootstrapped samples. See figure 8-1 for description of populations.

Metalaxyl resistance

In contrast to the frequent resistance to metalaxyl in the 1988/89 collections, most isolates in the 1997/1998 collection were sensitive to metalaxyl (Figure 8-4). The frequency distribution of sensitivity to metalaxyl at 5 and 100 ppm over all populations was bimodal (Figure 8-4A). At 5 ppm all populations had a bimodal distribution (Figure 8-4B), whereas at 100 ppm the bimodality was present only for VALLEY and WSS populations (Figure 8-4C). Frequency distributions of metalaxyl resistance (at both concentrations of metalaxyl) for the three populations were not significantly different based on the Kruskall-Wallis test (Figure 8-4B, $\chi^2 = 3.55$, $P = 0.1697$; and Figure 8-4C, $\chi^2 = 5.54$, $P = 0.0627$).

Because the frequency distribution of sensitivity to metalaxyl in the 1997/98 collections differed from that in the 1988/89 collections, a portion of 1997/98 collections was tested again using a subset of 47 isolates from the 1997/98 collections.

Correlations between metalaxyl resistance (relative growth of isolates on metalaxyl amended medium relative to that on medium without metalaxyl) in the two independent experiments on the same isolates were significant at both 5 μg ml^{-1} (r = 0.66; $P < 0.001$) and 100 μg ml^{-1} (r = 0.58; $P < 0.001$). Frequency distributions of metalaxyl resistance of the two independent assays were not significantly different based on the Wilcoxon 2-Sample Test (Z = 1.371, $P = 0.1703$ at 5 μg ml^{-1}; Z = 0.368, $P = 0.7132$ at 100 μg ml^{-1}).

Effect of formulation of metalaxyl

Isolates of *P. infestans* responded similarly to the two Ridomil products, metalaxyl and mefenoxam. Correlations between responses to the two commercial formulations of metalaxyl and mefenoxam were highly significant ($P < 0.001$) at 5 μg ml^{-1} (r = 0.91) and 100 μg ml^{-1} (r = 0.96) active ingredient. Similarly, correlations between responses to reagent grade and the commercial formulation of metalaxyl were highly significant ($P < 0.001$) at 5 μg ml^{-1} (r = 0.94) and 100 μg ml^{-1} (r = 0.93) metalaxyl. Control treatments with reagent grade metalaxyl vs. commercial formulations of metalaxyl/mefenoxam consisted of DMSO amended Rye-A agar vs. plain Rye-A, respectively. DMSO amended Rye-A agar reduces growth of *P. infestans* relative to plain agar by 7%: $y = 0.93 x$ ($r^2 = 0.99$), where y and x are radial growth (mm) on DMSO amended and plain Rye-A agar, respectively.

Discussion

Our observations confirm previous reports (Goodwin *et al.*, 1992a; Tooley *et al.*, 1985) and expand the description of high genetic diversity of *P. infestans* in the central highlands of Mexico. The overall population maintains approximately equal frequencies of the A1 and A2 mating types and a large diversity of multi-locus genotypes even within single fields. Additionally, there was significant diversity among isolates of identical mating type and allozyme pattern; 65% or 85% of these isolates in the two populations had a unique genomic fingerprint pattern. Clearly, the few markers used in this study are dramatically insufficient to identify identical individuals. There was no evidence for dominance by any particular genotype or clonal lineage.

Isolates in these collections had allozyme alleles previously unreported from central Mexico. The *111 Gpi* allele, previously reported only for Los Mochis and Saltillo (Goodwin *et al.*, 1992a), was observed at low frequencies in both sampling periods. The *90 Gpi* allele, found in one isolate out of 41 from a 1983 sampling in the Toluca Valley (Goodwin *et al.*, 1992a), was not observed in our sampling. The *78* and *96 Pep* alleles detected in our study have not been reported previously for the Toluca Valley ((Goodwin *et al.*, 1992; Tooley *et al.*, 1985). We did not find any novel alleles that are not currently listed in the global marker database for *P. infestans* (Forbes *et al.*, 1988).

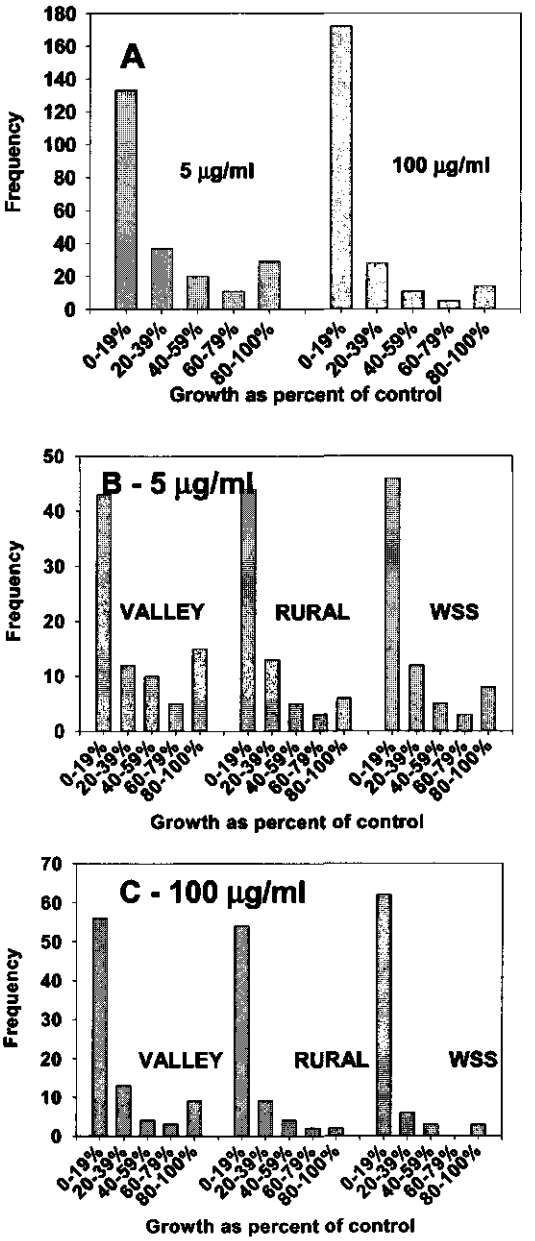

Figure 8-4. Relative growth of isolates of *Phytophthora infestans* on Rye-A agar amended with metalaxyl. **A,** Frequency distributions of relative growth of isolates from all three populations amended with metalaxyl [5 and 100 µg ml^{-1} (n = 230)], and of relative growth of isolates from VALLEY (n = 85), RURAL (n = 71) and WSS (n = 74) populations at **B,** 5 µg metalaxyl ml^{-1} and **C,** 100 µg metalaxyl ml^{-1}. See figure 8-1 for description of populations.

Our analysis distinguished the richness and evenness components of genotypic diversity, and indicated that changes in genotypic diversity were due mostly to changes in richness rather than evenness. For a population in which sexual reproduction is frequent and asexual reproduction is rare, high evenness would be expected. On the other hand, populations that reproduce mostly clonally with only an extremely low degree of sexual reproduction would likely be dominated by a few clones via a seasonal founder effect. Our finding of high evenness provides further support for the concept that sexual reproduction is common in the population of *P. infestans* in the highlands of central Mexico.

We found that there was very little sub-structuring of *P. infestans* populations according to host-management system in the region around Toluca. The WSS, VALLEY and RURAL populations differed from each other only slightly (G_{ST} = 0.173 in 1988/89 and 0.095 in 1997/98). In contrast to our expectation, our results did not support the hypothesis that the WSS population is genetically more diverse than either the RURAL or VALLEY population. Instead, genotypic diversity, richness and heterozygosity were greatest in the VALLEY population and were generally smallest in the WSS populations. There was no predominance by any genotype in any of the host-management systems. Based on the neutral markers used in this study, the WSS population was more distantly related to the VALLEY and RURAL populations than VALLEY and RURAL populations were to each other.

The selectable trait, metalaxyl resistance, had a very different frequency distribution in 1997-98 than in 1988-89. The proportion of isolates that grew more than 40% of the control on 5 and 100 μg ml^{-1} metalaxyl amended medium was much lower for isolates collected in 1997/1998 (13 %) than for isolates collected in 1988 and 89 (60%). The frequency for 1997-98 is similar to frequencies of metalaxyl resistance observed in central Mexico in the early 1980's (Matuszak *et al.*, 1994). It is unlikely that introduction of mefenoxam between the two sampling periods could have resulted in lowering the proportion of isolates sensitive to metalaxyl. We detected no differences in frequency distributions of resistance to either of the two formulations of Ridomil. It might be that farmers have learned the dangers of applying metalaxyl too often and are now applying it on average less than once per season (J. Morgado, NOVARTIS Agro, Mexico, *pers. comm.*).

The similarity in frequency of metalaxyl (mefenoxam) resistance among isolates from the VALLEY, RURAL and WSS systems in both collections was initially unexpected. The wild species are not treated with fungicide so selection for metalaxyl resistance does not occur on these plants. Instead, the occurrence of similar metalaxyl resistance frequencies in all three systems suggests that gene flow is common.

Observations on the chronology of epidemics within a field season and geographical relationships among the populations are consistent with our observation that WSS populations are genetically less diverse than RURAL or VALLEY populations. Epidemics within the central valley usually start at the beginning of July when rainfall occurs almost daily (Grünwald *et al.*, 2000c). However, epidemics of late blight on wild

Solanum species rarely occur before the end of August or beginning of September. In addition to the chronological patterns, the geographic relationship might also play a role in structuring populations. Wild *Solanum* species occur at the edges of forests, RURAL potato fields and sometimes at the edge of commercial fields.

We therefore hypothesize that the WSS populations in the late 20th century were most likely derived from RURAL and/or VALLEY populations. This hypothesis is supported by observations that (1) genetic diversity is significantly lower in the WSS population compared to the RURAL/VALLEY populations, and (2) that the frequency distribution of metalaxyl resistance is not significantly different between the WSS and the RURAL/VALLEY populations. It seems likely that the situation at the end of the 20th century is different than it was in the middle of the 20th century when large-scale potato production was first introduced to the highlands of central Mexico. In the mid-20th century, wild species were regarded as the source of *P. infestans* that infected commercial potatoes. Decades of intensive potato production could have altered the situation so that at the end of the 20th century, populations of *P. infestans* associated with intensive commercial potato production dominate the entire region, including the wild species.

Acknowledgements

PICTIPAPA (Programa Internacional Cooperativo del Tizón Tardío de la Papa), the CEEM (Cornell-Eastern Europe-Mexico) Potato Late Blight Project and HPA (Dutch Potato Industry) funded this work. We thank PICTIPAPA for use of their infrastructure; ICAMEX (Instituto de Investigación y Capacitación Agropecuaria, Acuícola y Forestal del Estado de México) and INIFAP (Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias) for providing us with research equipment, as well as laboratory and greenhouse space; CIMMYT (Centro Internacional de Mejoramiento de Maíz y Trigo) for their continuous support. We thank Telesforo Zavala for providing us with two reference isolates; José Gomez Nieva and many potato growers in the Toluca Valley for letting us sample their fields; and the Centro Medico Toluca for use of their X-ray processor. We thank Tobin L. Peever for many suggestions improving the manuscript.

9

The population structure of *Phytophthora infestans* from the Toluca Valley in central Mexico suggests genetic differentiation between populations from cultivated potato and wild *Solanum* species

W. G. Flier, N. J. Grünwald, L. P. N. M. Kroon, A. K. Sturbaum, T.B.M. van den Bosch, E. Garay-Serrano, W. E. Fry and L. J. Turkensteen

To be submitted

Summary

The population structure of *Phytophthora infestans* in the Toluca Valley of central Mexico was assessed using 170 isolates collected from cultivated potatoes and the native wild *Solanum* species *S. demissum* and *S.* x *edinense*. All isolates were analyzed for mtDNA haplotype and AFLP multilocus fingerprint genotype. Isolate samples were monomorphic for mtDNA haplotype as all isolates tested were of the Ia haplotype. A total of 158 multilocus AFLP genotypes were identified among the 170 *P. infestans* isolates included in this study. *Phytophthora infestans* populations sampled in the Toluca Valley in 1997 were highly variable, and almost every single isolate represented a unique genotype, based on the analysis of 165 dominant AFLP marker loci. Populations of *P. infestans* collected from the commercial potato-growing region in the valley, the subsistence potato production area along the slopes of the Nevado de Toluca and from native *Solanum* species on the forested slopes of the extinct volcano showed a high degree of genetic diversity. The number of polymorphic loci varied from 20.0% to 62.4% for isolates collected from the field station and wild *Solanum* species. On average, 81.8% (135) of the AFLP loci were found to be polymorphic. Heterozygosity varied between 7.7 and 19.4%. Significant differentiation was found at the population level, between strains originating from cultivated potatoes and wild *Solanum* species (P = 0.001 to 0.022). Private alleles were observed in individual isolates collected from all three populations, with numbers of unique dominant alleles varying from 9 to 16 for isolates collected from commercial potato crops and native *Solanum* species respectively. Four AFLP markers were exclusively found present in isolates collected from *S. demissum*. Indirect estimation of gene flow between populations indicated restricted gene flow between both *P. infestans* populations from cultivated potatoes and wild *Solanum* hosts. There was no evidence found for the presence of sub-structuring at the sub-population (field) level. We hypothesize that population differentiation and genetic isolation of *P. infestans* in the Toluca Valley is driven by host specific factors (i.e. R-genes) widely distributed in wild *Solanum* species and random genetic drift.

Introduction

The central highlands of Mexico, which include the Toluca Valley, are considered to be the centre of origin of the oomycete *Phytophthora infestans* (Mont.) de Bary, causing potato late-blight (Reddick, 1943). Genetic diversity of virulence (Mills & Niederhauser, 1953; Niederhauser *et al.*, 1954; Tooley *et al.*, 1986; Rivera-Peña, 1990b; Turkensteen, 1993), allozymes (Tooley *et al.*, 1985; Goodwin *et al.*, 1992a), and RFLP loci (Goodwin *et al.*, 1992a) has been extensively studied using the *P. infestans* population in the Toluca Valley. Both mating types can be found in approximately a 1:1 ratio (Goodwin *et al.*, 1992a) and oospores are commonly found in potato crops ((Niederhauser 1956; Gallegly & Galindo, 1958) and native *Solanum* species (Flier *et al.*, 2001b). The population of *P. infestans* in the Toluca Valley of central Mexico is therefore believed to be the most diverse in the world (Tooley *et al.*, 1985; Fry & Spielman, 1991; Goodwin *et al.*, 1992a). The Toluca Valley is located at an altitude of 2,600m and mountain ridges surround the valley itself (Grünwald *et al.*, 2000b). On the southwest the valley is delimited by the volcano Nevado de Toluca, reaching an altitude of 4,690 m. Potatoes

are grown until about 3,500m of altitude and wild species of *Solanum* occur at the edges of pine and *Abies* forests up to about 3,800m altitude (Rivera-Peña & Molina-Galan, 1989; Rivera-Peña 1990a,b,c,d).

The *P. infestans* meta-population in the Toluca valley can be differentiated into three populations based on host-plant and potato production systems. In the central valley, high input potato fields are prevalent, while low input potato production is found on the lower slopes of the volcano. In addition, *P. infestans* infects several wild *Solanum* species native to central Mexico (Rivera-Peña & Molina-Galan, 1989).

Reports of specialised strains with differential pathogenicity to potato and tomato (Turkensteen, 1973; Lebreton *et al.*, 1999) fuelled speculation about the presence of host preference and specialization within *P. infestans*. Recently, Ordoñez *et al.* (2000) provided evidence suggesting the presence of population differentiation and host specificity in *P. infestans*. They reported on an Ecuadorian *Phytophthora* A2 population (closely resembling *P. infestans*) from a native *Solanum* species. This A2 population appears to be strictly isolated from potato strains by host-plant specificity.

While host-plant specialisation has been reported for these systems, limited information is available on presence and implications of population differentiation and isolating mechanisms in *P. infestans* from the Toluca Valley. Earlier reports (Fry & Spielman, 1991; Matuszak *et al.*, 1994; Grünwald *et al.*, 2000b Grünwald *et al.*, in preparation) used the selectable marker for metalaxyl-resistance and neutral *Pep* and *Gpi* allozyme markers to explore the possibility of population differentiation of *P. infestans* in the Toluca Valley. Metalaxyl resistant strains were found on commercial potato crops (heavily treated with metalaxyl) and native *Solanum* species (not treated with metalaxyl) in similar frequencies, suggesting that strains migrate freely from potato crops to native *Solanum* species and back. Allozyme data indicated that heterogeneity and genetic diversity in *P. infestans* populations declined from valley to rural and wild *Solanum* species. Little support was found for the hypothesis that the native hosts *Solanum demissum* and *S.* x *edinense* act as differential host plant species harbouring specialised populations of *P. infestans*. Some indications for the presence of isolating mechanisms in *P. infestans* populations from the Toluca Valley have been reported (Flier *et al.*, 2001b). Significant differences in oospore production were observed between *in vitro* matings of *P. infestans* strains collected from potato and *S. demissum*. Isolates originating from the native host *S. demissum* produced significantly more oospores in crosses with compatible strains collected from the same host species as compared to crosses with isolates collected from cultivated potatoes.

Our previous studies provided only few indications for the presence of host specific populations of *P. infestans* in the Toluca Valley (Grünwald *et al.*, 2000b, Flier *et al.*, 2001b). Sufficient neutral DNA markers had until now not been deployed to measure genetic diversity and gene flow between host specific populations of *P. infestans* to determine whether population differentiation is likely to occur in the centre of diversity of the late blight pathogen. The objectives of this study were to: (i) characterize the genetic structure of *P. infestans* populations in the Toluca Valley using

mitochondrial DNA haplotype and AFLP markers to determine genetic diversity and gene flow of *P. infestans* at its centre of diversity; and (ii) to determine whether populations of *P. infestans* on native *Solanum* species are merely derived from populations on cultivated potatoes or whether they should be regarded as host-specific sub-populations of the total Valley population.

Materials and methods

Definitions

An operational definition of population was used as described in Grünwald *et al.* (2000a). Populations of *P. infestans* were defined by both host composition and potato growing practice in regions in which they were sampled: (1) large potato fields in the central valley, where the late blight susceptible cultivars Alpha and Atlantic are grown (VALLEY), (2) small fields containing cultivar mixtures of locally grown potato cultivars locally known as "papas criollas" or commercial varieties (mostly Alpha and Rosita) grown by subsistence farmers on the slopes of the volcano (RURAL), and (3) patches of *S. demissum* (200-5000 plants per population) and *S.* x *edinense* (usually individual plants within "Criolla fields") (WSS). Individual sampling sites (fields or patches of wild *Solanum* species) sampled within one of the three defined populations were considered to be sub-populations of assigned populations.

Sources and cultivation of isolates

A total of 371 isolates were collected from the three populations WSS, VALLEY and RURAL in 1997 (Table 9-1). Collections were made in three areas in the Toluca Valley using a hierarchical sampling strategy described previously (Grünwald *et al*, 2001). The sampling scheme consisted of isolating 10-15 isolates each from at least 10 fields in the valley (VALLEY), on the slopes of the volcano (RURAL) and 10 patches of wild *Solanum* species (WSS). Each isolate originated from a randomly selected single leaf lesion of *Solanum tuberosum*, *S. demissum*, or *S.* × *edinense*. These populations were characterized previously using mating type, isozyme, metalaxyl and RFLP markers (Grünwald *et al*, 2000a,b; chapter 8). We used 170 isolates that were available in the *Phytophthora infestans* isolate collection at Plant Research International and assumed they represent a random subsample of the isolates collected during multiple field collections in 1997. Isolation and culture were as described previously (Flier *et al.*, 2001b; chapter 8).

DNA extraction

Isolates were grown for 10 to 14 days at 20 °C in pea broth (with 200 mg l^{-1} ampicillin added) prepared by autoclaving 120 g of frozen peas in 1 l of water. The peas were removed by filtering through cheesecloth and the broth was autoclaved again. The

mycelium grown on pea broth, harvested, lyophilised and stored at –80 °C. Lyophilised mycelium (10 to 20 mg) was ground in microcentrifuge tubes with a pestle and sterile sand. Total DNA was extracted using the Puregene kit (Gentra/Biozym, Landgraaf, the Netherlands) according to manufacturers instructions. DNA was dissolved in 100 µl of TE (10 mM Tris-HCl [pH 8.0], 1mM EDTA [pH 8.0]) and stored at –20 °C.

Mitochondrial DNA (mtDNA) haplotypes

The P1 (1,118 bp), P2 (1070 bp), P3 (1308 bp) and P4 (964 bp) regions of the mitochondrial genome were amplified using primers and methods described by Griffith & Shaw (1998). Reactions were performed in a PTC200 thermocycler (MJ Research/Biozym). Digestion of PCR products with the restriction enzymes *Cfo*I, *Msp*I and *Eco*RI results in restriction fragment band patterns that can be classified into four different mtDNA haplotypes: Ia, Ib, IIa and IIb (Griffith & Shaw, 1998).

Fluorescent amplified fragment length polymorphisms (AFLP)

DNA (250 ng) was digested in a 50 µl reaction volume with *Eco*RI (10U) and *Mse*I (10U) for 6 hours at 37 °C in restriction ligation buffer (10 mM Tris/Ac [pH 7.5], 10 mM MgAc, 50 mM Kac, 5 mM DTT, 50 ng μl^{-1} BSA). Digestion was confirmed on agarose gels. Restriction fragments were ligated to *Mse*I adapters (5'GACGATGAGTCCTGAT/CTACTCAGGACTAGC 5') and EcoRI adaptors (5' CTCGTAGACTGCGTACC/CATCTGACGCATGGTTAA 5') using 0.1 µM *Eco*RI adapter, 1.0 µM *Mse*I adapter, 0.2 mM ATP and 2.4 U T4 DNA ligase (Amersham Pharmacia Biotech, Uppsala, Sweden) (Baayen *et al.*, 2000). Ligation was performed overnight at 10-12 °C and the ligation products were diluted 10 times with filtered ultra pure water. Nonselective PCR amplification was performed using primers E00 (5' GACTGCGTACCAATTC) and Mse00 (5' GATGAGTCCTGAGTAA) for all restriction fragments. Non-selective PCR amplifications were performed in a PTC200 thermocycler (MJ Research/Biozym) as described previously (Bonants *et al.*, 2000). The amplified restriction fragment products were checked on 1.0% agarose gels.

Selective PCR was performed in a 50 µl reaction volume with 5 µl of 20 X diluted amplification products as described previously (Baayen *et al.*, 2000), but with 200 µM dNTP and 5 ng of Cy5-labeled fluorescent Eco21 primer (5' CTCGTAGACTGCGTACC), and 30 ng of Mse16 (5' GATGAGTCCTGAGTAACC) primer. Products were loaded on Sequagel (Biozym) polyacrylamide gels and run on an ALFexpress automatic sequencer (Amersham). Conditions were 1500 V, 60 mA, 35 W, and 55 °C. On each gel, 36 samples were loaded together with flanking Cy5-labelled fluorescent 50 bp ladders (Amersham) and two reference isolates (PIC96001 and US-1-VK6C).

Data analysis

Each isolate was classified into a mtDNA haplotype after visualization of the restriction fragments on agarose gels using ethydium bromide under UV illumination. AFLP patterns were analysed using Imagemaster ID software (Pharmacia Biotech), manually correcting for faint bands and exclusion of controversial bands. A total of 158 distinct and reproducible AFLP bands were identified using the primers *Eco-21* and *Mse*-16. Bands were treated as putative single AFLP loci and a binary matrix containing the presence or absence of these reproducible bands was constructed and used for further analysis. The matrix is available from the first author upon request.

