Molecular genetic evidence for a new sexually reproducing population of *Phytophthora infestans* in Europe

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Proefschrift

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Authors abstract

In the early 1980s the old A1 mating type population of the potato late blight pathogen, *Phytophthora infestans*, was displaced by new A1 and A2 mating type isolates in Europe. Analyses of virulence characteristics and DNA fingerprint patterns of a large number of isolates revealed that with the introduction of new *P. infestans* isolates the level of genetic diversity in the population has increased dramatically. Experiments under controlled conditions and under natural conditions in the field demonstrated that oospores are formed in large numbers after inoculation of potato leaves with a mixture of A1 and A2 mating type isolates of *P. infestans*. Oospores in soil, exposed to natural weather conditions during the winter, remain viable for at least eight months. It is concluded that after the introduction of the new A1 and A2 mating type population in The Netherlands *P. infestans* reproduces sexually and forms oospores. It is likely that oospores play an important role in the epidemiology of potato late blight.

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Stellingen

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1. Vóór 1980 werd de aardappelziekte in Nederland door een andere *Phytophthora infestans* populatie veroorzaakt dan ná 1980.
   Dit proefschrift

2. De *Phytophthora infestans* populatie die ná 1980 in Nederland voorkomt en bestaat uit isolaten met het A1 en het A2 paringstype, is afkomstig uit Mexico.
   Dit proefschrift

3. In Nederland kunnen oösporen van *Phytophthora infestans* onder natuurlijke omstandigheden minstens één jaar in de grond overleven.
   Dit proefschrift

4. De introductie van een nieuwe populatie waarin zowel isolaten met het A1 als het A2 paringstype voorkomen, heeft geleid tot het optreden van sexuele voortplanting van *Phytophthora infestans* in Nederland.
   Dit proefschrift

5. Evolutionair gezien heeft een populatie die zich geslachtelijk kan voortplanten voordelen ten opzichte van een populatie die zich alleen maar ongeslachtelijk kan voortplanten.
   Muller, H.J., 1964. Mutation Research 1: 2-9.

6. Alle populaties zijn gedoemd uit te sterven.
   Lynch, L. & Gabriel, W., 1990. Evolution 44: 1725-1737.

7. Sommige wetenschappelijke hypothesen worden niet verworpen maar sterven langzaam.

8. Het huidige systeem van "peer review" in de wetenschap verlengt de levensduur van bestaande paradigma's.

9. Het publieke debat wordt niet gestuurd door gebeurtenissen maar door de verslaggeving van gebeurtenissen.

10. Gebrek aan een eigen smaak leidt tot het volgen van de mode.

11. Kationen kunnen nooit negatief zijn.

12. Netwerken en net werken zijn uiterst belangrijk in de huidige wetenschap.

13. "Down under" betekent bovenaan.

Stellingen behorend bij het proefschrift "Molecular genetic evidence for a new sexually reproducing population of *Phytophthora infestans* in Europe"

Wageningen, 15 april 1994 André Drenth

The severity of Phytophthora infestans *epidemics and the public attention they attracted brought the scientific discipline of plant pathology into prominence and acceptance.*

Robertson, N.F., 1991. The challenge of *Phytophthora infestans*. p 1-30 in: Ingram, D.J. & Williams, P.H. (eds.). *Phytophthora infestans*, the cause of late blight of potato. Advances in Plant Pathology. Vol. 7. Academic Press, London.

The research described in this thesis was carried out at the Department of Phytopathology, Wageningen Agricultural University, Wageningen, The Netherlands, the DLO Research Institute for Plant Protection (IPO-DLO), Wageningen, The Netherlands and the Department of Plant Pathology, Cornell University, Ithaca, New York, USA. It was financially supported by the Agricultural Research Department (DLO-NL) and the DLO Research Institute for Plant Protection (IPO-DLO), Wageningen, The Netherlands.