Statistical analyses were conducted with POPGENE 1.31, available at no cost from the University of Alberta, Canada and TFPGA (Tools for Population Genetic Analyses, version 1.3) available at no cost from Mark P. Miller at Northern Arizona University, Flagstaff, AZ). Each AFLP band was assumed to represent the dominant genotype at a single locus while the absence of that same band represents the alternate homozygous recessive genotype. We assumed a diploid model with 2 alleles per locus and estimated the frequency of the recessive allele by a Taylor expansion estimator (Lynch & Milligan, 1994), which proves an alternative and apparently less biased estimator as compared to the square root of the frequency of "blanks" seen at a particular AFLP locus.

Genotypic diversity analysis was used to determine the distribution of genetic diversity among populations (VALLEY, RURAL an WSS) and among subpopulations of *P. infestans* collected from patches of *S. demissum* or *S. x edinense*. Heterozygosity and percent polymorphic loci (95% criterion) was estimated for populations and WSS subpopulations. Genotypic diversity was calculated using Shannon's information index (Shannon & Weaver, 1949). Pair-wise measures of Rogers' modified genetic distance and population differentiation using Nei's coefficient of differentiation (G_{st}) (Nei, 1973; Slatkin & Barton, 1989) were calculated using POPGENE.

Population structure was analysed using *F* statistics (Weir & Cockerham, 1984) in order to test the significance of the different statistics for the null hypothesis of no differentiation at the corresponding hierarchical level. Permutation and re-sampling tests (jackknifing and bootstrapping) were carried out according to calculate estimates for standard errors. In our analysis, we used a two-level hierarchy with corresponding statistics F_{pop} and F_{site} for sites within populations and isolates within sites, respectively. Populations were defined according to their geographic location and potato production system.

Differentiation among populations was estimated using an exact test (Raymond & Rousset, 1995) and by indirect estimation of gene flow using G_{ST} with $Nm = ¼(1- G_{ST}) / G_{ST}$ (Nei, 1973; Slatkin, 1987), where *N* is the effective population size, *m* is the immigration rate, and *Nm* is the average number of migrants among populations per generation (Slatkin, 1987).

Cluster analysis of multilocus AFLP genotypes was based on allele frequencies observed for each population. A phenogram was constructed using the unweighted pair-group

method of averages (UPGMA) algorithm from a Rogers' modified genetic distance matrix. Bootstrap sampling (1000 replicates) was performed for parsimony analysis of the constructed phenogram (Felsenstein, 1985).

Table 9-1. Sample sizes and indicators of genotypic diversity for four Mexican *Phytophthora infestans* field populations sampled in the Toluca Valley in 1997 based on AFLP fingerprinting. A total of 165 dominant marker loci were obtained.

Population	Location	n^a	g^b	h_0^c	P^d	H^e	Metalaxyl tolerance[f]		
							Sensitive	Intermediate	Resistant
VALLEY	Field station	12	12	2.485	20.0	0.077	10	1	1
	Commercial fields	41	41	3.714	44.2	0.153	28	7	6
RURAL	low input landraces	35	34	3.545	38.2	0.192	27	5	3
WSS	Wild *Solanum* species	80	75	4.270	62.4	0.194	71	9	2
Overall		170	158	5.023	81.8	0.210	136	22	12

[a] n = population size
[b] g = number of multilocus AFLP genotypes in population
[c] h_0 = Shannon index
[d] p = Percentage of polymorphic loci (99 % criterion)
[e] H = Average unbiased proportion heterozygosity
[f] Data from Grünwald *et al.* (in prep.).

Results

Variation revealed by markers

mtDNA haplotypes. All 170 isolates from the three populations were of Ia haplotype.
AFLP fingerprinting. A total of 158 distinct multilocus AFLP genotypes were found among 170 *P. infestans* isolates included in this study (Table 9-1). In general, *P. infestans* populations sampled in the Toluca Valley in 1997 were highly variable, and almost every single isolate represented a unique genotype, based on the analysis of 165 dominant AFLP marker loci. Populations of *P. infestans* collected from the commercial potato-growing region in the valley, the subsistence potato production area along the slopes of the Nevado de Toluca and from wild *Solanum* species on the forested slopes of the volcano showed a high degree of genetic variability. The number of polymorphic loci (based on the 95% criterion) varied from 20.0% to 62.4% for isolates collected from the field station and wild *Solanum* species respectively (Table 9-1). Overall, 81.8% (135) of the AFLP loci were found to be polymorphic. Heterozygosity obtained by indirect estimates of allele frequencies based on a Taylor expansion (Lynch & Milligan, 1994) varied between 7.7 and 19.4% (Table 9-1). Again, isolates collected from wild

Solanum hosts showed the highest level of heterozygozity, while isolates from the experimental plots at the field station showed only half of the variation as compared to isolates from commercial fields and locally grown potato cultivars. Genotypic diversity measurements revealed a similar pattern of genetic diversity between field populations with the highest genotype diversity for the isolates collected on native *Solanum* species (Table 9-1).

Most parameters describing the genetic variation at the sub-population level were very similar, based on the results of the fine-grain sampling of *P. infestans* isolates from 5 patches of native *Solanum* species (Grünwald *et al.*, 2000b), mainly consisting of *S. demissum* and in the case of patch 3 also including isolates collected from *S. x edinense* (Table 9-2). Sample sizes varied between 2 and 32 isolates per patch for patch 9 and 3 respectively. Heterozygozity and the level of genotypic diversity were comparable between the six distinguished sub-populations of *P. infestans* collected from *S. demissum* and *S. x edinense* (Table 9-2). The number of polymorphic loci varied between sub-populations (Table 9-2), but is clearly associated with the number of unique genotypes in a sample ($r = 0.961$).

Metalaxyl sensitivity. Metalaxyl resistant or intermediate tolerant isolates were observed for all four *P. infestans* populations sampled (Grünwald *et al.*, 2000b; chapter8) (Table 9-1). Metalaxyl tolerance was found in isolates originating from wild *Solanum* species. Resistance to metalaxyl was detected in isolates from all patches except patch 4, with relatively high numbers of intermediate or resistant isolates originating from *S. x edinense* (Table 9-2). A total of 4 isolates out of 13 collected from *S.* x *edinense* were found to be resistant or intermediate while 7 out of 67 isolates from *S. demissum* were determined to be less sensitive to metalaxyl (Table 9-2). Pearson's χ^2test revealed a positive association between host plant species and metalaxyl insensitivity ($P = 0.05$).

Population differentiation

Results based on the test for population differentiation indicate strong population sub-structuring between *P. infestans* populations originating from cultivated potato and wild *Solanum* species (Table 9-3). The χ^2 test statistic probabilities for the pair-wise comparisons between VALLEY RURAL and WSS populations lead to rejection of the null hypothesis stating an absence of population differentiation in the case of VALLEY vs. WSS ($P = 0.001$) and RURAL vs. WSS ($P = 0.022$). Genotypic diversity measures were similar for 28 pairs of sub-populations of *P. infestans* in the Toluca Valley (Table 9-4). Genetic identity, as presented by Nei's genetic identity index, revealed only limited differences between the populations included in this study. Based on Rogers' genetic distance coefficients VALLEY and RURAL populations are more closely related to each other than to sub-populations of *P. infestans* collected from wild *Solanum* species (WSS-population) (Figure 9-1).

Table 9-2. Sample sizes and indicators of genotype diversity for six Mexican sub-populations of *Phytophthora infestans* collected on wild *Solanum* host plant species in the Valley of Toluca in 1997 based on AFLP fingerprinting. A total of 165 dominant marker loci were obtained.

	Host species	n^a	g^b	h_o^c	P^d	H^e	Metalaxyl tolerance		
Sub-population							**Sensitive**	**Intermediate**	**Resistant**
Patch 1	*S. demissum*	23	21	3.015	35.2	0.122	22	1	0
Patch 3	*S. demissum*	19	18	2.871	37.6	0.143	17	1	1
Patch 3	*S. x edinense*	13	12	2.458	27.3	0.111	9	3	1
Patch 4	*S. demissum*	18	17	2.813	30.9	0.114	18	0	0
Parch 7	*S. demissum*	5	5	1.609	21.8	0.098	2	3	0
Patch 9	*S. demissum*	2	2	0.693	18.8	0.104	1	1	0
Overall		80	75	5.023	62.4	0.192	69	9	2

[a] n = population size
[b] g = number of genotypes in population
[c] h_o = Shannon index
[d] p = Percentage of polymorphic loci (99% criterion)
[e] H = Average unbiased proportion heterozygosity
[f] Unpublished data.

Table 9-3. Pairwise population differentiation in *Phytophthora infestans*. Below the diagonal probabilities for each pairwise comparison using an exact test (Raymond & Rousset, 1995). Above diagonal, estimates of pairwise number of migrants (*Nm*).

Population	**VALLEY**	**RURAL**	**WSS**
VALLEY		4.417	1.053
RURAL	1.000		0.785
WSS	0.001	0.022	

Table 9-4. Matrix of genetic similarity and distance coefficients for *Phytophthora infestans* populations. Below the diagonal, Rogers' modified genetic distance; above the diagonal, Nei's genetic identity.

Population		Field station	Comm. fields	Criolla's	Patch 1	Patch 3	Patch 4	Patch 7	Patch 9
VALLEY	Field station		0.985	0.991	0.957	0.959	0.950	0.950	0.915
VALLEY	Comm. fields	0.129		0.983	0.970	0.976	0.961	0.957	0.932
RURAL	Criolla's	0.105	0.129		0.961	0.957	0.948	0.947	0.915
WSS	Patch 1	0.204	0.168	0.192		0.976	0.952	0.955	0.946
WSS	Patch 3	0.201	0.152	0.198	0.153		0.947	0.944	0.936
WSS	Patch 4	0.220	0.190	0.219	0.212	0.222		0.964	0.933
WSS	Patch 7	0.226	0.206	0.227	0.213	0.233	0.193		0.956
WSS	Patch 9	0.298	0.264	0.293	0.243	0.26	0.266	0.231	

Rare alleles were observed in individual isolates collected from all three populations, with the number of unique dominant alleles varying from 9 to 16 for VALLEY and WSS respectively. Four AFLP markers were exclusively found present in isolates collected from *S. demissum* (varying from 7 to 11 unique genotypes per marker). Sampling effects could not explain the observed differences in frequency of the four AFLP alleles (Chi-square based goodness of fit test, not shown). Indirect estimation of gene flow between populations using *Nm* as a measurement of population differentiation indicated restricted gene flow between both *P. infestans* populations from potato (VALLEY and RURAL) and wild *Solanum* hosts (WSS) (Table 9-5). On average, less than 1 migrant per generation was estimated between potato and *S. demissum* and *S. x edinense* populations (Table 9-5). Low differentiation was measured between VALLEY and RURAL *P. infestans* populations (Table 9-5).

Gene flow was generally greater between VALLEY and RURAL populations as compared to the WSS population when fine-grain comparisons between pairs of sub-populations were made (Table 9-4). Significant population subdivision was detected within the WSS population. Patch 3 and 4 showed a significant differentiation (P = 0.001) using the exact test for population differentiation. In addition, significant differences were detected between most WSS patches and the VALLEY and RURAL populations (Table 9-4). Most of the population sub-structuring could be attributed to differences at the population level, while only certain WSS patches were found to differentiate at the sub-population level (Table 9-4, Figure 9-2).

Table 9-5. Probabilities of pair-wise population differentiation and migration in *Phytophthora infestans*. Below the diagonal probabilities for each pairwise comparison using an exact test (Raymond and Rousset, 1995). Above diagonal, estimates of pairwise number of migrants (*Nm*).

Population	Sub-population	Field station	Comm. fields	Criolla's	Patch 1	Patch 3	Patch 4	Patch 7	Patch 9
VALLEY	Field station		4.050	5.069	2.003	1.440	2.162	0.866	0.633
VALLEY	Comm. fields	1.000		13.261	3.821	3.403	2.387	1.127	0.895
RURAL	Criolla's	1.000	1.000		3.626	2.667	2.647	1.058	0.739
WSS	Patch 1	1.000	0.996	0.999		5.250	2.461	1.249	0.895
WSS	Patch 3	0.999	0.944	0.464	1.000		1.614	0.965	0.778
WSS	Patch 4	1.000	0.366	0.210	0.958	0.001		1.564	0.863
WSS	Patch 7	1.000	1.000	1.000	1.000	0.999	1.000		1.177
WSS	Patch 9	1.000	1.000	1.000	1.000	1.000	1.000	1.000	

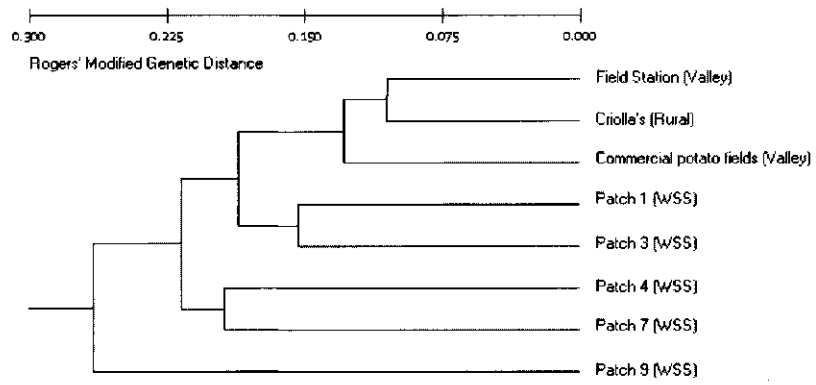

Figure 9-1. Cluster analysis of 8 sub-populations of *Phytophthora infestans* in Toluca Valley using 165 putative AFLP loci. Distance was estimated using Rogers' Modified Genetic Distance coefficients ((Wright, 1978). Dendrogram drawn by UPMGA clustering.

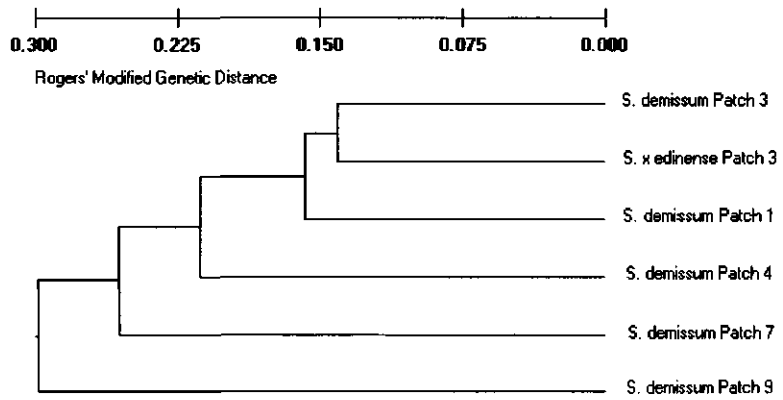

Figure 9-2. Cluster analysis of 6 populations of *Phytophthora infestans* collected from patches of *Solanum demissum* or *S. x edinense* from the Toluca Valley using 165 putative AFLP loci. Distance was estimated using Rogers' Modified Genetic Distance coefficients (Wright, 1978). Dendrogram drawn by UPMGA clustering.

Discussion

Isolate diversity

The first goal of our study was to use mtDNA and AFLP markers to characterize the genetic structure of *P. infestans* populations located in the Toluca Valley, central Mexico. AFLP markers used in this study revealed a high degree of genetic variability in the three *P. infestans* populations sampled in the Toluca Valley. Almost every single isolate represented a unique genotype based on 165 dominant AFLP markers. Our observations confirm previous reports (Goodwin *et al.*, 1992a; Matuszak *et al.*, 1994; Grünwald *et al.*, 2000a) of high genetic diversity of *P. infestans* in central Mexico and add to the extensive evidence that support the hypothesis of central Mexico being the centre of diversity and probably the centre of origin of the late blight pathogen (Reddick 1939; Fry & Spielman, 1991; Goodwin *et al.*, 1992a).

No variation was found for mtDNA markers as only the Ia haplotype was detected. Our results confirm that Ia is the predominant mtDNA haplotype present in *P. infestans* populations in the highlands of central Mexico. Together with haplotype IIa and IIb (Carter *et al.*, 1991; Griffith & Shaw, 1998), type Ia has been found among isolates of the diverse genotypes that migrated worldwide during the 1970s (Fry *et al.*, 1992). The tight association between haplotype Ia and variable, often sexually reproducing *P. infestans* populations provides strong circumstantial evidence for Ia representing the ancestral type of mitochondrial haplotype in *P. infestans*.

Intermediately metalaxyl resistant or resistant isolates were detected in all three populations. Metalaxyl resistance was fairly common among isolates collected from *S.*

demissum (Matuszak *et al.*, 1994; Grünwald *et al.*, 2000b; chapter 8) and was also detected in *P. mirabilis* isolates growing along roadsides and in natural vegetation in the Toluca Valley in 1998 and 1999 (Flier & Grünwald, unpublished).

Differentiation of P. infestans populations

We tested the hypothesis that populations of *P. infestans* on native *Solanum* species are merely derived from populations on cultivated potatoes. This hypothesis implies a decrease in genotypic diversity in the WSS population. We did not find evidence for reduced genetic diversity among *P. infestans* strains collected from native *Solanum* species based on AFLP marker data. Instead, sub-populations of *P. infestans* on wild *Solanum* hosts were among the most variable populations found in the Toluca Valley. Our results are therefore not consistent with the earlier report (Grünwald *et al.*, 2000b; chapter 8), which suggested that WSS populations are less diverse than RURAL or VALLEY populations based on mating type and Pep and Gpi allozyme markers. A difference in mutation rate might serve as an explanation for the greater genetic diversity found between *P. infestans* strains by AFLP fingerprinting as compared to allozyme analyses.

Insensitivity to metalaxyl proved not to be a very informative marker for gene flow studies in *P. infestans* populations from Toluca Valley. Metalaxyl insensitive isolates were collected from all three populations and relatively high numbers of intermediate or resistant isolates were detected in *P. infestans* collections from *S. x edinense*. The slightly higher frequency of metalaxyl resistant strains found on *S.* x *edinense* compared to collections from *S. demissum* might be explained by the difference in ecological habitats between the two native host species. *S.* x *edinense* is commonly found in rural, low-input potato fields in which they mingle with the Criolla potato crops. In contrast, *S. demissum* is usually found under pine trees in natural habitats. It is therefore very likely that *P. infestans* isolates from *S. x edinense* have had a higher chance of repeated exposure to metalaxyl as compared to isolates from *S. demissum*. It has been shown (Davidse *et al.*, 1989) that repeated exposure to metalaxyl will soon lead to the occurrence of phenylamide resistance in field populations of *P. infestans*. The presence of metalaxyl resistant isolates on native *Solanum* species has been repeatedly used to argue against the presence of genetic differentiation between *P. infestans* from potato and native *Solanum* hosts (Fry & Spielman, 1991; Matuszak *et al.*, 1994). The hypothesis of panmixis can however not explain the presence of considerable levels of metalaxyl resistance in *P. mirabilis* (8.7% of *P. mirabilis* isolates being intermediate or resistant, n = 69). Based on neutral marker data, Goodwin *et al.* (1999) concluded that *P. infestans* and *P. mirabilis* should be regarded as separate species. Gene flow was estimated at a *N*m value of 0.38, indicating highly restricted or no genetic exchange between the two species. The use of metalaxyl resistance as an indirect marker for gene flow between *P. infestans* populations on different host plant species is therefore not unambiguous. Our suggestion in an earlier report (Grünwald *et al.*, 2000b) stating that

gene-flow between VALLEY, RURAL and WSS populations of *P. infestans* based on metalaxyl resistance data is common, should perhaps be reconsidered.

The presence of a relatively large number of private AFLP alleles in the WSS population suggests that gene flow between *P. infestans* populations on native *Solanum* species and cultivated potatoes has been restricted. Exact test for population differentiation based on multilocus genotypes and indirect estimation of gene flow between populations using *Nm* as a measurement of population differentiation confirmed this view. On average, migration was less than 1 migrant per generation between *P. infestans* populations from potatoes and *S. demissum* or *S. x edinense* populations. No restrictions in gene flow were measured between *P. infestans* populations from cultivated potatoes (VALLEY and RURAL). The measurements of population differentiation combined with the observed *Nm* values suggest that the *P. infestans* population on native *Solanum* species tend to evolve independent from the populations that can be found on cultivated potatoes (Slatkin & Barton, 1989), which can eventually lead to sympatric speciation (Bush, 1969).

We considered two alternative hypotheses to explain the existing genetic differentiation between *P. infestans* strains from potato crops and native *Solanum* hosts. One hypothesis is that genetic differentiation between *P. infestans* populations can be attributed to spatial and temporal features of late blight epidemics. This cause of genetic subdivision develops only when gene flow is restricted and populations are separated for long periods of time (Berg & Lascoux, 2000). None of these two limiting requirements are in concordance with our current knowledge of potato production in the Toluca Valley. During the 1950s, commercial potato production was intentionally introduced to the Toluca Valley (Niederhauser, 1962). Geographical isolation between native *Solanum* species cultivated potatoes is very limited, and lies often in the order of magnitude of 0.1 to a few km. Late blight epidemics within the valley usually start at the beginning of July (Grünwald *et al.*, 2000b) in the central valley while we never observed epidemics of late blight on native *Solanum* species before the end of August or beginning of September. The delayed onset of late blight epidemics on native *Solanum* species temporal effects tend to support the hypothesis that WSS populations are derived from VALLEY and/or RURAL populations, which is obviously not supported by our AFLP marker data. We conclude that it is therefore not sufficient to explain the observed differentiation between *P. infestans* populations based on spatial and temporal effects.

A second hypothesis to explain sub-structuring is that genetic isolation mechanisms prevent panmixis between populations of *P. infestans* from the Toluca Valley. Host specific compatibility genes, involved in host specificity as well as reproductive barriers might contribute to this type of isolation. At least 11 race-specific virulence genes (R-genes) have been identified in native *Solanum* species from the central highlands of Mexico (Black *et al.*, 1953; Malcolmson & Black, 1966) and it is generally believed that this these 11 R-genes are only the tip of the iceberg. The presence of specific resistance genes (and the numerous combinations of R-genes in

individual *Solanum* genotypes) could serve as an explanation for the observed reduction in gene flow between *P. infestans* from cultivated potatoes and native *Solanum* species. Additional evidence supporting the view that R-genes and host specificity contribute to population differentiation is presented by Ordoñez *et al.* (2000). They reported on an Ecuadorian *Phytophthora* A2 population closely resembling *P. infestans,* which appears to be strictly isolated from potato strains by host-plant specificity. In addition, significant differences in oospore production were observed between *in vitro* matings of isolates collected from potato and *S. demissum* (Flier *et al.*, 2001b). Isolates originating from the native host *S. demissum* produced significantly more oospores in crosses with compatible strains collected from the same host species as compared to crosses with isolates collected from cultivated potatoes. Isolation mechanisms based on host specificity and reproductive success urge for strategies that facilitate the survival of the pathogen on alternative hosts during the dry season when foliage of both potato crops and wild hosts is not present. Extensive monitoring of late blight epidemics on wild tuber-bearing *Solanum* species in the Toluca Valley during the 1980s led to the conclusion that hibernation of mycelium in infected tubers of native *Solanum* species cannot be regarded as a common source of initial inoculum for *P. infestans* (Rivera-Peña, 1990a). There is however strong evidence that oospores, produced in leaflets of *S. demissum* with multiple late blight lesions, might contribute to the survival during the 'host-free' period (Flier *et al.*, 2001b).

Ongoing sympatric speciation in P. infestans?