IPO-DLO

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Contents

Chapter 1

Scope of this thesis

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In 1845 a strange disease appeared on potatoes in Europe and revealed itself as a new and formidable enemy of mankind. It destroyed the staple food supply of a human society and this caused unprecedented suffering and death (Large, 1940; Woodham-Smith, 1962). A lively debate concerning the cause of the disease started. Many people at that time thought that a little bit of mould on the diseased plants was not important. However, the Reverend M.J. Berkeley put forward the revolutionary theory that a mould might be the cause and not, as was initially thought, the consequence of the disease (Berkely, 1845). In 1845, Dr Montagne described the fungus and called it *Botrytis infestans* (Mont.). In 1861, Anton de Bary proved that, indeed, this fungus was responsible for potato late blight and renamed it *Phytophthora infestans* (Mont.) de Bary (de Bary, 1876). *P. infestans* became the type species of the newly created genus *Phytophthora* ("plant destroyer"). The rapid spread of *P. infestans* over Europe in 1845, as documented by Bourke (1964), demonstrated the potential of *P. infestans* to migrate and cause disease. This extraordinary late blight epidemic marked the beginning of plant pathology as a science.

Since 1845 late blight in potatoes has been a serious threat to potato cultivation and presently the disease is still difficult to control. Large amounts of protectant fungicides are used to control the disease. A few years ago in The Netherlands, the annual costs of late blight control were estimated to be 100 million guilders (ca. 55 million US $) (Davidse *et al.*, 1989). Fungicides are not only very costly, but they also have an undesirable impact on the environment.

P. infestans is heterothallic with two known mating types, A1 and A2. When hyphae of A1 and A2 mating type isolates interact, sexual propagation, by means of oospore formation, may take place. Oospores are thick walled, hard hulled spores, which enable the fungus to survive for many years in soil outside the living host plant. In central Mexico a high level of genetic diversity exists in populations of *P. infestans* and in several species of its *Solanaceous* hosts. This led to the postulation that co-evolution between the pathogen and its host species occurred in central Mexico (Reddick and Crosier, 1933). *P. infestans* isolates with the A2 mating type and large numbers of oospores were first discovered in central Mexico in 1956 (Niederhauser, 1956; Gallegly & Galindo, 1958; Smoot *et al.*, 1958). Initially, only A1 mating type isolates escaped from central Mexico and spread worldwide. In the absence of A2 mating type isolates, *P. infestans* was confined to asexual propagation and, in order to survive between growing seasons, the biotrophic pathogen had to survive as mycelium in potato tubers.

In 1980, serious outbreaks of late blight occurred throughout Europe. *P. infestans* isolates resistant to a new fungicide, metalaxyl, appeared (Davidse *et al.*, 1981). Extensive sampling schemes were implemented to study the occurrence and spread of resistant isolates (Davidse *et al.*, 1989). In 1984 it was reported that isolates with the A2 mating type were present in Switzerland since 1981 (Hohl & Iselin, 1984). This finding called for mating type screening of field samples and isolates of *P. infestans* maintained in culture collections. The screenings

revealed that in addition to A1 mating type isolates, A2 mating type isolates had been present in Europe since at least 1980. The discovery that A2 mating type isolates of *P. infestans* were present in Europe raised questions concerning the occurrence of sexual reproduction and the role of oospores in the potato late blight disease. The aim of the research described in this thesis was to determine whether *P. infestans* reproduces sexually under field conditions in The Netherlands and, if so, to elucidate the role of oospores in the epidemiology of potato late blight.

The occurrence, the spread and the frequency of A2 mating type isolates in populations of *P. infestans* have been analyzed in several European countries (chapter 2). Moreover, allozyme markers have been used to analyze the *P. infestans* populations that occurred in Europe before and after the introduction of A2 mating type isolates. It was shown that the old A1 mating type population, present before 1980, has been replaced by a new population, consisting of A1 and A2 mating types isolates (chapter 3). The rapid appearance of A2 mating type isolates of *P. infestans* in various continents led to an international effort to obtain a global picture of the migration of the new *P. infestans* population (Fry *et al.*, 1992, 1993).

A non-biased approach was called for to demonstrate the occurrence of sexual reproduction. One of the differences between sexually and asexually reproducing populations is the level of genetic diversity in the populations. Genetic diversity can be determined using genetic markers. In 1989, when the research described in this thesis started, the number of markers available to identify *P. infestans* isolates was very limited. It mostly concerned biologically significant markers, such as virulence and resistance to the fungicide metalaxyl. Such phenotypic markers are under strong selection pressure in agricultural systems. Therefore, it is likely that they provide biased estimates of the genetic structure and diversity in the pathogen population. Moreover, with these markers it is impossible to determine whether particular alleles arose independently in different fungal isolates or whether they originated from a common ancestor (McDonald & McDermott, 1993). Genetic diversity can be measured more accurately with selection-neutral genetic markers such as DNA markers. With DNA fingerprinting numerous neutral genetic markers can be analyzed in one experiment. The moderately repetitive DNA probe RG-57, which is a DNA fragment randomly selected from a genomic library of *P. infestans*, recognizes many unlinked loci dispersed over the genome. The genomic fragments hybridizing to RG-57 show independent Mendelian inheritance and they are stable through asexual reproduction. DNA fingerprinting with probe RG-57 was used to identify individual genotypes of *P. infestans*, and to distinguish sexually reproduced hybrid progeny from their parents and selfings (chapter 4).

The genetic diversity in the Dutch *P. infestans* population, as it occurred in 1989, was determined at different geographic levels: among-regions, among-fields within-regions, and within-fields. Initially, allozymes, mating type and resistance to metalaxyl were used as markers. It appeared that with these markers only a limited level of genetic diversity could be detected. The number of available polymorphic allozyme markers for *P. infestans*, glucose phosphate isomerase (*Gpi*) and peptidase (*Pep*), proved to be to small to detect individual genotypes (chapter 5). DNA fingerprint probe RG-57 and a mitochondrial DNA probe were used to unravel

the genetic diversity in more detail. With these tools numerous different genotypes were identified among the collected isolates and their geographic distribution in The Netherlands was determined with a precision which was not possible before (chapter 6).

The observation of the existence of a high level of genetic diversity among isolates collected within one year indicated that besides vegetative spores, the sexual stage (i.e. oospores) contributes to the propagation of the *P. infestans* population in the field. This raised the question whether the high level of genetic diversity coincided with the introduction of new A1 and A2 mating type isolates around 1980. Isolates collected before 1980 and after 1980 were analyzed for virulence characteristics and RG-57 DNA fingerprint patterns. Survival as mycelium in potato tubers only, will result in more or less similar genotypes throughout time. In contrast, sexual reproduction via oospores, which survive in soil between seasons, would yield different genotypes throughout time. The results obtained demonstrated that after 1980 a dramatic increase in the level of genetic diversity showed up and it appeared that every year the *P. infestans* population consisted mostly of entirely new genotypes not found before. In addition, it was shown unambiguously that oospores can survive in soil and cause new infections the next year. Based on the high level of genetic diversity within and between years, and the survival of oospores in soil, it is concluded that *P. infestans* propagates sexually under field conditions in The Netherlands (chapter 7).

In order to elucidate the role of oospores in the epidemiology of potato late blight the production and survival of oospores and their ability to infect potato leaves, were analyzed in more detail. The production of oospores in leaves of potato and tomato plants was studied in growth chamber and field experiments. Oospores present in soil, exposed to natural weather conditions, were tested for survival and infectivity. A bioassay was developed to obtain late blight infections from soil infested with oospores. With the help of RG-57 DNA fingerprint analyses infections originating from oospores could be distinguished from infections caused by contamination or by mycelium overwintering in potato tubers (chapter 8).

REFERENCES

Berkeley, M.J., 1845. Observations, botanical and physiological, on the potato murrain. Journal of the Horticultural Society 1: 9-34.