Restricted gene flow and reported differences in reproductive success between *P. infestans* populations from native *Solanum* species and cultivated potatoes support the hypothesis of population differentiation and eventually sympatric speciation (Kondrashov & Kondrashov, 1999) in local populations of *P. infestans* in central Mexico. Ordoñez *et al.* (2000) provided evidence in concordance with our hypothesis that sympatric speciation; the origin of two or more new species from a single local ancestral population without geographical isolation is possible in populations of *P. infestans.* It has been stated that the speciation process can be accelerated by either resource use through host adaptation (strong selection for virulence genes in the pathogen to neutralize R-genes in *S. demissum*) or random genetic drift (Berlocher, 1998; Dieckmann & Moebeli, 1999). The presence of recurrent population bottlenecks at the end of the season has a pronounced effect on the differentiation for neutral genes and the time to reach equilibrium (Berg & Lascoux, 2000). It is likely that *P. infestans* populations in populations of *S. demissum* undergo severe bottlenecks at the end of each growing season, so it is expected that random genetic drift plays an important role in shaping very restricted sized populations of *P. infestans.*

We postulate that host specific populations of *P. infestans* are at the beginning of sympatric speciation process. In order to test this hypothesis, we propose a model describing the subsequent stages of isolation and speciation in *P. infestans* that can be validated through future theoretical and experimental experimentation.

A model developed by Bush (1969) predicts evolution of host specific forms in sympatry and stated that continued adaptation to hosts could eventually produce strong reproductive isolation. Berlocher (1998) described a set of hypothetical stages of sympatric speciation based on true fruit flies (*Rhagoletis pomonella* species group), which is often seen as a model system for sympatric speciation (Mayr, 1963; Futuyma & Mayer, 1980). Adapting the Bush-Berlocher model to the ecology of *P. infestans* in Toluca Valley we propose the following 4-stage model. Starting from panmixis, a number of genotypes emerge that are adapted to a specific host genotype (i.e. specific virulence). Significant gene flow from the pool of genotypes on potato is prevented through temporal isolation based on the onset of the epidemics and strong selection by means of R-genes in the (novel) host plant species. During this stage (I), no reproductive barriers (other than host-plant fidelity) exist and no differences in allozyme allele frequencies are observed. The following stage (II) is marked by restricted gene flow between the two populations and the presence of a small amount of reproductive isolation. Differences in host specificity becoming more pronounced as genetic distances increase and allele frequencies start to differ for host-specific populations. When isolation proceeds, reproductive success is reduced in both F1 and subsequent backcrosses (stage III). Genetic distances are greater than in stage II. Species-specific allozymes exist, but are not fixed, which allows for low levels of gene flow between the two diverging populations. At stage IV, the isolation of both populations is completed. This stage is marked by great genetic divergence, no gene flow with relatives and fixed, species-specific allozymes.

The best way to test this hypothesis is through gene flow studies and estimation of differential reproductive success using host specific *P. infestans* populations In Central and South America that are suspected to have gone through severe bottlenecks as well as showing rare neutral marker alleles.

Acknowledgements

We thank PICTIPAPA (Programa Internacional Cooperativo del Tizón Tardío de la Papa), ICAMEX (Instituto de Investigación y Capacitación Agropecuaria, Agrícola y Forestal del Estado de México) and INIFAP (Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias) for the use of their infrastructure and for providing us with research equipment. The invaluable help of Hector Lozoya and José Gomez is acknowledged. The suggestions and comments on the manuscript given by Hans van der Beek and Rolf Hoekstra (WAU) are highly appreciated. PICTIPAPA, HPA, the CEEM project and the Dutch Ministry of Agriculture, Fisheries and Nature Management funded this work.

10

Phytophthora ipomoeae, a new homothallic species causing leaf blight on *Ipomoea longipedunculata* in the Toluca Valley of central Mexico

W. G. Flier, N. J. Grünwald, L. P.N. M. Kroon, T. B.M. van den Bosch, E. Garay-Serrano and P. J. M. Bonants

Submitted to Mycological Research

Summary

A *Phytophthora* species was found on blighted foliage of *Ipomoea longipedunculata*, a morning glory species native to the highlands of central Mexico. Based on host range, morphology, allozymes, mitochondrial DNA haplotype and rDNA sequences it is concluded that a new *Phytophthora* species, *P. ipomoeae*, is the causal agent of leaf blight disease on *I. longipenduculata*.

Introduction

The central highlands of Mexico, which include the Toluca Valley, are thought to be the centre of origin and are the centre of diversity of both *Phytophthora infestans* (Mont.) De Bary, causal agent of potato late blight (Niederhauser, 1991; Goodwin *et al.*, 1992a) on wild and cultivated *Solanum* spp, and the closely related species *P. mirabilis* Galindo & Hohl. *P. mirabilis* causes leaf blight on the host-plant *Mirabilis jalapa*, which is commonly known as four o' clock (Galindo & Hohl, 1985). The genus *Mirabilis* is in the family *Nyctaginaceae*, a family that is not closely related to the *Solanaceae*.

Several lines of evidence point to the fact that *P. mirabilis* and *P. infestans* evolved from one common ancestor. Based on morphology alone, these two species cannot be distinguished, except for the fact that they are host specific. Isozyme and RFLP analysis have shown these two species to be of unique and distinguishable genotypes (Goodwin *et al.*, 1999). Goodwin (1996) postulated that both of these species evolved sympatrically in the central highlands of Mexico, as both host plants co-occur in close proximity. It is thought that reproductive isolation and host plant specificity resulted in sympatric speciation (Goodwin *et al.*, 1999).

Throughout the summer of 1999, several daytrips were undertaken by the first and second author in order to collect *P. infestans* and *P. mirabilis* from both native and cultivated host plant species. In July 1999, several isolates were collected from *Ipomoea longipedunculata* Hemsl. (Sánchez Sánchez, 1968) showing symptoms of blighted leaves and stems. A total of four diseased plants were found on a hill, locally known as the 'Cerro de Metepec'. *I. longipedunculata* is a native species of central Mexico and is occasionally found climbing shrubs and deciduous trees in shrub/woodland areas. The disease symptoms observed on *I. longipedunculata* were marked by purple black or brownish lesions in the centre of the leaf (Figure 10-1) and the upper part of the stem. Under conditions of high relative humidity, sporangiophores bearing numerous sporangia appeared on the underside of the leaf.

The purpose of this paper was to test the hypothesis that the isolates collected from *I. longipedunculata* belong to a new *Phytophthora* species, closely related to *P. infestans* and *P. mirabilis* (Waterhouse, 1963). Morphology, host specificity, allozymes patterns and rDNA sequences were studied to test the hypothesis. For convenience, we will refer to the *Phytophthora* isolates obtained from *I. longipedunculata* as *Phytophthora ipomoeae* throughout this paper.

Materials and methods

Isolation

Isolates were obtained from single lesions on leaves of *Ipomoea longipedunculata* Hemsl. (Figure 10-1) growing on a hill in the village of Metepec in the Valley of Toluca. Pieces of tissue adjacent to the sporulating region of the lesion were cut out and surface sterilised by soaking them in 80% ethanol for 10 seconds and 0.5% sodium hypochlorite for 3 minutes followed by a rinse in sterile tap water for 1 min. Sterilised pieces were plated on Rye A agar (Caten & Jinks, 1968) supplemented with ampicillin (200 mg l^{-1}), Benlate (50% WP, 100 mg l^{-1}), PCNB (75% WP, 67 mg l^{-1}), polymixin B (50 mg l^{-1}) and rifampicin (20 mg l^{-1}) (Forbes, 1997). The six isolates obtained were maintained on Rye A agar at room temperature (20 ± 1 °C) with transfers every three to four months.

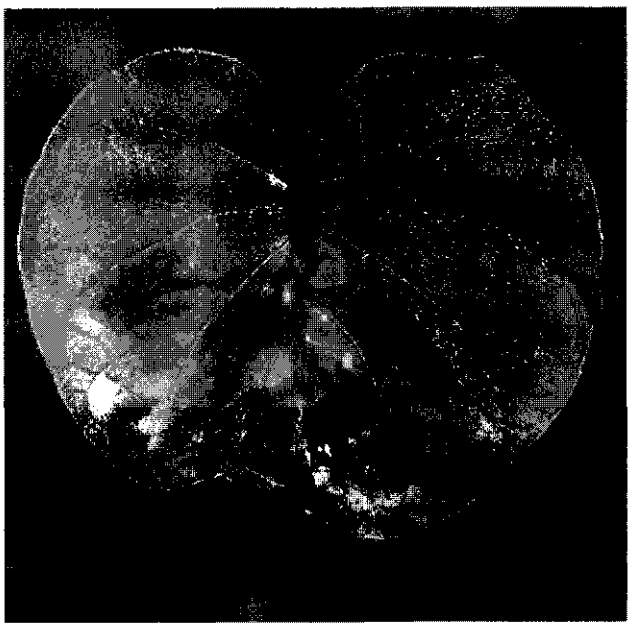

Figure 10-1. Blight symptoms on Ipomoea longipedunculata caused by Phytophthora infestans.

In planta formation of oospores

Infected leaflets were examined for the presence of oospores. Leaflets with single lesions were incubated for 2 days at room temperature in water agar (10 gl^{-1}) Petri dishes. Leaflets were then clarified in boiling ethanol (96% v/v) for 5 minutes, bleached in 1% sodium hypochlorite for at least 6 hours and mounted on microscope slides. Clarified leaflets were examined for the presence of oospores using a bright-field microscope at a magnification of 10 x 10.

Reference isolates

The six *Phytophthora* isolates obtained from *Ipomoea longipedunculata* were compared with 6 isolates of *P. infestans* collected in June and July 1999 from cultivated potato (*Solanum tuberosum* L.) and the wild host species *S. stoloniferum* Schlechtd. et Bché and 6 isolates of *P. mirabilis* collected from *Mirabilis jalapa* Linn. These isolates were collected in the Valley of Toluca within an area of approx. 2 km^2 around the village of Metepec, Estado de México, Mexico. In addition, a single isolate of *P. phaseoli* was obtained from the Centraal Bureau voor Schimmelcultures (CBS), Utrecht, the Netherlands. Characteristics of the strains used in this study are listed in Table 10-1. All reference isolates were maintained on rye A agar and stored under liquid nitrogen (Flier & Turkensteen, 1999).

Table 10-1. Isolates of *Phytophthora* used in morphology and allozyme comparisons.

Strain	Source	*Phytophthora* species	Host	Origin	Mating type
PIC99010	*PICTIPAPA*[a]	P. infestans	Solanum tubersosum	Metepec, México	A2
PIC99012	PICTIPAPA	P. infestans	*Solanum tubersosum*	Metepec, México	A1
PIC99050	PICTIPAPA	P. infestans	*Solanum tubersosum*	Metepec, México	A1
PIC99180	PICTIPAPA	*P. infestans*	*Solanum stoloniferum*	Metepec, México	A1
PIC99181	PICTIPAPA	P. infestans	Solanum stoloniferum	Metepec, México	A1
PIC99182	PICTIPAPA	*P. infestans*	*Solanum stoloniferum*	Metepec, México	A1
PIC99105	PICTIPAPA	P. mirabilis	Mirabilis jalapa	Metepec, México	A2
PIC99111	PICTIPAPA	*P. mirabilis*	*Mirabilis jalapa*	Metepec, México	A2
PIC99121	PICTIPAPA	*P. mirabilis*	*Mirabilis jalapa*	Metepec, México	A2
PIC99128	PICTIPAPA	*P. mirabilis*	*Mirabilis jalapa*	Metepec, México	n.a.
PIC99131	PICTIPAPA	*P. mirabilis*	*Mirabilis jalapa*	Metepec, México	A2
PIC99153	PICTIPAPA	*P. mirabilis*	*Mirabilis jalapa*	Metepec, México	A2
CBS556.88	*CBS*[b]	P. phaseoli	unknown	unknown	S.F.[c]
PIC99193	PICTIPAPA	P. ipomoeae	Ipomoea longipedunculata	Metepec, México	S.F.[c]
PIC99194	PICTIPAPA	*P. ipomoeae*	*Ipomoea longipedunculata*	Metepec, México	S.F.[c]
PIC99164	PICTIPAPA	*P. ipomoeae*	*Ipomoea longipedunculata*	Metepec, México	S.F.[c]
PIC99165	PICTIPAPA	*P. ipomoeae*	*Ipomoea longipedunculata*	Metepec, México	S.F.[c]
PIC99167	PICTIPAPA	*P. ipomoeae*	*Ipomoea longipedunculata*	Metepec, México	S.F.[c]
PIC99169	PICTIPAPA	*P. ipomoeae*	*Ipomoea longipedunculata*	Metepec, México	S.F.[c]

[a] Isolates from the PICTIPAPA culture collection

Morphology

Colony morphology and growth rate were compared on Rye A agar (RA), a minimal medium (MM) (Kamoen *et al.*, 1994) and four reference media: Cherry decoction agar (CA), Potato Dextrose Agar (PDA) V-8 Agar and Oatmeal Agar (OA). Pieces of mycelial agar (plugs of 5mm in diameter) were taken from the margins of actively growing 10-d-

old colonies, placed in the centre of Petri dishes (9 cm diameter) and incubated at 20 °C. Four replicate plates were used. Radial growth was measured after 7 days in two perpendicular directions per plate with an electronic marking gauge (Mitutoyo Absolute digimaster, Veenendaal, the Netherlands). Growth rates were corrected for plug diameter. Dimensions of sporangia for all four *Phytophthora* species and oospores of the homothallic species *P. phaseoli* and *P. ipomoeae* were measured for cultures grown on RA. All data are based on at least 30 measurements for each isolate. Oospore dimensions for *P. infestans* and *P. mirabilis* were measured from a mating of PIC99010 x PIC99012 and PIC99111 x PIC99124 on RA, respectively (Table 10-1).

Metalaxyl resistance

Response to the fungicide metalaxyl for the four *Phytophthora* species was determined by growing isolates *in vitro* on metalaxyl amended Rye A agar (Forbes, 1997). Radial growth of an isolate on RA amended with 5 or 100 µg ml^{-1} reagent grade metalaxyl (Novartis Agro Benelux B.V., Roosendaal, the Netherlands) was compared to radial growth in the absence of metalaxyl. Agar plugs (5mm diameter) were transferred to the centre of a Petri dish (9 cm diameter) and incubated at 20 °C in the dark for 8 days. Mean radial growth was measured as described earlier and averaged for each of 4 replications in the non-amended treatment and 2 replications in the metalaxyl-amended treatments. Radial growth was corrected for plug diameter. To determine metalaxyl resistance, all readings were separated into three classes for relative growth on 5 or 100 µg ml^{-1} metalaxyl (= growth relative to the DMSO amended control). Classes were defined as: Sensitive = 0 – 40% growth (on both 5 and 100 µg ml^{-1} metalaxyl), Intermediate = 0 – 40% growth on 100 µg ml^{-1} metalaxyl and > 40% growth on 5 µg ml^{-1} metalaxyl, and Resistant = > 40% growth on both 5 and 100 µg ml^{-1} metalaxyl.

Host specificity

Host specificity of a sub-set of *Phytophthora* isolates was tested by inoculating detached leaves of potato cv. Bintje, tomato cv. Moneymaker, *M. jalapa* and *I. longipedunculata* as well as tuber slices of potato cv. Bintje and sweet potato cv. A26/7 (*Ipomoea batata*). Detached leaflets, inoculated with six 10 µl droplets of sporangial inoculum (1,0 x 10^4 sporangia ml^{-1}) on the lower side of each leaf, were placed in Petri dishes (15 cm diameter) filled with 15 ml of 2% water agar. Inoculum was prepared from two-week-old cultures grown on RA. Petri plates with the inoculated leaves were wrapped in transparent polythene bags and incubated for 14 days at 15 °C at a light intensity of 12 Wm^{-2}, 16 hours light. Disease symptoms were assessed on a 0-3 scale (0 = no symptoms, 1 = small confined necrotic spots HR reaction, 2 = expanding lesions with few sporangia, 3 = abundant sporulation present). Disease symptoms were recorded at day 7, 10 and 14. The experiment was repeated twice. Tubers of potato cv. Bintje and sweet potato were surface sterilized in 5% sodium hypochlorite for 5 minutes, rinsed in tap water and wiped dry with Kleenex tissue paper. Slices of approximately eight mm

thick were cut and placed in Petri dishes (9 cm diameter); one slice in each Petri dish. A total of five slices were inoculated for each isolate. Slices were inoculated with a single 10 µl droplet of a sporangial inoculum (1,0 x 10^4 sporangia ml^{-1}), incubated for 10 days in closed Petri dishes in an incubator at 15 °C and 85% RH in the dark and evaluated for the presence of mycelium on the tuber slice surface. Presence of mycelium was considered as an indicator for the level of compatibility between the host and the pathogen. Disease symptoms were assessed on a 0-3 scale (0 = no symptoms; 3 = abundant mycelium present).

Allozyme analysis

Allozyme genotype for glucose-6-phosphate isomerase (*Gpi*, EC 5.3.1.9) and peptidase (*Pep*, EC 3.4.3.1) was determined using cellulose acetate plates (Goodwin *et al.*, 1995a).

Mitochondrial DNA haplotypes

Mitochondrial DNA (mtDNA) was amplified using four sets of primers designed to amplify specific regions (P1 to P4) of the mitochondrial genome of *P. infestans* (Griffith & Shaw, 1998). PCR was performed according to Ordoñez *et al.* (2000). PCR products were digested with the restriction enzymes *Cfo*I (P1), *Msp*I (P2), and *Eco*RI (P3 & P4). Ten µl of the amplified product was digested with 1 unit of the restriction enzyme for 4 hours. The digested products were run on a 1.8% agarose gel in TBE buffer at 10 V cm^{-1} and visualised with ethidium bromide under UV light.

rDNA amplification and sequencing

Isolates of *P. infestans* (PIC97757), *P. mirabilis* (G4-6), *P. phaseoli* (CBS556.88) and *P. ipomoeae* (PIC99169) were taken from liquid nitrogen storage, revitalized on RA plates for 2 weeks and grown for 10 to 14 days at 20 °C in clear pea broth. Mycelium was harvested and lyophilised (Ordoñez *et al.*, 2000). DNA was isolated using the Puregene kit (Gentra/Biozym, Landgraaf, the Netherlands) according to the manufacturer's instructions with slight modifications. ITS-PCR was performed using primers ITS1 and ITS4 (White *et al.*, 1990; Cooke *et al.*, 2000). The ribosomal DNA (rDNA) internal transcribed spacer region 1 (ITS1) and 2 (ITS2) products were directly sequenced on an ABI3700 automatic sequencer (Perkin-Elmer, Nieuwerkerk a/d IJssel, the Netherlands). ITS sequences from various *Phytophthora* species, *Pythium aphanidermatum* and *Achlia bisexualis* were obtained from GenBank, based on reports by Crawford *et al.* (1996) and Cooke *et al.* (2000) (Table 10-5).

All sequences were aligned to identify any dissimilar nucleotides or deletions using the ClustalW method of Dambe Version 4.0 (Data Analysis in Molecular Biology and Evolution) (Xia, 2000), available on-line at no cost from Xuhua Xia at the University

of Hong Kong, China. Phylogenetic inference was based on the Neighbour-joining method in Dambe.

Results

Morphology and growth characteristics of Phytophthora ipomoeae

All six isolates from *Ipomoea longipedunculata* grew well with radial growth rates of approx 10 mm d^{-1} on solid agar media such as Rye A agar (RA), Cherry decoction agar (CA), Potato Dextrose Agar (PDA) and Oatmeal Agar (OA). Restricted radial growth of approx 5 mm d^{-1} was observed on Minimal Medium Agar (MM). Growth inhibition was observed on V-8 Agar (V8) with an average radial growth rate of 0.5 mm d^{-1} (data not shown). Colonies on MM exhibited a petaloid to rosaceous colony morphology while growth on nutrient-rich media like RA, CA, PDA and OA resulted in rather undefined fluffy aerial growth of mycelium. Hyphae were non-septate and moderately or freely branching with a hyphal diam ranging from 3.8 to 7.5 µm. Hyphal swellings on solid agar media were rarely observed.

P. ipomoeae isolates sparsely formed sporangiophores on the solid agar media tested. The long, aerial sporangiophores branched in a compound-sympodial and intermediate fashion. Occasionally, swellings were present at the primordial sites of sporangia. Sporangia were semi-papillate, caduceus with a short pedicel, mainly ellipsoid (Figure 10-2). At times, ovoid sporangia were noticed. Shape and dimension of sporangia varied considerably within and between isolates of *P. ipomoeae*, but no consistent differences between isolates were observed. Length of sporangia ranged from 35.0 to 47.4 µm (average 39.0 µm), breadth ranged from 17.5 to 26.7 µm (average 20.8 µm), with a length:width ratio of 1.90 (Table 10-2). Zoospores were readily released (within 4 hours) from sporangia in a watery suspension at 10 °C. On average 6.9 zoospores per sporangium were produced (range 4 to 8 zoospores).

Sexual structures were abundantly produced on RA, sparsely in OA, and were absent in CA, PDA V8 and MM. Distribution of sexual structures in solid agar media was under-dispersed, they were mainly found in clusters of approximately 50-100 oospores. Antheridia were amphigynous, with an average length of 19.0 µm (range 17.5 to 20 µm) and a length:width ratio of 1.3. Oogonia were spherical, smooth-walled, with an average diameter of 32.3 µm on RA. Oospores were smooth-walled, aplerotic to nearly plerotic, transparent to yellow (Fig. 10-3b). Oospore dimensions ranged from 25.0 to 32.5 µm in diam (average 28.8 µm). Oospores were readily formed in single cultures and isolates are consequently considered to be homothallic (Table 10-2).

Metalaxyl resistance

Insensitivity to metalaxyl was not detected in strains of *P. ipomoeae, P. mirabilis* or *P. phaseoli,* while *P. infestans* isolates PIC99012 and PIC99050 (both collected from potato) were classified as resistant and intermediate respectively (Table 10-2).

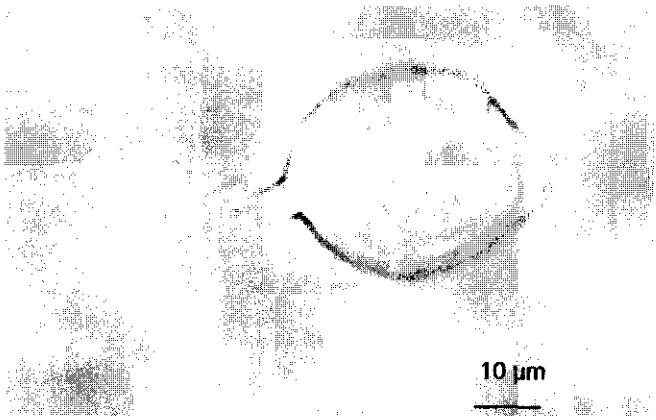

Figure 10-2. *Phytophthora ipomoeae* sporangium on minimal medium (400 x).

In planta formation of oospores

Oospores were observed in 12 out of 17 leaflets of *I. longipedunculata* inoculated with single isolates. Amphigynous antheridia and aplerotic oospores were present in clarified leaf samples (Figure 10-3a). Oospores of *P. ipomoeae* were present sparsely to abundantly in leaf tissue, and showed a clumped distribution and a preference for oospore formation near the major veins of the leave. Oospore diameters varied between oospores and leaf samples, but were in the range of 24 to 37 µm with an average of 28 µm.

Host specificity

P. ipomoeae is host specific for the range of hosts tested in this study. *P. ipomoeae* isolates rarely formed small, necrotic (< 2 mm diameter) spots on potato and tomato leaflets in the detached leaflet bioassays (Table 10-3). However, no sporangiophores were observed during the incubation period of 14 days. No disease symptoms were observed when tuber slices of potato cv. Bintje or leaves of *Mirabilis jalapa* and sweet potato (*I. batata*) were inoculated. Inoculation with either of the two *P. ipomoeae* isolates on *I. longipedunculata* led to sporulating lesions (Table 10-3). Isolates of *P. infestans* were found pathogenic on both potato and tomato, on which abundant sporulation occurred after 5-8 days after inoculation. Both potato and *M. jalapa* leaf tissues were colonised by *P. mirabilis,* isolate PIC99111 causing necrotic flecking and small sporulating lesions on Bintje leaflets 10 days after inoculation. No disease symptoms were observed when *P. phaseoli* was inoculated on the host plant species included.

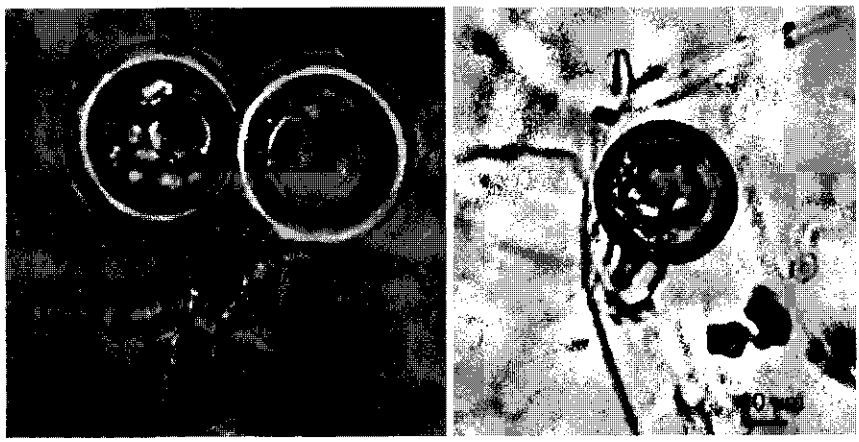

Figure 10-3. Oospores of *Phytophthora ipomoeae*: In clarified leaflets (A), in Rye A agar medium (B).

Allozyme, mtDNA and rDNA diversity

Genetic marker studies were applied to investigate the taxonomic status of *P. ipomoeae*. The *Pep* banding patterns of *P. ipomoeae* isolates consisted of the 78 and 96 alleles, with four isolates being homozygous for the *Pep* 78 allele. This allele was unique for isolates of *P. ipomoeae* while the *Pep* 96 allele was also found in *P. mirabilis*. The *Gpi* banding pattern of the *P. ipomoeae* isolates tested appeared to be 108/108 on cellulose-acetate gels, which is different from the predominant genotypes of both *P. infestans* (Grünwald *et al.*, submitted) and *P. mirabilis* (Flier *et al.*, in preparation) in the Toluca Valley. This *Gpi* allele was also detected in one *P. mirabilis* isolate. No isozyme data are available for *P. phaseoli*.

Restriction fragment length analysis of amplified mitochondrial DNA revealed that *P. infestans* isolates from both potato and *S. stoloniferum* were of the haplotype Ia, but isolates of *P. mirabilis*, *P. phaseoli* and *P. ipomoeae* are all marked by mtDNA haplotypes that are distinct from those reported for *P. infestans* (Griffith & Shaw, 1998) (Table 10-4). Amplification of mtDNA with each of the four primer sets in every case yielded a band that corresponded to published results for *P. infestans*. *P. mirabilis*, *P. phaseoli* and *P. ipomoeae* were characterised as haplotype Ia or Ib according to restriction analysis of the P1 mtDNA region. Digestion of the P3 region placed *P. mirabilis* and *P. ipomoeae* together into class I haplotypes and *P. phaseoli* into class II haplotypes. After digestion of the P2 and the P4 region, differences were detected between the described *P. infestans* haplotypes and *P. mirabilis*, *P. phaseoli* and *P. ipomoeae* haplotypes. Amplification of the P2 region and digestion with *Msp*1 produced a novel two-band pattern in *P. ipomoeae*, that included the reported 203 bp fragment characteristic for the IIA and IIB haplotype in *P. infestans* and a long novel fragment of approx 867 bp. (Table 10-4). *P. mirabilis* and *P. phaseoli* were characterised as IIa and Ia or IIb respectively. Restriction analysis of the P4 region yielded novel

haplotypes for *P. mirabilis*, *P. phaseoli* and *P. ipomoeae* not described before in *P. infestans*. In both *P. mirabilis* and *P. phaseoli*, restriction of the 964 bp P4 amplification product with *Eco*R1 failed, indicating the absence of at least two restriction sites when compared to *P. infestans*. The *P. ipomoeae* haplotype was characterised by a two-band pattern which consisted of the 209 and 755 bp fragments, recently described for Ecuadorian isolates similar to *P. infestans* but with a different host range (Ordoñez *et al.*, 2000).

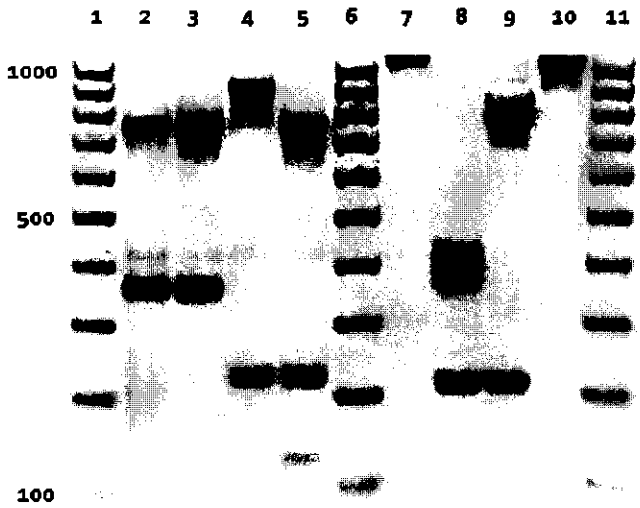

Figure 10-4. Mitochondrial DNA haplotypes produced after PCR amplification and digestion of the P2 and P4 mtDNA region of *P. phaseoli*, *P. infestans*, *P. ipomoeae* and *P. mirabilis*. Lane 1, 6 and 11; DNA ladder (numbers indicate size in base pairs); lane 2 to 5, restriction fragments of amplified P2 region of *P. phaseoli*, *P. infestans* (IA haplotype), *P. ipomoeae* and *P. mirabilis*; lane 7 to 10, restriction fragments of amplified P4 region of *P. phaseoli*, *P. infestans* (IA haplotype), *P. ipomoeae* and *P. mirabilis*.

Phenograms based on the ITS1 and ITS2 rDNA regions of 39 *Phytophthora* spp, *Pythium aphanidermatum* and *Achlya bisexualis* reveal that *P. ipomoeae* is closely related to group IV *Phytophthora* spp. (Figure 10-5 and 10-6). Characterisation of *P. ipomoeae* rDNA sequences supported the hypothesis that this species is closely related to other *Phytophthora* species within clade 1c (Cooke *et al.*, 2000) and Group IV (Waterhouse, 1963). The ITS1 region in *P. ipomoeae* was identical with the sequences found in both *P. infestans* and *P. mirabilis*, and differed only one base pair with *P. phaseoli*. One base-pair difference between *P. ipomoeae* and the other three species was detected when the ITS2 region was characterised.

Table 10-2. Growth rates, dimensions, metalaxyl resistance and allozyme alleles scored at the putative *Pep* and *Gpi* loci.

Isolate	*Phytophthora* species	Growth rate (mm/day)		Sporangium dimensions (µm)		Oospore diameter (µm)	Metalaxyl tolerance	Allozyme genotype	
		RA	MM	length	width			*Pep*	*Gpi*
PIC99010	*P. infestans*	5.8	0.0	35	23	-	Sensitive	100/100	86/122
PIC99012	*P. infestans*	3.4	0.0	30	19	-	Resistant	100/100	100/122
PIC99050	*P. infestans*	9.8	1.9	32	20	-	Intermediate	100/100	100/100
PIC99180	*P. infestans*	9.4	3.6	30	19	-	Sensitive	100/100	100/100
PIC99181	*P. infestans*	6.9	4.5	29	20	-	Sensitive	100/100	86/100
PIC99182	*P. infestans*	6.9	2.1	33	21	-	Sensitive	100/100	86/100
PIC99105	*P. mirabilis*	8.1	8.7	32	17	-	Sensitive	96/96	83/111
PIC99111	*P. mirabilis*	3.3	5.5	34	16	-	Sensitive	96/96	100/100
PIC99121	*P. mirabilis*	10.9	6.4	35	19	-	Sensitive	96/96	90/111
PIC99128	*P. mirabilis*	10.1	3.4	31	17	-	Sensitive	96/96	83/108
PIC99131	*P. mirabilis*	8.2	4.8	28	14	-	Sensitive	96/96	100/111
PIC99153	*P. mirabilis*	10.2	7.2	30	16	-	Sensitive	96/96	90/100
CBS556.88	*P. phaseoli*	1.5	0.0	24	15	21	Sensitive	n.a.	n.a.
PIC99193	*P. ipomoeae*	12.9	6.0	36	19	29	Sensitive	78/96	108/108
PIC99194	*P. ipomoeae*	12.0	5.6	39	24	27	Sensitive	96/96	108/108
PIC99164	*P. ipomoeae*	9.9	4.0	40	21	31	Sensitive	78/78	108/108
PIC99165	*P. ipomoeae*	11.2	5.3	38	22	27	Sensitive	78/78	108/108
PIC99167	*P. ipomoeae*	10.3	5.1	44	25	29	Sensitive	78/78	108/108
PIC99169	*P. ipomoeae*	10.1	5.7	40	21	30	Sensitive	78/78	108/108

Table 10-3. Pathogenicity of *Phytophthora* species on different host plant species as determined in bioassays.

Isolate	*Phytophthora* species	Sporangia production on different host species					
		Solanum tuberosum cv. Bintje1		*L. esulentum* cv. Moneymaker1	*M. jalapa1*	*I. batatas2*	*I. longipedunculata*
		Detached leaflets	Tuber slices				
PIC99010	*P. infestans*	3	3	3	0	0	0
PIC99181	*P. infestans*	3	2	3	0	0	0
PIC99111	*P. mirabilis*	1	0	0	2	0	0
PIC99128	*P. mirabilis*	0	0	0	3	0	0
PIC99193	*P. ipomoeae*	0	0	0	0	0	3
PIC99167	*P. ipomoeae*	0	0	0	0	0	2
CBS556.88	*P. phaseoli*	0	0	0	0	0	0

Table 10-4. Mitochondrial DNA haplotypes detected after PCR amplification and restriction fragment length analysis of 4 mtDNA regions.

			Phytophthora species						
			P. infestans				***P. mirabilis***	***P. phaseoli***	***P. ipomoeae***
Primer	**Restriction enzyme**	**Fragment**	**Haplotype Ia** **Ha*Pinf*Ia**	**Haplotype Ib**[1] **Ha*Pinf*Ib**	**Haplotype IIa**[1] **Ha*Pinf*IIa**	**Haplotype IIb**[1] **Ha*Pinf*IIb**	**Ha*Pmir*I**	**Ha*Ppha*I**	**Ha*Pipo*I**
P1	*Cfo*I	211	+	+	-	-	+	+	+
		907	+	+	-	-	+	+	+
		1118	-	-	+	+	-	-	-
P2	*Msp*I	79	-	+	-	-	-	-	-
		147	-	-	+	-	+	-	-
		203	-	-	+	-	+	-	+
		350	+	+	-	+	-	+	-
		641	-	+	-	-	-	-	-
		720	+	-	+	+	+	+	-
		867	-	-	-	-	-	-	+
		1070	-	-	-	-	-	-	-
P3	*Eco*RI	230	+	+	-	-	+	-	+
		1078	+	+	-	-	+	-	+
		1308	-	-	+	+	-	+	-
P4	*Eco*RI	209	+	+	-	-	-	-	+
		361	+	+	+	+	-	-	-
		394	+	+	-	-	-	-	-
		603	-	-	+	+	-	-	-
		755	-	-	-	-	-	-	+
		964	-	-	-	-	+	+	-

[1] Data from Griffith and Shaw, 1998.

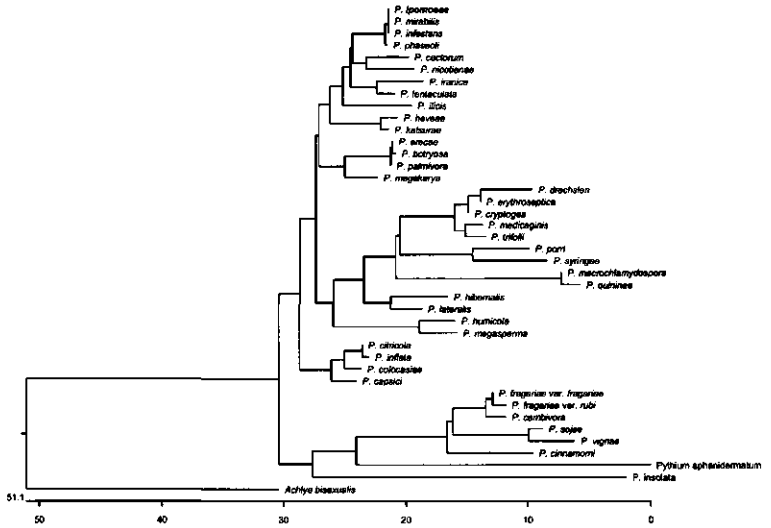

Figure 10-5. Phenogram based on the ITS 1 region of the genomic ribosomal RNA tandem gene repeat for 39 *Phytophthora* taxa, one *Pythium* and one *Achlia* species. The phenogram was constructed after DNA distance-based and neighbour-joining analysis of the data.

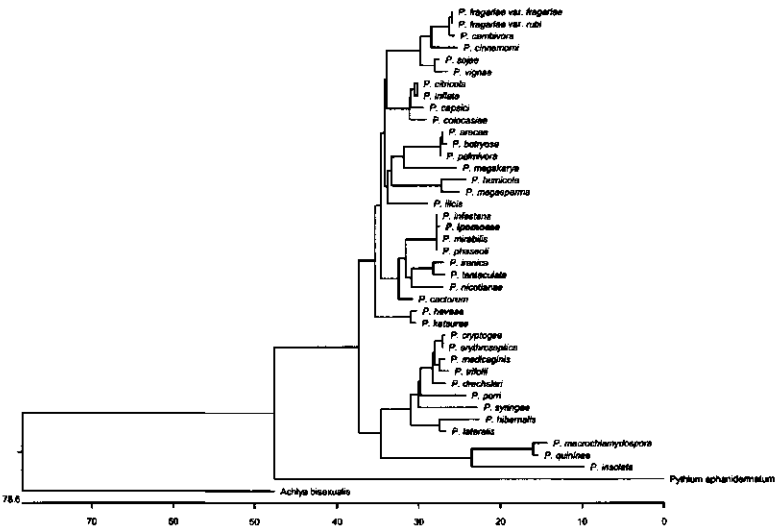

Figure 10-6. Phenogram based on the ITS 2 region of the genomic ribosomal RNA tandem gene repeat for 39 *Phytophthora* taxa, one *Pythium* and one *Achlia* species. The phenogram was constructed after DNA distance-based and neighbour-joining analysis of the data.

Table 10-5. Isolates of *Phytophthora* taxa and outgroups used for the rDNA study.

Species	Isolate	GenBank	Host	Reference
P. fragariae v. fragariae	IMI 330736	AF266762	Fragaria x ananassa	Cooke et al., 2000
P. fragariae rubi	IMI 355974	AF266761	*Rubus idaeus*	Cooke et al., 2000
P. macrochlamydospora	UQ205	-	*Glycine max*	Cooke et al., 2000
P. arecae	IMI 348342	AF266781	*Cocos nucifera*	Cooke et al., 2000
P. cactorum	IMI296524	AF266772	*Rubus idaeus*	Cooke et al., 2000
P. cambivora	IMI296831	AF266763	*Rubus idaeus*	Cooke et al., 2000
P. capsici	IMI352321	AF266787	*Piper nigrum*	Cooke et al., 2000
P. cinnamomi	UQ881	AF266764	*Syzygium aromaticum*	Cooke et al., 2000
P. citricola	IMI031372	AF266788	*Rubus idaeus*	Cooke et al., 2000
P. colocasiae	IMI368918	AF266786	*Colocasia esculenta*	Cooke et al., 2000
P. cryptogea	IMI045168	AF266796	*Lycopersicum esculentum*	Cooke et al., 2000
P. drechsleri	CBS292.35	-	-	Cooke et al., 2000
P. erythroseptica	ATTC36302	AF266797	*Solanum tuberosum*	Cooke et al., 2000
P. heveae	IMI180606	AF266770	Hevea brasiliensis	Cooke et al., 2000
P. humicola	IMI302303	AF266793	Citrus orchard soil	Cooke et al., 2000
P. ilicis	ILI1	AJ131990	Ilex aquilifolium	Cooke et al., 2000
P. inflata	IMI342898	AF266789	*Syringa*	Cooke et al., 2000
P. insolita	IMI288805	AF271222	Soil	Cooke et al., 2000
P. iranica	IMI158964	AJ131987	Solanum melongena	Cooke et al., 2000
P. katsurae	IMI360596	AF266771	*Cocos nucifera*	Cooke et al., 2000
P. lateralis	IMI040503	AF266804	*Chamaecyparus*	Cooke et al., 2000
P. medicaginis	UQ125	AF266799	*Medicago sativa*	Cooke et al., 2000
P. megasperma	IMI133317	AF266794	*Malus sylvestris*	Cooke et al., 2000
P. megakarya	IMI337104	AF266782	*Theobroma cacao*	Cooke et al., 2000
P. nicotianae	UQ848	AF266776	-	Cooke et al., 2000
P. palmivora	UQ1294	AF266780	*Theobroma cacao*	Cooke et al., 2000
P. phaseoli	CBS556.88	-	-	Cooke et al., 2000
P. porri	CBS782.97	AF266801	*Brassica chinensis*	Cooke et al., 2000
P. sojae	UQ1200	AF266769	*Glycine max*	Cooke et al., 2000
P. syringae	IMI296829	AF266803	*Rubus idaeus*	Cooke et al., 2000
P. tentaculata	CBS552.96	AF266775	*Chrysanthemum leucanth.*	Cooke et al., 2000
P. trifolii	UQ2143	AF266800	*Trifolium* spp.	Cooke et al., 2000
P. vignae	UQ136	AF266766	Vigna sinensis	Cooke et al., 2000
P. infestans	PIC97757	-	*Solanum demissum*	This paper
P. mirabilis	G4-6	-	*Mirabilis jalapa*	This paper
*P. ipomoea**	PIC99169	-	*Ipomoea longipedunculata*	This paper
Pythium aphanidermatum	UQ2071	AF271227	Root rot	Cooke et al., 2000
Achlya bisexualis	CBS 102.50	-	-	Crawford et al., 1996

Discussion

Disease symptoms of *P. ipomoeae* on leaves of *I. longipedunculata* closely resembled those of late blight on potatoes, but the absence of pathogenicity on potato or tomato of isolates collected from *I. longipedunculata* fuelled speculations about the taxonomic

status of these strains. Our genetic characterisations of the isolates confirm that these strains are very similar to *P. infestans*, *P. mirabilis* and *P. phaseoli*, which are found in the same geographical area of central Mexico.

Morphological and molecular data indicate that *P. ipomoeae* is very similar to the heterothallic species *P. infestans* and *P. mirabilis* and the homothallic species *P. phaseoli*. The colony growth rate of isolates collected from *Ipomoea* is somewhat higher than compared to the three species while sporangia dimensions are notably larger than those reported for the three group IV species examined. The isolates differ from most other species in taxonomic group IV by the presence of typical basal swellings on the sympodial sporangiophores which they only share with *P. phaseoli*, *P. mirabilis* and *P. infestans*. Sex organs found in cultures of *P. ipomoeae* are similar to those of *P. infestans*, *P. mirabilis* and *P. phaseoli*, the average oospore diameter being larger as compared to *P. mirabilis* and *P. phaseoli*. The isolates share the combination of amphigyny and homothallism with *P. phaseoli*. Allozyme analysis of six *P. ipomoeae* isolates revealed limited variability at the *Pep* locus and fixation of a single homozygous genotype at the *Gpi* locus. The *Pep* 78 allele was found predominantly in *P. ipomoeae*. This allele appears to be unique for *P. ipomoeae* since the *Pep* 78 allele was not detected in an extensive survey including numerous isolates of both *P. infestans* and *P. mirabilis* (Grünwald & Flier, unpublished) collected from the Toluca Valley. The 96 *Pep* allele has been reported for *P. infestans* (Goodwin *et al.*, 1999) and was also found in *P. mirabilis* (Flier, unpublished). Isolates of *P. ipomoeae* were homozygous and monomorphic at the *Gpi* locus. The *Gpi* 108 allele present in *P. ipomoeae* was only recently reported (Goodwin *et al.*, 1999) for *P. mirabilis* isolates collected in Texcoco, Mexico. In addition, the *Gpi* 108 allele has been found in *P. ilicis* (Goodwin *et al.*, 1999) but does not appear to be present in *P. infestans*. In *P. phaseoli*, only the 100 Gpi allele has been reported (Goodwin *et al.*, 1999). Allozyme data suggest only very limited gene flow between *P. ipomoeae* and the heterothallic sister-species *P. infestans* and *P. mirabilis*.

It has been reported that members of the genus *Ipomoea* serve as host plants for *Phytophthora* species. *Ipomoea hederacea* Jacq. has been reported as a host for *P. infestans* (Raj *et al.*, 1976) in India. Our limited attempt to infect a single sweet potato cultivar (*I. Batatas*) with the Mexican *Phytophthora* isolates failed, but the possibility of adaptation of *P. ipomoeae* strains to sweet potato is to be considered. So far, sweet potato production in Mexico is confined to tropical areas, while *P. ipomoeae* is known from the Toluca Valley, situated in the temperate highlands of central Mexico. However, more research is needed to elucidate the geographic distribution of both the pathogen and it's primary natural host species and therefore will lead to predictions about the likeliness of a future transfer of *P. ipomoeae* to sweet potato.

We now hypothesise that the central highlands of Mexico are the centre of origin of group IV *Phytophthora* species. The highlands of central Mexico presumably form the centre of origin of *P. infestans*, *P. mirabilis* and *P. phaseoli* (Brasier & Hansen, 1992). The shared morphological characters of the three species and *P. ipomoeae*

combined with the lack of ITS rDNA diversity found hints to the presence of a speciation hot-spot in the highlands of central Mexico with a common ancestry for all four species. Host specialisation and inter-specific hybridisation events could both serve as possible driving forces of speciation. It is however difficult to speculate on the exact origin of *P. ipomoeae*. In the genus *Phytophthora*, paragyny and heterothallism are considered ancestral to amphigyny and homothallism, heterothallic *Phytophthora* species being exclusively amphigynous (Brasier, 1983). Homothallic amphigynous species like *P. phaseoli* and *P. ipomoeae* are thought as secondary homothallics, evolved from their heterothallic ancestors (Brasier, 1983; Cooke *et al.*, 2000) by speciation events followed by differential evolutionary pressures (Brasier, 1983). Evidence is accumulating that inter-specific hybridisation in *Phytophthora* plays an important role in the exploitation of new host plant species (Ilieva *et al.*, 1998; Man in 't Veld *et al.*, 1998; Brasier *et al.*, 1999). Host specificity can provide the mechanism needed for reproductive isolation and might eventually lead to sympatric speciation. The abruptness of such a hybridisation event, followed by abundant asexual reproduction and oospore formation of the hybrid (homothallic) species on the new host could serve as an explanation for the accelerated speciation processes observed in *Phytophthora*.

The *Phytophthora* isolates from *Ipomoea longipedicella* are closely related to species in the taxonomic group IV of Waterhouse (Waterhouse, 1963; Stamps et al., 1990) and Clade 1c (Cooke *et al.*, 2000). At the same time, we have shown that the *Phytophthora* isolates collected from *I. longipedicella* do not fit any of the species descriptions based on the disparity of host range, differences in morphology, thalism, growth characteristics, allozyme patterns and mtDNA haplotypes. We therefore conclude that the *Phytophthora* strains isolated from *Ipomoea longipedunculata* belong to a new species for which we propose the name *Phytophthora ipomoeae*.

Species description

Phytophthora ipomoeae Flier *et* Grünwald. sp. nov.

Coloniis mycelialibus in Secal bene crescentibus. Cultura minima ad 11 °C, optima ad 20 °C et maxima ad 25 °C. Hyphae eseptatae et copiose ramosae, 3.8-7.5 µm diam. Sporangiophori aerii in agaro ramis composito-sympodialibus et indeterminatis, cum tumoribus in loco sporangiis emergentes. Sporangia semipapillata, ellipsoidea, subovoidalibus, caduca cum pedicella brevi, valore medio 39.0 µm longa (variatione 35.0-47.4 µm), ratione longitudinis/latitudinis 1.9, germinantia directe tubo germinativo vel indirecte cum zoosporis 4-8. Antheridia amphigyna, valore medio 19.0 µm longa, ratione longitudinis/latitudinis 1.3. Oogonia laevitunicata, valore medio 32.3 µm diam., basi attenuata. Oosporae laevitunicatae colore luteo raro aspersae, cavitatem oogonialem fere omnino impletes, valore medio 28.8 µm. Segregatus homothallicis. Hab. Solum in *Ipomoea longipedunculata* Hemsl. nota Mexico. Holotypus PIC98169 cultura viva CBS 109229

Mycelial colonies grow well on Rye A agar. Minimum growth at approx 11°C, optimum at 20 °C and maximum at 25 °C. Hyphae nonseptate and freely branching,

hyphal diam 3.8-7.5 µm, mostly 5.6 µm. Sporangiophore aerial, sparsely formed on rye agar with compound-sympodial and intermediate branches, with swellings where sporangia emerge. Sporangium semipapillate, ellipsoid or semi-ovoid, caduceus with short pedicel, on average 39.0 µm long (ranging from 35.0 to 47.4 µm), with a length/width ratio of 1.9, germinating directly with germ tubes or indirectly with 4-8 zoospores. Antheridia amphygynous, average length 19.0 µm, ratio of length/width 1.3. Oogonia smooth-walled, average diam 32.3 µm, with tapered base. Oospores smooth-walled, rarely tinted yellow, almost filling the oogonial cavity, average diam 28.8 µm. Isolates homothallic. Known host range restricted to *Ipomoea longipedunculata* Hemsl. Holotype specimen isolate PIC98169 has been deposited (CBS 109229).

Acknowledgements

The excellent technical assistance of Monique Goossens is acknowledged. We thank Rolf Hoekstra David Shaw and Hans van der Beek for stimulating discussions and useful comments on the manuscript. Thanks to all at PICTIPAPA, ICAMEX and INIFAP for facilitating our research in Mexico. This work was conducted as part of module three of the International Co-operative Program for Potato Late Blight (PICTIPAPA) and was supported by grants from CEEM, PICTIPAPA, the Dutch Potato Industry (HPA) and the Dutch Ministry of Agriculture, Fisheries and Nature Management.

11

Summarising discussion

The oomycete pseudofungus *Phytophthora infestans* (Mont.) de Bary, the causal organism of late blight, is considered to be one of the most devastating pathogens affecting potatoes and tomatoes worldwide. In Europe, the pathogen caused severe epidemics on potatoes after its introduction in 1845. With time, farmers learned to manage late blight in their fields. Late blight management became much more troublesome after the introduction of another *P. infestans* population of Mexican origin, which presumably took place in 1976. The present population of *P. infestans* in the Netherlands and an increasing number of other countries in Europe consists of both A1 and A2 mating type isolates. As a consequence, resting spores (oospores) may be formed in infected plant tissues after sexual reproduction of the pathogen. It was shown that oospores are able to survive under field conditions for at least one winter. Oospores are thought to play a significant role in initiating late blight epidemics in the Netherlands together with asexual initial inoculum sources like refuse piles and infected seed tubers. Sexual reproduction and the presence of functional oospores in potato fields lead to increased levels of genetic variation present in *P. infestans* populations. Strains marked by high levels of aggressiveness might be selected for during late blight epidemics, leading to an increase in 'pathogenic fitness'. The aim of the work described here is to elucidate the impact of the 'new' *P. infestans* population on late blight epidemiology and population biology. Therefore, sources and patterns of variation for pathogenicity in sexual *P. infestans* populations were studied and the impact of increased levels of aggressiveness on the stability of partial resistance to late blight was investigated.

Variation in *P. infestans*; sources and implications

Variation of aggressiveness in P. infestans populations

Isolates of *P. infestans* were collected in three potato-growing regions with different cultural practises in the Netherlands and were tested for their aggressiveness to tubers and leaves of potato cv. Bintje. For tubers, large differences in both infection efficiency and invasive ability were found between isolates of each of the three regional pathogen populations. Infection and subsequent spread of the fungus in tuber tissues were not found correlated. The most aggressive isolate of the displaced asexual population (VK 1.3.4) matches the average level of the new, sexual population in its ability to infect tubers. Consequently, the use of the old population strain VK 6C for tuber blight resistance screenings (that are conducted for the Dutch national list of recommended potato varieties) can no longer be considered to be representative for the present *P. infestans* population (chapter 2).

A comparative study on the variation for foliar aggressiveness in regional populations of *P. infestans* revealed considerable variation with respect to the components of aggressiveness assessed using a leaf-disc bioassay. Infection efficiency, latent period, maximal growth rate and sporulation density were used as determinants

of foliar aggressiveness. The three populations differed significantly in latent period, but not for maximal growth rate and infection efficiency. No association was found between mating type and aggressiveness. The components of foliar aggressiveness studied were not found correlated to either infection efficiency or invasive ability in tubers (chapter 3).

Oospore formation and viability

Oospores were observed in blighted leaflets and fruits of tomato and in leaflets of field crops and volunteer plants of potato in the Netherlands. It has been demonstrated that viable oospores can be extracted from blighted potato leaflets collected from commercial potato fields. Oospores were readily produced following inoculation of leaf discs of eight potato cultivars expressing different levels of partial resistance with a mixture of sporangia of A1 and A2 isolates. Highest numbers of oospores were produced in cultivars Bintje (susceptible) and Pimpernel (moderately resistant), and the lowest in Nicola (intermediate resistant). A simple mathematical model has been developed to describe the relationship between the number of lesions per leaflet and oospore incidence. The survival of oospores in a sandy and a light clay soil has been monitored during a six-year period using buried clay pots exposed to the local weather conditions. The sandy and clay soil remained infectious for 48 and 30 months respectively. Oospore derived infections of potato leaflets occurred within 84-92 hours after flooding the soil and ceased after 11 days. Soil samples remained infective if dried and re-flooded after two, but not more flooding events. (chapter 4).

In-vitro pairings of *P. infestans* isolates collected from cultivated potato and the native wild tuber bearing species *S. demissum* in the Toluca Valley showed considerable levels of variation for oospore production and oospore viability. Most crosses produced large numbers of oospores but a few yielded few or no oospores. Oospore viability was assessed using a tetrazolium based test, oospore plasmolysis and oospore germination. Viability ranged from 0 to 75 % based on the tetrazolium test, from 0 to 50% for the plasmolysis method and 0 to 11% for oospore germination. A positive correlation was found between oospore viability and number of oospores produced. Results revealed that oospore production and viability is dependent on the general combining ability and the specific combining ability of the parental strains. There was no consistent trend for preferential mating success between isolates from the same location or host although *P. infestans* isolates collected from *S. demissum* tend to produce more oospores in pairings with strains from the same host as compared to pairings with isolates from cultivated potato. Oospores were readily produced in naturally blighted leaflets of *S. demissum* collected from the slopes of the volcano 'Nevado de Toluca'. They were found in 10–20% of naturally infected *S. demissum* leaves. Leaflets with two lesions produced more oospores as compared to leaflets with three to five lesions in case oospores were found present (chapter5).

Stability of partial resistance to late blight in potato

Whole tuber and tuber slice inoculations revealed differential interactions in tuber blight attack between potato cultivars and *P. infestans* isolates. Incidence and severity was studied in a whole tuber assay while necrosis and mycelium coverage was evaluated in a tuber slice assay. Potato cvs Kartel and Producent showed low tuber blight incidence and limited spread of the pathogen while cvs Bintje and Astarte reacted more susceptible. A highly significant cultivar-by-year interaction was observed when tuber blight incidence was evaluated in two successive years. Differential responses occur as revealed by changing ranked order of cultivars after exposure to *P. infestans* strains. The results show that cultivar-by-isolate interactions existed for all components of tuber blight resistance studied. The presence of cultivar-by-isolate interaction provides indications that tuber blight resistance is not equally effective against all pathogenic strains of the pathogen. The results obtained provided evidence for the existence of races with respect to components of tuber blight resistance. The presence of specificity for components of tuber blight resistance and isolates of the present sexual population of *P. infestans* is thought to have a negative impact on the stability of tuber blight resistance (chapter 6).

Potato cultivars expressing different levels of partial resistance to foliar and tuber blight were evaluated for their resistance responses to selected highly aggressive strains of *P. infestans* in field and laboratory experiments in 1998 and 1999. Results disclosed significant differences in expression of foliar and tuber blight resistance against the pathogen strains tested. Analysis of variance revealed significant differential cultivar-by-isolate interactions for both foliar and tuber blight. The assessed levels of partial resistance in the foliage and tubers did not correlate well with foliar and tuber blight resistance ratings as presented in the Dutch national list of recommended potato varieties. No association was found between field and laboratory tuber blight resistance assessments. In field trials a significant relation between the rate of late blight infection in the foliage and tuber blight incidence was demonstrated. From the results it is concluded that cultivar-by-isolate interactions play a significant role in determining the outcome of interactions between partially resistant potato cultivars and *P. infestans* strains. The presence of specificity in interactions between *P. infestans* and partially resistant potato cultivars may affect the stability and the durability of partial resistance against late blight in potatoes. This stresses the need of a better insight in the adaptive ability of the pathogen in order to be able to predict durability of partially resistant potato cultivars exposed to variable *P. infestans* populations (chapter 7).

Patterns of variation in sexual reproducing P. infestans populations

The Toluca Valley contains the most diverse populations of *P. infestans* in the world and probably represents the centre of origin of this species. The relationships among populations of *P. infestans* on wild *Solanum* species, on local ("criolla") cultivars in low-input production systems, and on modern cultivars in high-input agriculture in the

central highlands of Mexico were studied using mating type, allozyme and metalaxyl resistance as markers. Isolates were sampled from the three different host-pathosystems in 1988 and 1989 (n = 179) and in 1997 and 1998 (n = 401). Analyses of genotypic diversity were based on mating type and two allozyme loci. Two groups of isolates from diverse host/management systems but monomorphic for these markers were further investigated via RFLP fingerprinting, and most individuals (65% and 85%) in the two subsets demonstrated to be unique which is in accordance with the predicted levels of isolate diversity in a sexual *P. infestans* population. Based on the selectable metalaxyl resistance marker it was concluded that the population structure in 1988-89 was similar to that in 1997-98, except that the frequency of metalaxyl resistance was 13% in 1997-98 and 60% in 1988-89. The populations from the three different pathodemes were only slightly different from each other, with greatest diversity found in the population from commercial cultivars (chapter 8).

The hypothesis that populations of *P. infestans* on wild *Solanum* species are now merely derived from populations on cultivated potatoes in the central highlands of Mexico near Toluca was tested using 170 isolates collected from cultivated potatoes and the native wild *Solanum* species *S. demissum* and *S.* x *edinense*. All isolates were analysed for mtDNA haplotype and AFLP multilocus fingerprint genotype. All isolates were shown to be monomorphic for mtDNA haplotype Ia. A total of 158 multilocus AFLP genotypes were identified among the 170 *P. infestans* isolates included in this study. Populations of *P. infestans* sampled in the Toluca Valley in 1997 were highly variable, and almost every single isolate represented a unique genotype, based on the analysis of 165 dominant AFLP marker loci. On average, 81.8% of the AFLP loci were found to be polymorphic. Heterozygosity varied between 7.7 and 19.4%. Significant differentiation was demonstrated at the population level between strains originating from cultivated potatoes and from wild *Solanum* species. Private alleles were observed in individual isolates collected from all three populations, with numbers of unique dominant alleles varying from 9 to 16 for isolates collected from commercial potato crops and native *Solanum* species respectively. Four AFLP markers were exclusively found present in isolates collected from *S. demissum*. No evidence was found supporting the hypothesis that populations of *P. infestans* on wild *Solanum* species are now derived from populations on cultivated potatoes. Indirect estimation of gene flow between populations indicated restricted gene flow between both *P. infestans* populations from cultivated potatoes and wild *Solanum* hosts. As a consequence, the working hypothesis was to be rejected. Now an alternative hypothesis has been put forward that population differentiation and genetic isolation of host specific *P. infestans* populations in the Toluca Valley is present. It is supposed that host specific resistance genes and random genetic drift form the driving forces behind population differentiation (chapter 9).

A new homothallic *Phytophthora* species closely resembling *P. infestans* and *P. mirabilis* was discovered in the Toluca Valley in Mexico. In 1999, six isolates of a *Phytophthora* species were obtained from blighted foliage of *Ipomoea*

longipedunculata, a morning glory species native to the highlands of central Mexico. Based on host range, morphology, allozymes, mitochondrial DNA haplotype and rDNA sequences it was concluded that a new *Phytophthora* species could be identified as the causal agent of leaf blight disease on *I. longipedunculata*. The name *Phytophthora ipomoeae* has been proposed and a species description was prepared. The type specimen has been deposited at the Centraal Bureau voor Schimmelcultures (CBS), Baarn, The Netherlands (chapter 10).

Conclusions and future perspectives

The research described in this thesis elucidated several aspects concerning the sources and implications of genetic variation in sexual populations of *P. infestans*. The results show (I) that considerable levels of variation for aggressiveness are being maintained in regional populations of *P. infestans* in the Netherlands, (II) oospores are readily produced in field crops and volunteer potato plants in the Netherlands, (III) that production and viability of oospores is highly dependent on combining abilities of parental strains, (IV) that oospores are able to survive in soils for three to four years, (V) that cultivar-by-isolate differential interactions play a significant role in interactions between partial resistant potato cultivars and *P. infestans* strains and (VI) that population differentiation and host specificity is present in *P. infestans* populations in its centre of origin and diversity.

From the results presented in this thesis, it is clear that the introduction of a sexually reproducing population of *P. infestans* in the Netherlands has had a major impact on late blight epidemics and population biology of the late blight pathogen. Oospores are nowadays occasionally produced in blighted potato crops and commonly on volunteers. It has been shown that oospores survive up to 48 months in the soil when exposed to local weather conditions. It seems therefore plausible to assume that oospores do form an alternative source of initial inoculum in most crop rotation schemes. Besides representing an additional source of initial inoculum, oospores act as the driving force behind the 'explosion' of genetic variation observed in recent years in the Netherlands. Sexual reproduction has therefore led to a genetically more diverse population of *P. infestans* in the Netherlands that is marked by an increased adaptability to host and environment.

Impact on the speed of late blight epidemics

It is conceivable that the presence of aggressive strains will lead to shorter infection cycles and a more rapid epidemic development of the disease. In moncyclic tests, the difference of these components for aggressiveness for the old and the new population are strikingly in favour of the new population. The combined effect of the components of increased aggressiveness on polycyclic late blight epidemics is dramatic. Comparison of infection efficiencies and sporulating capacity of isolates representing the old and the

new population of *P. infestans* in the Netherlands shows that isolates of the newly established population are able to infect potatoes at temperatures ranging from 3 to 27 °C while old population isolates caused infections from 8 to 23 °C (Flier *et al.*, unpublished). Recent results (Flier *et al.*, in preparation) show that isolates of the new population are marked by more rapid spore germination and host penetration, leading to shorter critical leaf wetness-periods. Under normal field conditions, isolates need only a few hours of leaf wetness (approx. 4 hours at 15 °C) to penetrate potato leaves instead of the 8 hours that was widely considered to be the minimum time needed for germination and infection. In 1999, we successfully inoculated a field crop under extremely high temperatures (max/min: 34°C/27°C) and observed a latent period of approximately 2.5 days under field conditions. Whether observations like this should be regarded as rare incidents or represent the current performance of *P. infestans* is still under debate, yet more and more evidence supporting the hypothesis of increased levels of pathogenic fitness is accumulating. The failure to achieve the reduction in fungicide use in potatoes aimed for by the Multi-Year Crop Protection Plan (MJP-G) is most likely to be attributed to the increased aggressiveness of the present population of *P. infestans* in the Netherlands.

The increased chance of infection at sub-optimal temperatures in combination with shorter leaf wetness periods will inflate the number of critical infection periods during the growing season of potato crops while shorter latent periods boost the speed of the epidemic. The window in which a potato grower has to act against late blight is narrowing. It has become extremely difficult to achieve a proper timing of fungicide applications. Only the implementation of late blight decision support systems will enable potato growers to apply fungicides using a proper timing while at the same time assisting growers to comply with the present urge to optimise fungicide inputs. In addition, decision support systems offer the opportunity to safely explore the use of reduced fungicide rates in combination with more resistant cultivars.

Impact on the level and stability of partial resistance in potato

The introduction in the Netherlands of a variable, sexually reproducing *P. infestans* population from its centre of origin in central Mexico has not only led to the presence of functional oospores and more pathogenic strains of the pathogen that speed up late blight epidemics. It also shows a malignant effect on the level of partial resistance in potato cultivars. Our results provided indications that in some cases partial resistance and its components can be considered as race-specific types of late blight resistance as we observed a change in ranking order of partially resistant cultivars after exposure to different *P. infestans* genotypes. Our findings indicate that stability and durability of partial resistance of an unknown number of potato cultivars is at stake.

Stability and durability are crucial characteristics needed for a successful implementation of partial resistance in late blight management strategies aimed to reduce fungicide inputs. The existence of race-specific effects raises the question whether the concept of partial resistance of potatoes against late blight is still tenable.

The existence of specificity implies that selection of partial resistance using only one or few isolates may lead to the selection of cultivars which will not express stable forms of partial resistance. It is not known to which extend race-specific interactions affect the stability of partial resistance as only a limited number of cultivars and isolates have been tested. The principal problem is that our present knowledge on the genetic and physiological background of the specific interaction between the pathogen and key components of partial resistance is insufficient. A future challenge is to identify and characterise the genetic mechanisms that are involved in the cascade of interactions determining the actual level of partial resistance that can be observed in the field. For the time being, we propose to expose cultivars and breeding materials to well-defined aggressive *P. infestans* strains that possess as many virulence factors as possible. Potato genotypes showing outstanding levels of late blight resistance should then be exposed to a highly variable field population of *P. infestans*. Only genotypes that express stable resistance to the variable pathogen population should be selected. Compatible isolates should be collected from those breeding materials for additional studies on the genetic background and stability of the resistance concerned. This approach will reduce the risk of selection for non-stable forms of partial resistance.

New hosts, new pathogens?

Results presented in this thesis demonstrated the presence of population differentiation in host specific *P. infestans* populations in the Toluca Valley of central Mexico. In addition, we report on the discovery of a new homothallic *Phytophthora* species collected from *Ipomoea longipedunculata* in the Toluca Valley. The new species appears to be closely related to *P. infestans* and *P. mirabilis*. Recently, we have found evidence for a broadening of host range of *P. infestans* in the Netherlands. During the last two years, we successfully isolated *P. infestans* from severely infected plants of *Solanum nigrum* (black nightshade), *S. dulcamara* (bittersweet) and *S. sisymbriifolium* (an experimentally grown potato cyst nematode trap plant). Oospores have been observed in diseased leaf tissue of *S. dulcamara* and *S. sisymbriifolium* collected in the field. It appears that the colonisation of new hosts followed by a subsequent specialisation on these hosts is a shared feature of Mexican and Dutch populations of *P. infestans*. The question is raised whether host specificity driven speciation is taking place in our own backyard.

In conclusion, potato growers, breeders and plant pathologists alike are facing a pathogen that appears to be more flexible, more variable and faster than we have been used to in the past. For the farmer, late blight appears to be less predictable. The implication of an integrated strategy to control late blight based on sophisticated late blight decision support systems will enable potato growers to explore stable forms of host resistance, dynamic fungicide use and cultural practises in an effort to protect their crops in an effective way while minimising inputs of fungicides for late blight control. To this date, daring multidisciplinary and unparalleled research initiatives are urgently needed to develop the tools needed to conquer late blight.

Samenvattende discussie

De oömyceet *Phytophthora infestans* (Mont.) de Bary, de veroorzaker van 'het kwaad' ofwel de aardappelziekte, wordt wereldwijd gerekend tot de belangrijkste pathogenen in de teelt van aardappelen en tomaten. Sinds de introductie van het pathogeen in Europa rond 1845 veroorzaakt *P. infestans* regelmatig grote problemen in de teelt van aardappelen. Naast een bescheiden inzet van partieel resistente rassen worden jaarlijks worden grote hoeveelheden fungiciden gebruikt om aardappelen te beschermen tegen de aardappelziekte. Problemen met de *Phytophthora* bestrijding namen toe na de introductie van een geheel nieuwe populatie van het pathogeen vanuit Mexico rond 1976. De huidige *P. infestans* populatie in Nederland bestaat uit isolaten die behoren tot het A1 en het A2 paringstype. Wanneer isolaten van het A1 en het A2 paringstype in geïnfecteerde plantdelen in elkaars nabijheid groeien, kunnen door middel van seksuele voortplanting rustsporen (oösporen) ontstaan. Deze rustsporen bleken gedurende ten minste één winter in de grond te kunnen overleven en waren staat zijn om in het volgende seizoen aardappelgewassen aan te tasten. Aangenomen wordt dat oösporen samen met aseksuele inoculum bronnen zoals afvalhopen en geïnfecteerde aardappelknollen een rol spelen bij het ontstaan van nieuwe aardappelziekte epidemieën. Verder werd aangenomen dat seksuele voortplanting en de vorming van functionele oösporen in aangetaste aardappelgewassen hebben geleid tot grotere genetische variatie in populaties van het pathogeen. Agressieve isolaten (isolaten met een hoger ziekteverwekkend vermogen) zouden mogelijk een selectief voordeel hebben en zullen daardoor gedurende het verloop van de epidemie in frequentie toenemen. Door de toename van agressieve isolaten ontstond een *P. infestans* populatie die wordt gekenmerkt door een hoger ziekteverwekkend vermogen. Om in de toekomst betere en meer effectieve bestrijdingsstrategieën tegen de aardappelziekte te kunnen ontwikkelen is een beter inzicht in de epidemiologie en de populatiebiologie van de huidige pathogeen populatie noodzakelijk. Het doel van het onderzoek beschreven in dit proefschrift is het bestuderen van bronnen van variatie in *P. infestans* en het ophelderen van het effect van de toegenomen genetische variatie op het optreden van *P. infestans*.

Variatie in *P. infestans*; bronnen en implicaties

Variatie in aggressiviteit in regionale P. infestans populaties

In een vergelijkende studie naar de variatie in agressiviteit binnen regionale *P. infestans* populaties werd een aanzienlijke fenotypische diversiteit aangetoond voor componenten van agressiviteit voor knol en loof (hoofdstuk 2 & 3. De knolpathogeniteit van het meest agressieve isolaat van de reeds verdrongen, aseksueel reproducerende pathogeen populatie dat beschikbaar is in de isolaatcollectie van Plant Research International bleek vergelijkbaar met het gemiddelde niveau van knolagressiviteit van de nieuwe, seksueel reproducerende pathogeen populatie. Onderzoek naar het voorkomen van variatie in agressiviteit door middel van een biotoets met bladponsjes

heeft opgeleverd dat er veel variatie in ziekteverwekkend vermogen bestaat in regionale populaties van het pathogeen. De gemiddelde loofagressiviteit van isolaten, afkomstig uit drie onderzochte regionale *P. infestans* populaties bleek overeenkomstig voor infectieefficiency, maximale groeisnelheid en sporenproductie. De gemiddelde latente periode van isolaten afkomstig van een volkstuinencomplex in Ede was significant langer dan die van isolaten uit Zuidelijk Flevoland of Drenthe. Er werd geen verband gevonden tussen agressiviteit en het paringstype van een isolaat en ook de diverse componenten van loof- en knolagressiviteit bleken niet of zwak gecorreleerd te zijn.

De rol van oösporen

In Nederland werden oösporen aangetroffen in aangetaste tomatenvruchten en tomatenblad en in blaadjes van aangetaste aardappelplanten die waren verzameld in aardappelopslag en commerciële percelen. De aanwezigheid van vitale oösporen in bladmonsters werd aangetoond door extractie van kiemkrachtige oösporen uit aangetaste blaadjes. Na inoculatie van bladponsjes met isolaten van verschillend paringstype werden oösporen van *P. infestans* aangetroffen in 8 verschillende aardappelrassen met verschillende niveau's aan partiële loofresistentie. Grote aantallen oösporen werden geproduceerd in bladponsjes van het vatbare ras Bintje en het partieel resistente ras Pimpernel. Een relatief lage oösporenproductie werd waargenomen voor het matig vatbare ras Nicola. De overleving van oösporen in een zand- en een lichte kleigrond bij blootstelling aan de natuurlijke weersomstandigheden werd gedurende een periode van zes jaar gevolgd. In een biotoets bleken oösporen uit de zandgrond maximaal 48 maanden, en oösporen uit de lichte klei maximaal 30 maanden infectieus te zijn. De eerste bladaantastingen vanuit oösporen werden 84-92 uur na inundatie van de grond waargenomen. Tijdens dergelijke behandelingen bleek de grond infectieus gedurende 11 dagen. Grondmonsters bleven infectieus gedurende maximaal twee cycli van inundatie gevolgd door drogen van de grond aan de lucht.

De variatie in productie en vitaliteit van oösporen werd onderzocht door kruisingen te maken tussen een aantal Mexicaanse isolaten afkomstig van aardappel en de wilde aardappelverwant *Solanum demissum*. De gevormde aantallen oösporen en de vitaliteit van deze sporen verschilden sterk per kruising. In de meeste kruisingen werden veel oösporen gevormd, maar in sommige oudercombinaties werden zeer weinig of geen oösporen geproduceerd. Een positief verband werd aangetoond tussen het aantal gevormde oösporen in een kruising en de vitaliteit van deze sporen. De productie en vitaliteit van oösporen bleek afhankelijk te zijn van algemene ('*General Combining Ability*', GCA) en de specifieke ('*Specific Combining Ability*', SCA) combinatiegeschiktheid van de ouderstammen. Isolaten afkomstig van *S. demissum* produceerden significant meer oösporen in kruisingen met compatibele isolaten welke ook afkomstig waren van *S. demissum* dan in kruisingen met isolaten afkomstig van aardappelpercelen. Voor het eerst werden er oösporen waargenomen in natuurlijk aangetaste blaadjes van de wilde aardappel soort *S. demissum*. Op twee vindplaatsen

van *S. demissum* werden oösporen aangetroffen in 10 tot 20% van de verzamelde blaadjes met twee of meer *Phytophthora*-lesies.

Stabiliteit van partiële resistentie tegen P. infestans

Om de invloed van de huidige, meer agressieve isolaten op het niveau en de stabiliteit van resistentie te kunnen bepalen werden aardappelrassen met verschillende niveaus van partiële resistentie geinoculeerd met isolaten van de oude en de nieuwe *P. infestans* populatie. Inoculatie-experimenten met hele knollen en knolschijfjes toonden aan dat knolinfectie en het invasief vermogen (de kolonisatie van knolweefsels door het pathogeen ná infectie) niet alleen bepaald wordt door de knolresistentie van het ras en het ziekteverwekkend vermogen van het pathogeen, maar in hoge mate ook door ras x isolaat specifieke factoren.

De stabiliteit van loof- en knolresistentie van een achttal aardappelrassen werd gedurende twee jaren onder laboratorium en veldomstandigheden geëvalueerd om een beter inzicht te verwerven over de gevolgen van de toegenomen variatie en agressiviteit van de ziekteverwekker voor de aardappelteelt en de resistentieveredeling. De mate van loofresistentie in met name cv. Bintje (vatbaar) en cv. Pimpernel (partieel resistent) in kunstmatig geïnfecteerde veldgewassen bleek sterk afhankelijk te zijn van het gebruikte isolaat, terwijl cv. Santé een meer stabiele resistentie reactie te zien gaf. Statistische analyses van veld- en laboratoriumproeven hebben aangetoond dat het uiteindelijke (waargenomen) resistentie niveau van een aardappelras met partiële resistentie niet alleen afhankelijk is van de agressiviteit van het pathogeen maar ook van een specifieke pathogeniteit van het *Phytophthora*-isolaat voor het betreffende ras. De experimenteel bepaalde loof- en knolresistentiecijfers van de getoetste rassen bleken niet of nauwelijks gecorreleerd te zijn met de *Phytophthora* resistentiecijfers in de Nederlandse rassenlijst (hoofdstuk 7). Het wijdverbreid aanwezig zijn van specificiteit in de interactie tussen partieel resistente aardappelrassen en *P. infestans* kan consequenties hebben voor de stabiliteit en duurzaamheid van partiële resistentie en noopt tot herbezinning over de wijze waarop resistentiecijfers voor *P. infestans* ten behoeve van de Nederlandse rassenlijst worden bepaald. Een beter inzicht in het adaptief vermogen van *P. infestans* is noodzakelijk om de duurzaamheid van partieel resistente rassen bij blootstelling aan de huidige variabele pathogeen populaties betrouwbaar te kunnen bepalen.

Variatiepatronen in seksueel reproducerende P. infestans populaties

Genetische variatie van *P. infestans* populaties afkomstig van de hooglanden van centraal Mexico werd onderzocht met behulp van paringstype, allozym en metalaxyl resistentie markers. Isolaten van *P. infestans* werden verzameld van wilde *Solanum* soorten, lokaal geteelde "Criolla" aardappelrassen in marginale productiesystemen en moderne cultivars in intensieve teeltsystemen in de vallei van Toluca. Deze vallei herbergt de meest variabele *P. infestans* populaties ter wereld en wordt in brede kring beschouwd als het oorsprongsgebied van deze soort. In 1988-1989 en 1997 werden

respectievelijk 179 en 401 isolaten verzameld uit de drie onderscheiden deelpopulaties waaraan analyses van genotypische diversiteit werden uitgevoerd. Twee groepen van isolaten afkomstig van verschillende productiesystemen die op basis van het paringstype en allozym genotype niet onderscheidbaar bleken werden nader geanalyseerd door middel van 'Restriction Fragment Length Polymorphism' markers (RFLP's). De meeste isolaten in beide groepen bleken unieke (65% en 85%) RFLP genotypen te bezitten, hetgeen in overeenstemming is met de verwachte diversiteit in een seksueel reproducerende *P. infestans* populatie. De populatiestructuur in 1988-1989 week niet sterk af van die in 1997 met uitzondering van het percentage metalaxyl resistente isolaten (60 versus 13%). De grootste genetische diversiteit werd aangetroffen voor isolaten uit de populatie afkomstig van aardappelcultivars uit het intensieve teeltsysteem (hoofdstuk 8).

De hypothese dat *P. infestans* populaties van wilde *Solanum* soorten grotendeels afkomstig zijn van de pathogeen populaties op gecultiveerde aardappelen in de centrale hooglanden van Mexico werd getoetst. In totaal werden 170 isolaten welke in 1997 werden verzameld op aardappelgewassen en wilde *Solanum* soorten gekarakteriseerd door middel van selectie neutrale mitochondriale (mtDNA haplotypes) en 'Amplified Fragment Length Polymorphism' (AFLP) DNA markers. Alle *Phytophthora*-isolaten bleken te behoren tot het Ia haplotype en er werden in totaal 158 verschillende genotypen onderscheiden op basis van 165 dominant overervende AFLP markers. Gemiddeld bleek 81,8% van de AFLP markers polymorf en varieerde het percentage heterozygotie tussen 7,7 en 19,4%. Er werd een significante differentiatie aangetoond tussen de *P. infestans* populatie afkomstig van wilde *Solanum* soorten en die afkomstig van gecultiveerde aardappel. Populatie specifieke AFLP markers werden waargenomen in isolaten afkomstig uit de drie bemonsterde populaties (variërend van 9 tot 16 markers in geval van isolaten afkomstig uit de intensieve aardappelteelt en wilde *Solanum* soorten). In totaal werden 4 AFLP markers gevonden van welke de aanwezigheid alleen werd aangetoond in isolaten afkomstig van *S. demissum*. Er werden geen aanwijzingen gevonden die de uitgangshypothese ondersteunen. Indirecte schattingen van 'gene-flow' tussen *P. infestans* populaties duiden op een zeer beperkte migratie van isolaten tussen populaties afkomstig van wilde *Solanum* soorten en de gecultiveerde aardappel. De beperkte uitwisseling van isolaten tussen deze populaties is in overeenstemming met de eerder gevonden differentiatie tussen pathogeen populaties van aardappel en wilde *Solanum* soorten (hoofdstuk 9). Populatie differentiatie en genetische isolatie van waardplant specifieke populaties van *P. infestans* in de vallei van Toluca worden verondersteld samen te hangen met het voorkomen van waardplant-specifieke resistentiegenen en 'genetic drift' (hoofdstuk 9).
Een nieuwe homothallische *Phytophthora* soort, nauw verwant aan *P. infestans* en *P. mirabilis* werd in 1999 ontdekt in de vallei van Toluca. Zes isolaten werden verzameld van aangetast bladeren van *Ipomoea longipedunculata*, een wilde grootbloemige winde endemisch voor centraal Mexico. Gebaseerd op vergelijkingen van waardreeks, morfologie, allozym markers, mtDNA haplotype en rDNA basenvolgorde werd

geconcludeerd dat het gaat om een nog niet eerder beschreven soort. De soort is beschreven als *Phytophthora ipomoeae* en een type-exemplaar is gedeponeerd bij het Centraal Bureau voor Schimmelcultures (CBS) in Baarn (hoofdstuk 10).

Conclusies en vooruitblik

Het hier beschreven onderzoek belicht aspecten betreffende de oorsprong en de gevolgen van genetische variatie in seksueel reproducerende populaties van *P. infestans*. Uit het onderzoek kan geconcludeerd worden dat (I) een aanzienlijke mate van variatie voor agressiviteit in stand wordt gehouden in regionale *P. infestans* populaties in Nederland, (II) dat oösporen in aanzienlijke aantallen worden gevormd in aangetaste aardappelgewassen en vooral op opslagplanten, (III) dat de vorming en vitaliteit van oösporen sterk afhankelijk is van de combinatiegeschiktheid van de ouder-isolaten, (IV) dat oösporen tussen de drie en de vier jaar kunnen overleven in de grond, (V) dat differentiële ras x isolaat interacties een belangrijke rol spelen in de ontwikkeling van de aardappelziekte op aan aantal partieel resistente rassen en dat (VI) waardplant specificiteit en populatie differentiatie een rol spelen in de totstandkoming van de populatiestructuur van *P. infestans*.

De invloed van de huidige populatie op de snelheid van epidemieën van de aardappelziekte

De resultaten van het promotie onderzoek maakt duidelijk dat de introductie van een seksueel reproducerende *P. infestans* populatie een grote invloed heeft gehad op epidemieën van de aardappelziekte en de populatiebiologie van het pathogeen. Oösporen worden thans regelmatig gevormd in aangetaste veldgewassen en in aardappelopslag. Onderzoek heeft aangetoond dat deze oösporen tot 48 maanden kunnen overleven in de grond onder veldomstandigheden. Oösporen vormen zeer waarschijnlijk een alternatieve bron van initieel inoculum in aardappelen met een conventionele gewasrotatie. Naast hun rol als bron van eerste aantastingen vormen oösporen vooral de drijvende kracht achter de explosie van genetische variatie die recentelijk werd waargenomen. Het proces van seksuele reproductie heeft ertoe geleid dat door meiotische recombinatie de genetisch diverse populatie van *P. infestans* in Nederland wordt gekenmerkt door een toegenomen aanpassingsvermogen aan waardresistentie en het milieu.

Het is aannemelijk dat de aanwezigheid van meer agressieve isolaten heeft geleid tot een snellere ontwikkeling van de ziekte in het veld. Vergelijking van infectieefficiency en sporulatie-capaciteit van Nederlandse oude en nieuwe populatie isolaten laat zien dat isolaten van de huidige populatie in staat zijn om aardappelblad te infecteren bij temperaturen variërend tussen de 3 en 27 °C terwijl isolaten van de oude populatie infecties veroorzaken tussen 8 en 23 °C (Flier *et al.*, ongepubliceerd). Daarnaast worden isolaten van de nieuwe populatie gekenmerkt door een versnelde

sporenkieming en infectie van bladeren. Onder normale omstandigheden in het veld behoeft *P. infestans* thans slechts een beperkt aantal uren bladnat om infecties te veroorzaken (ongeveer 4 uur bij 15 °C) (Flier *et al.*, ongepubliceerd), dit in tegenstelling tot het verleden waar een bladnat periode van minimaal 8 uur als kritisch werd beschouwd.

In 1999 werd met succes een veldgewas geïnfecteerd na inoculatie onder extreem warme temperaturen (max/min: 34°C /27°C) en werd een latente periode waargenomen van ongeveer 2,5 dag (Flier *et al.*, ongepubliceerd). De toegenomen kans op infecties bij lage temperaturen in combinatie met kortere kritische bladnatperiodes zal het aantal infectiemomenten tijdens het teeltseizoen doen toenemen. Daarnaast wordt door de kortere latente periode de snelheid van de epidemie ook nog weer aanzienlijk versneld. De speelruimte van de aardappelteler wordt hierdoor aanzienlijk beperkt en maakt het gebruik van lagere fungicide doses al dan niet in combinatie met meer resistente rassen of het verlengen van spuitintervallen een hachelijke zaak wanneer geen gebruik wordt gemaakt van een effectief geïntegreerd bestrijdingssysteem.

Invloed op het niveau en de stabiliteit van partiële resistentie

De introductie van een seksueel reproducerende *P. infestans* populatie vanuit het oorsprongsgebied van het pathogeen in centraal Mexico heeft niet alleen geleid tot het voorkomen van functionele oösporen, het optreden van meer agressieve isolaten en een snellere ontwikkeling van de ziekte, maar heeft ook een negatief effect op het niveau en de stabiliteit van partiële resistentie in aardappelen tegen de aardappelziekte. Onderzoek in het kader van dit promotieonderzoek heeft aangetoond dat specificiteit een belangrijke rol speelt in de interacties tussen *P. infestans* en partieel resistente aardappelrassen. Deze specificiteit kan leiden tot instabiliteit van partiële resistentie, en leidt daardoor tot een gehele of gedeeltelijke erosie van veldresistentie. In de Praktijk wordt erosie van resistentie opgemerkt als het meer of minder geleidelijk teruglopen van de resistentie in het veld.

Het optreden van specificiteit roept vragen op over het nut en de bruikbaarheid van partiële resistentie tegen de aardappelziekte omdat stabiliteit en duurzaamheid van partiële resistentie van doorslaggevend belang is voor een succesvolle implementatie en het gebruik ervan in geïntegreerde bestrijdingssystemen. Specificiteit impliceert dat partieel resistente rassen op een andere wijze getoetst dienen te worden. Het gebruik van één isolaat tijdens de selectieprocedure en de toetsing voor de rassenlijst kan leiden tot de selectie van aardappelrassen met instabiele vormen van partiële resistentie en daarom wordt een alternatief toetsingsprocedure voorgesteld. Van veel kruisingsmateriaal en raswaardige klonen is de genetische achtergrond van de resistentie onbekend. Het beste advies dat nu gegeven kan worden aan de aardappelkwekers is om deze nieuwe rassen of raswaardige klonen bloot te stellen aan goed gekarakteriseerde agressieve isolaten met een zo groot mogelijk aantal virulentiefactoren. Klonen die op basis van deze test als zeer resistent beoordeeld

worden, dienen daarna gedurende tenminste twee veldseizoenen getest te worden tegen een variabele populatie van het pathogeen (zoals aanwezig in bijv. zuidoost Drenthe). Gedurende de blootstelling van rassen en klonen aan de veldpopulatie kunnen compatibele isolaten worden verzameld van planten die aangetast worden. Rassen of klonen die worden gekenmerkt door een stabiele expressie van partiële resistentie worden geselecteerd en de (componenten van de) resistentie kan naderhand nog verder worden getoetst door inoculatie studies met compatibele *P. infestans* isolaten. Deze selectieprocedure zal naar verwachting een belangrijke bijdrage kunnen leveren aan het beschikbaar komen van rassen met stabiele vormen van partiële resistentie.

Nieuwe waarden, nieuwe pathogenen?

Het promotieonderzoek heeft aanwijzingen opgeleverd dat populatie differentiatie en waardplant specifieke *P. infestans* populaties voorkomen in het oorsprongsgebied van *P. infestans* in de hooglanden van centraal Mexico. Voorts werd in de Vallei van Toluca een nieuwe *Phytophthora* soort, *P. ipomoeae,* ontdekt en beschreven. Het optreden van waard specialisatie, sympatrische soortsvorming en ontstaan van nieuwe pathogenen vormt een ander belangrijk aspect van genetische variatie. Recentelijk zijn ook in Nederland aanwijzingen gevonden voor een verbreding van de waardreeks van *P. infestans*. Gedurende de laatste twee jaren is *P. infestans* met succes geïsoleerd uit natuurlijk geïnfecteerde planten van *Solanum nigrum* (zwarte nachtschade), *S. dulcamara* (bitterzoet) en *S. sisymbriifolium* (een experimenteel lokgewas voor aardappelcyste aaltjes). In het geval van *S. nigrum* en *S. dulcamara* werden tevens oösporen waargenomen in het aangetaste bladmateriaal.

Concluderend kan gesteld worden dat zowel telers als veredelaars en gewasbeschermers te maken hebben gekregen met een pathogeen dat zich flexibeler, variabeler, sneller en daardoor voor de aardappelteler minder voorspelbaar is gaan gedragen. Alleen echt geïntegreerde bestrijdingsstrategieën gebaseerd op betrouwbare ziektevoorspellingsmodellen zullen het voor de aardappelteler mogelijk maken om op een verantwoorde wijze duurzame vormen van waardresistentie, dynamisch fungiciden gebruik en cultuurmaatregelen in te passen in een bestrijdingsstrategie voor de aardappelziekte. Implementatie van dergelijke bestrijdingsstrategieën bieden op korte termijn de beste perspectieven om de effectiviteit van fungiciden te verhogen en tegelijkertijd het middelengebruik terug te dringen. Hiertoe is het noodzakelijk om snel essentiële basisgereedschappen te ontwikkelen door ambitieuze onderzoeksinitiatieven te ontplooien met een brede benadering van het *Phytophthora*-probleem.

References

Al-Kherb SM, Fininsa C, Shattock RC, Shaw DS (1995). The inheritance of virulence of *Phytophthora infestans* to potato. *Plant Pathology* **44**: 552-562.

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

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

Anonymous (1996). CIP in 1995. The International Potato Center Annual Report. Lima, Peru.

Bain RA, Möller K (1999). Factors influencing potato tuber infection by *Phytophthora infestans*. Proceedings of the Workshop on the European network for development of an integrated control strategy of potato late blight. Uppsala, Sweden, 9-13 September 1998, pp. 210-227.

de Bary A (1876). Researches into the nature of the potato fungus, *Phytophthora infestans*. *Journal of the Royal Agricultural Society of England*, Series 2, **12**: 239-269.

Baayen RP, O'Donnel K, Bonants PJM, Cigelnik E, Kroon LPNM, Roebroeck EJA, Waalwijk C (2000). Gene genealogies and AFLP analyses in the *Fusarium oxysporum* complex identify monophyletic and nonmonophyletic formae speciales causing wilt and rot disease. *Phytopathology* **90**: 891-900.

Berg LM, Lascoux M (2000). Neutral genetic differentiation in an island model with cyclical parthenogenesis. *Journal of Evolutionary Biology* **13**: 488-494.

Berlocher SH (1998). Can sympatric speciation via host or habitat shift be proven from phylogenetic and biogeographic evidence? In: *Endless forms, species and speciation* (ed. D. J. Howard & S. T. Berlocher), pp. 99-113. Oxford Press: New York.

Bjor T (1987). Testing the resistance of potato genotypes to tuber blight. *Potato Research* **30**: 525-532.

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

Bhatia SK, Young RJ (1985). Reaction of potato tuber slices to *Phytophthora infestans* in relation to physical age. *American Potato Journal* **62**: 471-478.

Black W, Mastenbroek C, Mills WR, Peterson LC (1953). A proposal for an international nomenclature of races of *Phytophthora infestans* and of genes controlling immunity in *Solanum demissum* derivates. *Euphytica* **2**: 173-240.

Black W, Gallegly ME (1957). Screening of *Solanum* species for resistance to physiologic races of *Phytophthora infestans*. *American Potato Journal* **34**: 273-281.

Bonants PJM, Hagenaar-de Weerdt M, Man in 't Veld WA, Baayen RP (2000). Molecular characterization of natural hybrids of *Phytophthora nicotianae* and *P. cactorum*. *Phytopathology* **90**: 867-874.

Borchart DS, Welz HG, Geiger HH (1998a). Genetic structure of *Setosphaeria turcica* populations in tropical and temperate climates. *Phytopathology* **88**: 322-329.

Borchart DS, Welz HG, Geiger HH (1998b). Spatial and temporal variation of genetic marker patterns in *Setosphaeria turcica* populations from Kenya. *Journal of Phytopathology* **146**: 451-457.

Brasier CM (1983). Problems and prospects in *Phytophthora* research. In: *Phytophthora: Its Biology, Taxonomy, Ecology and Pathology* (DC Erwin, S Bartnicki-Garcia and PH Tsao, Eds.), pp 351-364. APS, St. Paul.

Brasier CM, Hansen EM (1992). Evolutionary biology of *Phytophthora*. Part II: Phylogeny, speciation and population structure. *Annual review of Phytopathology* **30**: 173-200.

Brasier CM, Cooke DEL, Duncan JM (1999). Origin of a new *Phytophthora* pathogen through interspecific hybridization. *Proceedings of the National Academy of Sciences USA*. **96**: 5878-5883.

De Bruyn HLG (1947). Het rassenprobleem bij *Phytophthora infestans*. *Vakblad voor Biologen* **27**: 147-152.

Bush GL (1969). Sympatric host race formation and speciation in frugivorous flies of the genus *Rhagoletis* (Diptera, Tephritidae). *Evolution* **23**: 237-251.

Caffier V, Brändle UE, Wolfe MS (1999). Genotypic diversity in barley powdery mildew populations in northern France. *Plant Pathology* **48**: 582-587.

Campbell CL, Madden LV (1990). Introduction to plant disease epidemiology. John Wiley & Sons, NewYork

Carson ML (1987). Assessment of six models of host-pathogen interaction in horizontal pathosystems. *Phytopathology* **77**: 241-246.

Carter DA, Archer SA, Buck KW, Shaw DS, Shattock RC (1991). DNA polymorphism in *Phytophthora infestans*: the UK experience, pp. 272-294. In JA Lucas, RC Shattock, DS Shaw, LR Cooke (eds.), *Phytophthora*. Cambridge University Press Cambridge, United Kingdom.

Caten CE, Jinks JL (1968). Spontaneous variability of single isolates of *Phytophthora infestans*. I. Cultural variation. *Canadian Journal of Botany* **46**: 329-348.

Caten CE (1970). Spontaneous variability of single isolates of *Phytophthora infestans*. II . Pathogenic variation. *Canadian Journal of Botany* **48**: 897-905.

Caten CE (1974). Intra-racial variation in *Phytophthora infestans* and adaptation to field resistance for potato blight. *Annals of Applied Biology* **77**: 259-270.

Chen RS, McDonald BA (1996). Sexual reproduction plays a major role in the genetic structure of populations of the fungus *Mycosphaerella graminicola*. *Genetics* **142**: 1119-1127.

Chen RS, Boeger JM, McDonald BA (1994). Genetic stability in a population of a plant pathogenic fungus over time. *Molecular Ecology* **3**: 209-218.

Cohen Y, Farkash S, Reshit Z, Baider A (1997). Oospore production of *Phytophthora infestans* in potato and tomato leaves. *Phytopathology* **87**: 191-196.

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

Conner AJ, Keizer LCP, Mlynarova L, Stiekema WJ, Nap JP, Eberhart SA, Gardner CO (1998). Evaluation of diallel analysis using beta-glucuronidase activity from transgenes in *Nicotiana tabacum*. *Euphytica* **102**: 161-168.

Cooke DEL, Drenth A, Duncan JM, Wagels G, Brasier CM (2000). A molecular phylogeny of *Phytophthora* and related oomycetes. *Fungal Genetics and Biology* **30**: 17-32.

Correll DS (1962). The potato and its wild relatives. Texas Research Foundation, Renner, TX.

Corsten LCA, Denis JB (1990). Structuring interaction in two-way tables by clustering. *Biometrics* **46**: 207-215.

Crawford AR, Bassam BJ, Drenth A, Maclean DJ, Irwin JAG (1996). Evolutionary relationships among *Phytophthora* species deduced from rDNA sequence analysis. *Mycological Research* **100**: 437-443.

Crute IR, Norwood JM, Gordon PL (1987). The occurrence, characteristics and distribution in the United Kingdom of resistance to phenylamide fungicides in *Bremia lactucae* (lettuce downy mildew). *Plant Pathology* **36**: 297-315.

Davidse LC, Looijen D, Turkensteen LJ, van der Wal D (1981). Occurrence of Metalaxyl-resistant strains of *Phytophthora infestans* in the Netherlands. *European Plant Protection Organisation Bulletin* **15**: 403-409.

Davidse LC, Henken J, Dalen A van, Jespers AKB, Mantel BC (1989). Nine years of practical experience with phenylamide resistance in *Phytophthora infestans* in the Netherlands. *Netherlands Journal of Plant Pathology* **95**, supplement 1: 197-213.

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

Dieckmann U, Moebeli M (1999). On the origin of species by sympatric speciation. *Nature* **400**: 354-357.

Dorrance AE, Inglis DA (1998). Assessment of laboratory methods for evaluating potato tubers for resistance to late blight. *Plant Disease* **82**: 442-446.

Drenth A, Goodwin SB, Fry WE, Davidse LC (1993a). Genotypic diversity of *Phytophthora infestans* in The Netherlands revealed by DNA polymorphisms. *Phytopathology* **83**: 1087-1092.

Drenth A, Turkensteen LJ, Govers F (1993b). The occurrence of the A2 mating type of *Phytophthora infestans* in the Netherlands; significance and consequences. *Netherlands Journal of Plant Pathology* **99**, supplement 3: 57-67.

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

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

Duncan JM (1999). *Phytophthora*- an abiding threat to our crops. *Microbiology Today* **26**: 114-116.

Ebskamp AGM, Stolk H, Bonthuis H (1998). 73rd List of varieties of field crops 1998. Centrum voor Plantenveredelings- en Reproductieonderzoek (CPRO-DLO), Wageningen.

Ebskamp AGM, Stolk H, Bonthuis H (1999). 74rd List of varieties of field crops 1999. Centrum voor Plantenveredelings- en Reproductieonderzoek (CPRO-DLO), Wageningen.

Van Eeuwijk FA (1995). Multiplicative interaction in generalized linear models. *Biometrics* 51: 1017-32.

Van Eeuwijk FA (1996). Between and beyond additivity and non-additivity: the statistical modelling of genotype by environment interaction in plant breeding. Ph.D. thesis, Wageningen Agricultural University, Wageningen, The Netherlands.

Elguezabal JF (1993). Influencia del genotipo de papa en la produccion de oosporas de *Phytophthora infestans* (Mont.) de Bary. Montecillo, Mexico: Collegio de Postgraduados, PhD thesis.

Elliot CG (1983). Physiology of sexual reproduction in Phytophthora. In: Erwin DC, S Bartnicki-Garcia, PH Tsao (eds.), *Phytophthora: Its Biology, Taxonomy, Ecology and Pathology*. St. Paul, USA: The American Phytopathological Society, pp. 71-80.

Erwin DC, Ribeiro OK (1996). Phytophthora diseases worldwide. APS Press, St. Paul.

Fabritius AL, Judelson HS (1997). Mating-type loci segregate aberrantly in *Phytophthora infestans* but normal in *Phytophthora parasitica*: implications for models of mating-type determination. *Current Genetics* **32**: 60-65.

Falconer DS, Mackay TFC (1996). Quantitative Genetics. Longman, Harlow, UK.

Felsenstein J (1985). Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* **39**: 783-791.

Flier WG, Turkensteen LJ, Mulder A (1998). Variation in tuber pathogenicity of *Phytophthora infestans* in the Netherlands. *Potato Research* **41**: 345-354.

Flier WG and Turkensteen LJ (1999). Foliar aggressiveness of *Phytophthora infestans* in three potato growing regions in the Netherlands. *European Journal of Plant Pathology* **105**: 381-388.

Flier WG, Turkensteen LJ, van den Bosch GBM, Vereijken FG, Mulder A (2001a). Differential interaction of *Phytophthora infestans* on tubers of potato cultivars with different levels of blight resistance. Plant Pathology, in press.

Flier WG, Grünwald NJ, Fry WE, Turkensteen LJ (2001b). Formation, production and viability of oospores of *Phytophthora infestans* from potato and *Solanum demissum* in the Toluca Valley, Central Mexico. *Mycological Research*, in press.

Forbes, GA (1997). Manual for laboratory work on *Phytophthora infestans*. Centro Internacional de la Papa. CIP training manual. Quito, Ecuador.

Forbes GA, Goodwin SB, Drenth A, Oyarzun P, Ordoñez ME, Fry WE (1988). A global marker database for *Phytopthora infestans*. *Plant Disease* **82**: 811-818.

Freunds RJ, Littell RC (1991). SAS System for Regression. Second Edition Cary, NC: SAS Institute Inc.

Frinking HD, Davidse LC, Limburg H (1987). Oospore formation by *Phytophthora infestans* in host tissue after inoculations with isolates of opposite mating type found in the Netherlands. *Netherlands Journal of Plant pathology* **93**: 147-149.

Fry WE (1975). Integrated effects of polygenic resistance and a protective fungicide on development of potato late blight. *Phytopathology* **65**: 908-911.

Fry WE, Spielman LJ (1991). Population Biology. In: DS Ingram, PH Williams (eds.), *Advances in Plant Pathology Volume 7; Phytophthora infestans, the cause of late blight of potato.* pp. 171-192. Academic Press: London, UK.

Fry WE, Drenth A, Spielman LJ, Mantel BT, Davidse LC, Goodwin SB (1991). Population genetic structure of *Phytophthora infestans* in The Netherlands. *Phytopathology* **81**: 1330-1336.

Fry WE, Goodwin SB, Matuszak JM, Spielman LJ, Milgroom MG, Drenth A (1992). Population genetics and intercontinental migrations of *Phytophthora infestans*. *Annual Review of Phytopathology* **30**: 107-130.

Fry WE, Goodwin SB (1997). Resurgence of the Irish potato famine fungus. *Bioscience* **47**: 363-371.

Futuyma DJ, Mayer GC (1980). Non-allopatric speciation in animals. *Systematic Zoology* **29**: 254-271.

Galindo J, Gallegly ME (1960). The nature of sexuality in *Phytophthora infestans*. *Phytopathology* **50**: 123-128.

Galindo J, Hohl HR (1985). *Phytophthora mirabilis*, a new species of *Phytophthora*. *Sydowia* **38**:87-96.

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

Gallegly ME (1964). West Virginia 63, a new home-garden tomato resistant to late blight. *Bulletin West Virginia University Agricultural Experimental Station* **490**:15-16.

Gallegly ME (1968). Genetics of pathogenicity of *Phytophthora infestans*. Annual Reviews of *Phytopathology* **56**: 375-396.

Goodwin, SB, Spielman LJ, Matuszak JM, Bergeron SN, Fry WE (1992a). Clonal diversity and genetic differentiation of *Phytophthora infestans* populations in northern and central Mexico. *Phytopathology* **82**: 955-961.

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

Goodwin SB, Maroof MAS, Allard RW, Webster RK (1993). Isozyme variation within and among populations of *Rhynchosporium secalis* in Europe, Australia and the United States. *Mycological Research* **97**: 49-58.

Goodwin SB, Cohen BA, Fry WE (1994). Panglobal distribution of a single clonal lineage of the Irish potato famine fungus. *Proceedings of the National Academy of Sciences USA* **91**: 11591-11595.

Goodwin SB, Schneider RE, Fry WE (1995a). Use of cellulose-acetate electrophoresis for rapid identification of allozyme genotypes of *Phytophthora infestans*. *Plant Disease* **79**: 1181-1185.

Goodwin SB, Sujkowski LS, Dyer AT, Fry BA, Fry WE (1995b). Direct detection of gene flow and probable sexual reproduction of *Phytophthora infestans* in Northern North America. *Phytopathology* **85**: 473-479.

Goodwin SB (1996). Origin and ecology of *Phytophthora infestans*. *Revista Mexicana de fitopatologia* **14**: 143-147.

Goodwin SB, Legard DE, Smart CD, Levy M, Fry WE (1999). Gene flow analysis of molecular markers confirms that *Phytophthora mirabilis* and *P. infestans* are separate species. *Mycologia* **91**: 796-810.

Gower JC, Hand DJ (1996). Biplots. *Monographs on Statistics and Applied Probability* **54**. Chapman & Hall, London, UK.

Gregory PH (1948). The multiple-infection transformation. *Annals of Applied Biology* 35: 412-417.

Griffith GW, Shaw DS (1998). Polymorphisms in *Phytophthora infestans*: Four mitochondrial haplotypes are detected following PCR amplification of DNA from pure cultures or from host lesions. *Applied and Environmental Microbiology* **64**: 4007-4014.

Groth JV, Roelfs AP (1987). The concept and measurement of phenotypic diversity in *Puccinia graminis* on wheat. *Phytopathology* **77**: 1395-1399.

Grünwald NJ, Fry WE (2000a). Analysis of genotypic diversity in population genetic studies should include richness, evenness and diversity. *Phytopathology* **90**: S31.

Grünwald NJ, Flier WG, Turkensteen LJ, Fry WE (2000b). Populations of *Phytophthora infestans* in the Toluca valley on wild *Solanum* species are not different from those on cultivated potatoes. *Phytopathology* **90**: S31.

Grünwald NJ, Rubio-Covarrubias OA, Fry WE (2000c). Potato late blight management in the Toluca valley: forecasts and resistant cultivars. *Plant Disease* **84**: 410-416.

Grünwald NJ, Flier WG, Sturbaum AK, Garay-Serrano E, Van den Bosch GBM, Smart CD, Matuszak, J, Turkensteen LJ, Fry WE (2001). Genetic population structure of *Phytophthora infestans* within the Toluca valley of Central Mexico. *Phytopathology* (submitted).

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

Hawkes JG (1990). The Potato: Evolution, biodiversity and genetic resources. Washington DC.: Smithosian Institution Press.

Heddergott H (1957). Über 100 jahre Krautfäulebekämpfung. *Der Kartoffelbau* **8**: 82-84.

Hermansen A, Hannukkala A, Hafskjold Nearstad R, Brurberg MB (2000). Variation in populations of *Phytophthora infestans* in Finland and Norway: mating type, metalaxyl resistance and virulence phenotype. *Plant Pathology* **49**: 11-22.

Hermsen JGT, Ramanna MS (1973). Double-bridge hybrids of *Solanum bulbocastanum* and cultivars of *Solanum tuberosum*. *Euphytica* **22**: 457-466.

Hohl HR, Iselin K (1984). Strains of *Phytophthora infestans* from Switzerland with A2 mating type behaviour. *Transactions of the British mycological Society* **83**: 529-530.

Holden JHW (1977). Fifty-sixth annual report April 1976 to March 1977. Scottish Plant Breeding Station, Pentlandfield, pp. 66-97.

Hooker WJ (1981). Compendium of potato diseases. American Phytopathological Society, St. Paul, MN.

Ilieva E, Man in 't Veld WA, Veenbaas-Rijks W, Pieters R (1998). *Phytophthora multivesiculata*, a new species causing rot in Cymbidium. *European Journal of Plant Pathology* **104**: 677-684.

Inglis DA, Johnson DA, Legard DE, Fry WE, Hamm PB (1996). Relative resistances of potato clones in response to new and old populations *of Phytophthora infestans*. *Plant Disease* **80**: 575-578.

James WC (1974). Assessment of plant diseases and losses. *Annual Review of Phytopathology* **12**: 27-48.

James RV, Fry WE (1983). Potential for *Phytophthora infestans* populations to adapt to potato cultivars with rate-reducing resistance. *Phytopathology* **73**: 984-988.

Jenns AE, Leonard KJ (1985). Reliability of statistical analyses for estimating relative specificity in quantitative resistance in a model host-pathogen system. *Theoretical and Applied Genetics* **69**: 503-513.

Jiang J, Erwin DC (1990). Morphology, plasmolysis, and tetrazolium bromide stain as criteria for determining viability of *Phytophthora infestans*. *Mycologia* **82**: 107-113.

Judelson HS, Spielman LJ, Shattock RC (1995). Genetic mapping and non-Mendelian segregation of mating type loci in the Oomycete, *Phytophthora infestans*. *Genetics* **143**: 501-512.

Judelson HS (1996a). Chromosomal heteromorphism linked to the mating type locus of the oomycete *Phytophthora infestans*. *Molecular and General Genetics* **252**: 155-161.

Judelson HS (1996b). Genetic and physical variability at the mating type locus of the oomycete, *Phytophthora infestans*. *Genetics* **144**: 1005-1013.

Judelson HS (1997). Expression and inheritance of sexual preference and selfing potential in *Phytophthora infestans*. *Fungal Genetics and Biology* **21**: 188-197.

Kadish D, Cohen Y (1992). Overseasoning of metalaxyl-sensitive and metalaxyl-resistant isolates of *Phytophthora infestans* in potato tubers. *Phytopathology* **82**: 887-889.

Kamoun S, Young M, Förster H, Coffey MD, Tyler BM (1994). Potential role of elicitins in the interaction between *Phytophthora* species and tobacco. *Applied Environmental Microbiology*. **60**: 1593-1598.

Kondrashov AS, Kondrashov FA (1999). Interactions among quantitative traits in the course of sympatric speciation. *Nature* **400**: 351-354.

Krebs CJ (1989). Ecological Methodology. New York: HarperCollins Publishers.

Kulkarni RN, Chopra VL (1982). Environment as the cause of differential interaction between host cultivars and pathogenic races. *Phytopathology* **72**: 1384-1386.

Lacey J (1967). Susceptibility of potato tubers to infection by *Phytophthora infestans*. *Annals of Applied Biology* **59**: 257-264.

Lambert DH, Currier AI (1997). Differences in tuber rot development for North American clones of *Phytophthora infestans*. *American Potato Journal* **74**: 39-43.

Langcake P (1974). Sterols in potato leaves and their effect on growth and sporulation of *Phytophthora infestans*. *Transactions of the British Mycological Society* **63**: 573-586.

Lapwood DH (1977). Factors affecting the field infection of potato tubers of different cultivars by blight (*Phytophthora infestans*). *Annals of Applied Biology* **85**: 23-42.

Lapwood DH, Mckee RK (1961). Reaction of tubers of R-gene potato clones to inoculation with specialised races of *Phytophthora infestans*. *European Potato Journal* **4**: 3-9.

Latin RX, MacKenzie DR, Cole H Jr., (1981). The influence of host and pathogen genotypes on the apparent infection rates of potato late blight epidemics. *Phytopathology* **71**: 82-85.

Lebreton L, Lucas J-M, Andrivon D (1999). Aggressiveness and competative fitness of *Phytophthora infestans* isolates collected from potato and tomato in France. *Phytopathology* **89**: 679-686.

Lee TY, Mizubuti E, Fry WE (1999). Genetics of metalaxyl resistance in *Phytophthora infestans*. *Fungal Genetics and Biology* **26**: 118-130.

Legard DE, Lee TY, Fry WE (1995). Pathogenic specialization in *Phytophthora infestans:* aggressiveness to tomato. *Phytopathology* **85**: 1356-1361.

Levene H (1949). On a matching problem in genetics. *Annals of Mathematical Statistics* **20**: 91-94.

Leonard KJ, Moll RH (1979). Durability of general resistance: evaluation of cultivar x isolate interactions. In: Proceedings of Symposia 9th International Congress of Plant Protection, Washington DC, Volume I Plant Protection: Fundamental aspects, pp. 190-193.

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

Livingston RJ (1976). Diurnal and seasonal fluctuations of organisms in a north florida estuary. *Estuarine and Coastal Marine Science* **4**: 373-400.

Lozoya-Saldaña H, Hernández A, Flores R, Bamberg J (1997). Late blight on wild *Solanum* species in the Toluca valley in 1996. *American Potato Journal* **74**: 445.

Ludwig JA, Reynolds JF (1988). Statistical Ecology: a Primer on Methods and Computing. New York: John Wiley & Sons.

Lynch M, Milligan BG (1994). Analysis of population genetic structure with RAPD markers. *Molecular Ecology* **3**: 91-99.

Mackay GR (1996). Fighting Late Blight. *CIP Circular* **22**: 1-5.

Magurran AE (1988). Ecological diversity and its measurement. Princeton: Princeton University Press.

Malcolmson JF, Black W (1966). New R genes in *Solanum demissum* Lindl. and their complementary races of *Phytophthora infestans* (Mont.) de Bary. *Euphytica* **15**: 199-203.

Man in 't Veld WA, Veenbaas-Rijks WJ, Ilieva E, De Cock AWAM, Bonants PJM, Pieters R (1998). Natural hybrids of *Phytophthora nicotianae* and *Phytophthora cactorum* demonstrated by isozyme analysis and random amplified polymorphic DNA. *Phytopathology* **88**: 922-929.

Matuszak JM, Fernandez Elquezabal J, Villarreal Gonzalez M, Fry WE (1994). Sensitivity of *Phytophthora infestans* populations to metalaxyl in Mexico: distribution and dynamics. *Plant Disease* **78**: 911-916.

Mayr E (1963). Animal species and evolution. Belknap Press, Cambridge, Massachusetts.

McCullagh P, Nelder JA (1989). Generalized Linear Models. *Monographs on Statistics and Applied Probability* 37. Chapman & Hall, London, UK.

McDonald BA, Mundt CC, Chen RS (1996). The role of selection on the genetic structure of pathogen populations: evidence from field experiments with *Mycosphaerella graminicola* on wheat. *Euphytica* **92**: 73-80.

Milgroom MG (1995). Analysis of population structure in fungal plant pathogens. In: JF Leslie, RA Frederiksen (eds.) Disease Analysis through Genetics and Biotechnology. Ames: Iowa State University Press.

Mills WR, Niederhauser JS (1953). Observations of races of *Phytophthora infestans* in Mexico. *Phytopathology* **43**: 454-455.

Mucharromah, Kuc J (1995). The effect of sterols on the compatibility or incompatibility of potato tuber discs and foliage to *Phytophthora infestans* and *Helminthosporium carbonum*, and the induction of resistance by arachidonic acid. *Physiological and Molecular Plant Pathology* **47**: 1-12.

Mooi JC (1964). A laboratory method for testing tubers of potato varieties for 'field resistance' to late blight. Proceedings of the 2nd Triennial Conference of the European Association for Potato Research, Pisa, p. 195.

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

Müller K, Black W (1952). Potato breeding for resistance to blight and virus diseases during the last hundred years. *Zeitschrift für Pflantzenzüchtung* **31**: 305-318.

Müller K, McDermott JM, Wolfe MS, Limpert E (1996). Analysis of diversity in populations of plant pathogens: The barley powdery mildew pathogen across Europe. *European Journal of Plant Pathology* **102**: 385-395.

Nei M (1973). Analysis of Gene Diversity in Subdivided Populations. *Proceedings of the National Academy of Sciences USA* **70**: 3321-3323.

Nei M (1978). Estimation of average heterozygosity and genetic distance from a small number of individuals. *Genetics* **89**: 583-590.

Nelson RR (1979). The evolution of parasitic fitness. In: Horsfall JB, Cowling EB (eds.) Plant disease. An advanced treatise. Vol. 4, pp. 23-46. Academic Press, New York.

Niederhauser JS (1956). The blight, the blighter, and the blighted. *Transactions of the New York Academy of Sciences* **19**: 55-63.

Niederhauser JS (1962). Evaluation of multigenic 'field resistance' of the potato to *Phytophthora infestans* in 10 years of trails at Toluca, Mexico. *Phytopathology* **52**: 746.

Niederhauser JS, Cervantes J, Servin L (1954). Late Blight in Mexico and it's implications. *Phytopathology* **44**: 406-408.

Niederhauser JS (1991). *Phytophthora infestans*: The Mexican connection. In JA Lucas, RC Shattock, DS Shaw, LR Cooke (eds), *Phytophthora*. Cambridge: Cambridge University Press.

Ordoñez ME, Hohl HR, Velasco JA, Ramon MP, Oyarzun PJ, Smart CD, Fry WE, Forbes GA, Erselius LJ (2000). A novel population of *Phytophthora*, similar to *P. infestans*, attacks wild *Solanum* species in Ecuador. *Phytopathology* **90**: 197-202.

Parlevliet JE, Zadoks JC (1977). The integrated concept of disease resistance: a new view including horizontal and vertical resistance in plants. *Euphytica* **26**: 5-21.

Pathak N, Clarke DD (1987). Studies on the resistance of the outer cortical tissues of the tubers of some potato cultivars to *Phytophthora infestans*. *Physiological Molecular Plant Pathology* **31**: 123-132.

Payne RW, Lane PW, Digby PGN, Harding SA, Leech PK, Morgan GW, Todd AD, Thompson R, Tunnicliffe-Wilson G, Welham SJ, White RP (1993). *Genstat 5 Release 3 Reference Manual*. Clarendon Press: Oxford UK.

Peters RD, Platt HW, Hall R, Medina M (1999). Variation in aggressiveness of Canadian isolates of *Phytophthora infestans* as indicated by their relative abilities to cause potato tuber rot. *Plant Disease* **83**: 652-661.

Pielou EC (1975). Ecological Diversity. New York: Wiley.

Pietkiewicz JB, Jellis GJ (1976). Testing potato tubers for resistance to blight (*Phytophthora infestans* (Mont.) De Bary) in the laboratory. *Biuletyn Instytutu Ziemniaka* **2**: 129-134.

Pittis JE, Shattock RE (1994). Viability, germination and infection potential of oospores of *Phytophthora infestans*. *Plant Pathology* **43**: 387-396.

van der Plank JE (1968). Disease Resistance in Plants. Academic Press, New York.

van der Plank JE (1971). Stability of resistance to *Phytophthora infestans* in cultivars without R genes. *Potato Research* **14**: 263-270.

Platt HW, Tai G (1998). Relationship between resistance to late blight in potato foliage and tubers of cultivars and breeding selections with different resistance levels. *American Journal of Potato Research* **75**: 173-178.

Pooni HS, Jinks JL, Singh RK (1984). Methods of analysis and the estimation of the genetic parameters from a diallel set of crosses. *Heredity* **52**: 243-253.

Raj SA, Bhattacharyya SK, Sharma SR (1976). New hosts for *Phytophthora infestans* in nature. *Indian Phytopathology* **29**: 342-343.

Raymond ML, Rousset F (1995). An exact test for population differentiation. *Evolution* **49**: 1280-1283.

Reddick D (1939). When came *Phytophthora infestans*? *Chronica Botanica* **5**: 410-411.

Rivera-Peña A, Molina-Galan J (1989). Wild tuber-bearing species of *Solanum* and incidence of *Phytophthora infestans* (Mont.) de Bary on the Western slopes of the volcano Nevado de Toluca. 1. *Solanum* species. *Potato Research* **32**: 181-195.

Rivera-Peña A (1990a). Wild tuber-bearing species of *Solanum* and incidence of *Phytophthora infestans* (Mont.) de Bary on the Western slopes of the volcano Nevado de Toluca. 2. Distribution of *Phytophthora infestans*. *Potato Research* **33**: 341-347.

Rivera-Peña A (1990b). Wild tuber-bearing species of *Solanum* and incidence of *Phytophthora infestans* (Mont.) de Bary on the Western slopes of the volcano Nevado de Toluca. 3. Physiological races of *Phytophthora infestans Potato Research* **33**: 349-355.

Rivera-Peña A (1990c). Wild tuber-bearing species of *Solanum* and incidence of *Phytophthora infestans* (Mont.) de Bary on the western slopes of the volcano Nevado de Toluca. 4. Plant development in relation to late blight infection *Potato Research* **33**: 469-478.

Rivera-Peña A (1990d). Wild tuber-bearing species of *Solanum* and incidence of *Phytophthora infestans* (Mont.) de Bary on the Western slopes of the volcano Nevado de Toluca. 5. Type of resistance to *P. infestans*. *Potato Research* **33**: 479-486.

Sánchez Sánchez O (1968). La Flora del Valle de Mexico, Sexta edición, México D.F., 519 pp.

Schepers HTAM (2000). The development and control of *Phytophthora infestans* in Europe in 1999. Proceedings of the Workshop on the European network for development of an

integrated control strategy of potato late blight. Oostende, Belgium, 29 September –2 October 1999, pp. 10-18.

Schumann GL (1991). Plant deseases: Their biology and social impact. The American Phytopathological Society, APS Press, St. Paul Mn.

Schwinn FJ, Margot P (1991). Control with chemicals. In: DS Ingram, PH Williams (eds.), Advances in Plant pathology vol. 7. Academic Press, London, pp.193 – 224.

Searle SR, Casella G, McCulloch CE (1992). Variance Components. John Wiley & Sons, New York.

Shannon CE, Weaver W (1949). The Mathematical Theory of Communication. University of Illinois Press, Urbana, IL.

Shattock RC (1976). Winter survival of field isolates of *Phytophthora infestans* in seed tubers and development of primarily infected plants. Proceedings of the Federation of British Plant Pathologists: *Annals of Applied Biology* **84**: 273-274.

Shattock RC (1977). The dynamics of plant diseases. In Cherret JM Sagar GR (eds.), Origins of pest, parasite, disease and weed problems, pp. 83-107. Blackwell Scientific Publications, Oxford, UK.

Shattock RC, Tooley PW, Fry WE (1985). Determination of recombination, segregation and selfing in single-oospore cultures of *Phytophthora infestans* by isozyme analysis. *Phytopathology* **75**: 1309-1310.

Shattock RC, Tooley PW, Fry WE (1986). The genetics of *Phytophthora infestans*: identification of recombination, segregation and selfing by isozyme analysis. *Phytopathology* **76**: 410-413.

Shaw DS (1987). The breeding system of *Phytophthora infestans*: The role of the A2 mating type. In PR Day, GJ Jellis (eds.) *Genetics and Plant Pathogenesis*, pp. 161-174. Blackwell: Oxford, UK.

Shaw DS (1991). Genetics. In DS Ingram, PH Williams (eds.) *Advances in Plant Pathology Volume 7; Phytophthora infestans, the cause of late blight of potato*, pp. 131-170. Academic Press: London, UK.

Shaw DS (1996). Genetic Analysis in the Oomycetous Fungus: *Phytophthora infestans*. In CJ Bos (ed.) *Fungal Genetics, Principles and Practice*, pp. 407-417. Marcel Dekker, Inc: New York.

Simpson EH (1949). Measurement of diversity. *Nature* **163**: 688.

Slatkin M (1987). Gene flow and the geographic structure of natural populations. *Science* **236**: 787-792.

Slatkin M, Barton NH (1989). A comparison of three indirect methods for estimating average levels of gene flow. *Evolution* **43**: 1349-1368.

Smoot JJ, Gough FJ, Lamey HA, Eichenmuller JJ, Gallegly ME (1958). Production and germination of oospores of *Phytophthora infestans*. *Phytopathology* **48**: 165-171.

Spielman LJ, McMaster BJ, Fry WE (1989). Dominance and recessiveness at loci for virulence against potato and tomato in *Phytophthora infestans*. *Theoretical and Applied Genetics* **77**: 823-838.

Spielman LJ, Sweigard JA, Shattock RC, Fry WE (1990). The genetics of *Phytophthora infestans*: segregation of allozyme markers in F2 and backcross progeny and the inheritance of virulence against potato resistance genes R2 and R4 in F1 progeny. *Experimental Mycology* **14**: 57-69.

Spielman LJ, Drenth A, Davidse LC, Sujkowski LJ, Gu WK, Tooley PW, Fry WE (1991). A second world-wide migration and population displacement of *Phytophthora infestans*? *Plant Pathology* **40**: 422-430.

Stamps GJ, Waterhouse GM, Newhook FJ, Hall GS (1990). Revised tabular key to the species of *Phytophthora*. *Mycological Papers* **162**, 28 pp.

Stewart HE, McCalmont DC, Wastie RL (1983). The effect of harvest date and the interval between harvest and inoculation on the assessment of the resistance of potato tubers to late blight. *Potato Research* **26**: 101-107.

Stewart HE, Wastie RL, Bradshaw JE (1996). Susceptibility to *Phytophthora infestans* of field- and glasshouse-grown potato tubers. *Potato Research* **39**, 283-288.

Stokes ME, Davis CS, Koch GG (1995). Categorical Data Analysis Using the SAS System. Cary, NC: Sas Institute Inc.

Sujkowski LS, Goodwin SB, Dyer AT, Fry WE (1994). Increased genotypic diversity via migration and possible occurrence of sexual reproduction of *Phytophthora infestans* in Poland. *Phytopathology* **84**: 201-207.

Thakur RP, Shetty KG (1993). Variation in pathogenicity among single-oospore isolates of *Sclerospora graminicola*, the causal organism of downy mildew in pearl millet. Plant Pathology **42**: 715-721.

Tooley PW, Fry WE (1985). Field assessment of fitness of isolates of *Phytophthora infestans*. *Phytopathology* **75**: 982-988.

Tooley PW, Fry WE, Villareal Gonzalez MJ (1985). Isozyme characterisation of sexual and asexual *Phytophthora infestans* populations. *Journal of Heredity* **76**: 431-435.

Tooley PW, Sweigard JA, Fry WE (1986). Fitness and virulence of *Phytophthora infestans* isolates from sexual and asexual populations. *Phytopathology* **76**: 1209-1212.

Tooley PW (1988). Use of uncontrolled freezing for liquid nitrogen storage of *Phytophthora* species. *Plant Disease* **72**: 680-682.

Toxopeus HJ (1958). Some notes on the relations between field resistance to *Phytophthora infestans* in leaves and tubers and ripening time in *Solanum tuberosum* subsp. *tuberosum*. *Euphytica* **7**: 123-130.

Toxopeus HJ (1961). On the inheritance of tuber resistance of *Solanum tuberosum* to *Phytophthora infestans* in the field. *Euphytica* **10**: 307-814.

Toxopeus HJ (1964). Treasure digging for blight resistance in potatoes. *Euphytica* **13**: 206-222.

Turkensteen LJ (1973). Partial resistance of tomatoes against *Phytophthora infestans*, the late blight fungus. Agricultural Research Report No. 810, Research Institute for Plant Protection, Wageningen, the Netherlands.

Turkensteen LJ (1993). Durable resistance of potatoes against *Phytophthora infestans*. In Th Jabobs, JE Parlevliet (eds.) Durability of Disease Resistance, pp. 115-124. Kluwer Academic Publishers, Dordrecht, the Netherlands.

Turkensteen LJ, Flier WG, Baarlen P van (1996). Evidence for sexual reproduction of *Phytophthora infestans* in an allotment garden complex in the Netherlands. 13th Triennial Conference of the European Association for Potato Research. Abstracts of Conference Papers, Posters and Demonstrations, 356-7.

Turkensteen LJ, Flier WG, Mulder A, Roosjen Js (1997). Toegenomen agressiviteit van de aardappelziekte en de MJP-G doelstellingen. *Aardappelwereld* **51**: 16-20.

Turkensteen LJ, Flier WG, Wanningen R, Mulder A (2000). Production, survival and infectivity of oospores of *Phytophthora infestans*. *Plant Pathology* **49**: 688-696.

Umaerus V, Umaerus M (1994). Inheritance of resistance to Late Blight. In: Bradshaw JE, Mackay GR, (eds), Potato Genetics. CAB International, Wallingford, pp.365-402.

Varis E, Pietita L, Koikkalainen K (1996). Comparison of conventional, integrated and organic potato production in field experiments in Finland. *Acta Agriculturae Scandinavica Section B, Soil and Plant Science* **46**: 41-48.

Vleeshouwers VGAA, van Dooijeweerd W, Govers F, Kamoun S, Colon LT (2000). The hypersensitive response is associated with host and nonhost resistance to *Phytophthora infestans*. *Planta* **210**: 853-864.

Wastie RL, Caligari PDS, Stewart HE, Mackay GR (1987). A glasshouse progeny test for resistance to tuber blight (*Phytophthora infestans*). *Potato Research* **30**: 533-538.

Wastie RL (1991). Breeding for resistance. In DS Ingram, PH Williams (eds.) Advances in Plant Pathology vol. 7, pp.193-224. Academic Press, London,.

Waterhouse GM (1963). Key to the species of *Phytophthora* de Bary. *Mycological Papers* **92**, 22pp.

Weir BS, Cockerham CC (1984). Estimating F-statistics for the analysis of population structure. *Evolution* **38**: 1358-1370.

White TJ, Bruns T, Lee S, Taylor J (1990). Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: PCR protocols: A guide to methods and applications (Innis MA, Gelfand DH, Sninsky JJ, and White TJ (eds.) pp. 315-322. Academic Press, San Diego.

Wright S (1978). Evolution and the Genetics of Populations.Volume 4. Variability Within and Among Natural Populations. University of Chicago Press, Chicago.

Xia X (2000). Data analysis in molecular biology and evolution. Kluwer Academic Publishers, Dordrecht, the Netherlands.

Yeh FC, Boyle TJB (1997). Population genetic analysis of co-dominant and dominant markers and quantitative traits. *Belgian Journal of Botany* **129**: 157.

Yeh FC, Yang RC, Boyle TJB, Ye ZH, Mao JX (1997). POPGENE, the user-friendly shareware for population genetic analysis. Canada: Molecular Biology and Biotechnology Center, University of Alberta

Zadoks JC, Schein RD (1979). Epidemiology and plant disease management. Oxford University Press, New York, pp. 54-55.

Zaag DE van der (1956). Overwintering en epidemiologie van *Phytophthora infestans*, tevens enige nieuwe bestrijdingsmogelijkheden. *Tijdschrift Plantenziekten* **62**: 89-156.

Zan K (1962). Activity of *Phytophthora infestans* in soil in relation to tuber infection. *Transactions of the British mycological Society* **45**: 205-221.

Zwankhuizen MJ, Frinking HD (1996). Epidemiology of *Phytophthora infestans* in Southern Flevoland (the Netherlands). Proceedings of the 13th Triennial Conference of the European Association for Potato Research, Veldhoven, pp. 352-353.

Zwankhuizen MJ, Govers F, Zadoks JC (1998). Development of potato late blight epidemics: disease foci, disease gradients, and infection sources. *Phytopathology* **88**: 754-763.

Zwankhuizen MJ, Govers F, Zadoks JC (2000). Inoculum sources and genotypic diversity of *Phytophthora infestans* in Southern Flevoland, the Netherlands. *European Journal of Plant Pathology* **106**: 667-680.

Nawoord

Dit was eens maar nooit weer!!!! Het schrijven van dit proefschrift was een unieke ervaring die mij bij tijd en wijle snikken en grimlachjes heeft veroorzaakt. Toch waren er ook vele leuke momenten die ik koester. De geboorte van dit proefschrift is geen verdienste van de promovendus alleen, het is een activiteit waar de grote inzet en hulp van vele, vele mensen hebben geleid tot dit boekje dat u nu in uw handen heeft. Een aantal personen wil ik hierbij met name bedanken.

Beste Rolf, als promotor wil ik je bedanken voor de unieke wijze waarop je mijn immer uitdijende werk hebt begeleid. Mijn reisgedrag maakte regelmatig overleg vaak moeilijk, maar je was altijd bereid om op te treden als kritisch klankbord. Je rustige en nuchtere kijk op de populatiebiologie van Phytophthora was voor mij heel waardevol. Dear Bill, I'm very grateful for your continuous encouragement and support. Our blight interactions in many places around the globe have been very important to me and have been decisive for the choice of new late blight research topics. Your patience must have been put to the test several times when yet another drafty manuscript arrived from Holland; thank you very much. Beste Lo, je bent voor mij in de afgelopen 10 jaar veel meer geweest dan respectievelijk chef, collega en co-promotor. Je brede kennis op het gebied van aardappelziekten en fenomenale ervaring met het Phytophthora onderzoek in zowel Nederland als het verre buitenland heeft mij in grote mate gevormd. Vismaat, reisgezel, mentor en goede vriend, ik denk dat je iets van jezelf terug zult vinden in dit proefschrift. Hopelijk hebben we nu meer tijd om samen forel en vlagzalm te belagen.....

Nik, partner in 'blight delight', our blight adventures in Mexico and Ecuador always have been accompanied by Corona, Modelo Negro or red Chilean wine. Our partnership has been a very fruitful one and formed the basis of several manuscripts included in this thesis. Thank you so much for making me feel part of your family in Metepec. I would like to thank Hector, Allejandro, Anna, Edith and all the folks at PICTIPAPA for their hospitality and their continuous support during my fieldwork in Mexico. I am very grateful to ICAMEX and INIFAP for providing research equipment, a laboratory and greenhouse facilities.

Het uitvoeren van het internationale werk en het schrijven van dit proefschrift was alleen mogelijk wanneer de zaken 'thuis' goed werden georganiseerd. De Phytophthora-groep op de Binnenhaven heeft dit alles mogelijk gemaakt. Hierbij wil ik Marieke, Petra, Trudy, Geert, Henry en Laurens bedanken voor jullie bijdrage aan het onderzoek en de onvergetelijke steun in moeilijke tijden. Helen, Maarten en Monique hebben als studenten ook hun steentje bijgedragen, bedankt hiervoor! Trudy, altijd

bescheiden als het gaat om je eigen werk, jij bent de stille kracht achter vele veldproeven en onmisbaar als mijn persoonlijke 'DNA-wizard'. Het doet me daarom heel veel plezier dat ik je heb kunnen strikken als paranimf. Hopelijk zijn er ditmaal geen fotografen in de zaal!

Graag wil ik hierbij alle (oud)-collega's van het IPO en Plant Research International bedanken voor alle hulp en (dikwijls) stimulerende discussies. Helaas kan ik niet iedereen bij name noemen, maar een aantal van hen wil ik niet onvermeld laten. Cor, Nyckle en Piet, de wijze waarop jullie als afdelingshoofd en business-unit-manager mij de ruimte hebben geboden om dit onderzoek te verrichten heb ik hogelijk gewaardeerd. Hans, dankbaar doelwit voor mijn grappen en grollen, je was altijd bereid om manuscripten te voorzien van waardevol commentaar en je doorzag vele van mijn statistische dwalingen. De informele discussies over waard-pathogeen interacties met jou heb ik altijd als verfrissend en prettig ervaren. Maarten en Jan en al het personeel van de kassen- en proefvelddienst, jullie hebben het onmogelijke toch vaak mogelijk gemaakt. Ik hoop dat jullie mij kunnen vergeven voor het oprooien en doorsnijden van al die rotte knollen! Tina ben ik zeer erkentelijk voor de uiteindelijke vormgeving van dit proefschrift.

Het is al vaker opgemerkt, promovendi moeten woekeren met de toegemeten tijd, en vaak zijn familie en vrienden hiervan de dupe. Helaas vorm ik geen uitzondering op deze regel en dus is er nu alle reden om hen in het zonnetje te zetten.

Peter, mijn virtuele broer, alter-ego en collega 'ideale schoonzoon', onze wegen kruisen elkaar sinds 1987 toen we samen in een Betuws boerderijtje begonnen aan onze Wageningse periode. Je steun en goedlachse humor hebben me door moeilijke tijden heen geholpen. We hebben samen opgetrokken bij het schrijven van onze proefschriften (veel telefoontjes met gitzwarte humor en wederzijdse uitwisseling van 'loser-stories') en sluiten nu dus ook samen deze periode af. Ik ben blij dat jij samen met Trudy mijn paranimf wilt zijn. Jos en Marieke, bij jullie vond ik altijd een goed kop koffie en een luisterend oor, en kon even stoom afblazen wanneer dat nodig was. Nu is het mijn beurt, wanneer kan ik babysitten?

Lieve ma, we hebben samen een moeilijk jaar achter de rug. Je onbevangenheid en taai doorzettingsvermogen tijdens de lange en moeizame maanden in het ziekenhuis waren onvergetelijk en hebben ons beiden erdoorheen gesleept. Je hebt samen met pa altijd veel geduld gehad met die eigenzinnige snaak en ik zal de herinnering van jullie nestwarmte altijd blijven koesteren. Helaas kan pa dit alles niet meer meemaken, maar ik ben ontzettend blij dat jij bij mijn promotie aanwezig kunt zijn en draag daarom dit proefschrift aan jullie op.

Lieve Petra, jij hebt het meest geleden onder mijn 'Phytophthora-manie'. De afgelopen jaren waren voor ons beiden niet gemakkelijk, maar nu kijken we samen naar de toekomst.
Wat kan een mens zich nog meer wensen?

Curriculum vitae

Wilbert Flier werd geboren op 30 maart 1967 te IJsselstein. Na het doorlopen van de zandbak en de lagere school volgde hij de lagere landbouwschool te Montfoort. Daarna volgde een 4 jarige periode op het VHBO waarna hij in 1987 begon aan de HBO studie botanische laboratoriumtechniek op de toenmalige RHAS te Wageningen. Na het behalen van het HBO diploma in 1991 vond hij een baan als assistent onderzoeker bij de afdeling Fysiologie en Resistentie van het toenmalige IPO-DLO. In 1994 begon hij in zijn vrije tijd aan een studie Plantenveredeling aan de Landbouwuniversiteit te Wageningen. De ingenieursstudie, die *cum laude* werd afgerond in juni 1997, omvatte voornamelijk genetisch en evolutionair biologisch getinte vakken en werd afgesloten met een afstudeervak bij de vakgroep Plantenveredeling. In juni 1997 volgde zijn aanstelling als wetenschappelijk onderzoeker en projectleider bij de afdeling Mycologie & Bacteriologie van het IPO. Van 1997 tot 2000 werkte hij gedurende de zomermaanden in Mexico aan epidemiologisch en populatiebiologisch onderzoek aan *Phytophthora infestans*. Daarnaast werd in Nederland onderzoek verricht naar de stabiliteit van partiele resistentie tegen *P. infestans* in aardappel. Resultaten van zowel het Mexicaanse en Nederlandse onderzoek vormen de basis voor dit proefschrift.

List of publications

Turkensteen LJ, **Flier WG**, Baarlen P van (1996). Evidence for sexual reproduction of *Phytophthora infestans* in an allotment garden complex in the Netherlands. 13th Triennial Conference of the European Association for Potato Research, Veldhoven, The Netherlands. Abstracts of Conference Papers, Posters and Demonstrations, pp. 356-357.

Flier WG, Turkensteen LJ (1996). Aggressiveness of the new established population of *Phytophthora infestans* in The Netherlands. 13th Triennial Conference of the European Association for Potato Research, Veldhoven, The Netherlands. Abstracts of Conference Papers, Posters and Demonstrations, p. 598.

Flier WG, Turkensteen LJ, Mulder A (1998). Variation in tuber pathogenicity of *Phytophthora infestans* in the Netherlands. *Potato Research* **41**: 345-354.

Flier WG, Turkensteen LJ (1999). Sources of initial inoculum; relative importance, timing and implications for late blight epidemics. GILB Meeting, Quito, Ecuador. Proceedings of the Global Initiative on late blight Conference, p.80.

Flier WG, Grünwald NJ, Turkensteen LJ, Fry WE (1999). Production and viability of oospores produced by *Phytophthora infestans* isolates collected in the valley of Toluca, Central Mexico. 14th Triennial Conference of the European Association for Potato Research, Sorrento, Italy. Abstracts of Conference Papers, Posters and Demonstrations, pp. 56-57.

Flier WG, Turkensteen LJ, Bosch GBM van den, Raay HMG van (1999). Epidemiological parameters of the present *Phytophthora infestans* population in the Netherlands. 14th Triennial Conference of the European Association for Potato Research, Sorrento, Italy. Abstracts of Conference Papers, Posters and Demonstrations, pp. 58-59.

Flier WG, Turkensteen LJ, Mulder A, Bosch GBM van den (1999). Increased levels of aggressiveness to potato tubers by strains of *Phytophthora infestans*; epidemiological features and implications. 14th Triennial Conference of the European Association for Potato Research, Sorrento, Italy. Abstracts of Conference Papers, Posters and Demonstrations, pp. 108-109.

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

Turkensteen LJ, **Flier WG**, Wanningen R, Mulder A (2000). Production, survival and infectivity of oospores of *Phytophthora infestans*. *Plant Pathology* **49**: 688-696.

Flier WG, Grünwald NJ, Fry WE, Turkensteen LJ (2001). Formation, production and viability of oospores of *Phytophthora infestans* from potato and *Solanum demissum* in the Toluca Valley, Central Mexico. *Mycological Research*, in press.

Flier WG, Turkensteen LJ, van den Bosch GBM, Vereijken FG, Mulder A (2001). Differential interaction of *Phytophthora infestans* on tubers of potato cultivars with different levels of blight resistance. Plant Pathology, in press.

Grünwald NJ, **Flier WG**, Sturbaum AK, Garay-Serrano E, Bosch GBM van den, Smart CD, Matuszak JM, Turkensteen LJ, Fry WE (2001). Genetic population structure of *Phytophthora infestans* within the Toluca Valley of Central Mexico. (submitted).

Flier WG, Turkensteen LJ, Bosch GBM van den (2001). Stability of partial resistance in potato cultivars exposed to aggressive strains of *Phytophthora infestans*. (submitted).

Flier WG, Grünwald NJ, Kroon LPNM, Bosch GBM van den, Garay-Serrano E, Bonants PJM (2001). *Phytophthora ipomoeae*, a new homothallic species causing leaf blight on Morning Glory in the Toluca Valley of central Mexico. (submitted).

Notes