Bourke, P.M.A., 1964. Emergence of potato late blight, 1843-46. Nature 203: 805-808.

Davidse, L.C., Looyen, D., Turkensteen, L.J. & van der Wal, D., 1981. Occurrence of metalaxyl-resistant strains of *Phytophthora infestans* in Dutch potato fields. Netherlands Journal of Plant Pathology 87: 65-68.

Davidse, L.C., Henken, J., Dalen, A. van, Jespers, A.B.K. & Mantel, B.C., 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.

de Bary, A., 1861. Die gegenwärtig herrschende kartoffelkrankheit, ihre ursache und ihre verhütung. Arthur Felix, Leipzig. pp 75.

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

Fry, W.E., Goodwin, S.B., Matuszak, J.M., Spielman, L.J., Milgroom, M.G. & Drenth, A., 1992. Population genetics and intercontinental migrations of *Phytophthora infestans*. Annual Review of Phytopathology 30: 107-129.

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

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

Hohl, H.R. & Iselin, K., 1984. Strains of *Phytophthora infestans* with A2 mating type behaviour. Transactions of the British Mycological Society 83: 529-530.

Large, E.C., 1940. The advance of the fungi. Jonathan Cape Ltd. London. pp 488.

McDonald, B.A. & McDermott, J.M., 1993. Population genetics of plant pathogenic fungi. Bioscience 43: 311-319.

Niederhauser, J.S., 1956. The blight, the blighter, and the blighted. Transactions of the New York Academy of Science 19: 55-63.

Reddick, D. & Crosier, W., 1933. Biological specialization in *Phytophthora infestans*. American Potato Journal 10: 129-134.

Smoot, A.J., Gough F.J., Lamey H.A., Eichenmuller J.J. & Gallegly M.E., 1958. Production and germination of oospores of *Phytophthora infestans*. Phytopathology 48: 165-171.

Woodham-Smith, C., 1962. The great hunger. Harper & Row, New York, USA. pp 508.

Chapter 2

The occurrence of the A2 mating type of *Phytophthora infestans* in The Netherlands; significance and consequences

André Drenth, Lodewijk J. Turkensteen and Francine Govers

Adapted from: Netherlands Journal of Plant Pathology (1993) 99, supplement 3: 57-67

ABSTRACT

Phytophthora infestans (Mont.) de Bary, the causal agent of potato late blight, was first discovered in Europe in 1845. Until 1980, only A1 mating type isolates were known to occur in Europe. The absence of A2 mating type isolates restrained the fungus from sexual reproduction. In the early 1980s, A2 mating type isolates were discovered in Europe. Presumably a new introduction of *P. infestans* isolates originating from Mexico had taken place. In this chapter, the significance of the presence of A1 and A2 mating type isolates in The Netherlands is reviewed. Now that both mating types are present, sexual reproduction can occur and its consequences for the control of potato late blight are discussed.

INTRODUCTION

The first epidemic of *Phytophthora infestans* (Mont.) de Bary on potatoes in Europe in 1845 had disastrous effects on potato production. In Ireland the potato crop was destroyed in two successive years leading to a famine. As a consequence about one and a half million people died and another million emigrated, mainly to the United States (Large, 1940; Woodham-Smith, 1962). *P. infestans* spread over Europe within one year (Bourke, 1964) and was found in most potato growing areas of the world soon thereafter (Cox & Large, 1960).

De Bary (1876) named the causal agent of potato late blight *Phytophthora infestans*, after creating the genus *Phytophthora*. *P. infestans* is heterothallic with two known mating types, A1 and A2. Interaction between hyphae of opposite mating type results in the formation of oospores. Occasionally, the formation of limited numbers of oospores in agar media was reported (Clinton, 1911; Pethybridge & Murphy, 1913) but they did not germinate. It was not until 1956 that A2 mating type isolates and large numbers of oospores were found in potato leaves in the field in central Mexico. Gallegly and Galindo (1958) and Smoot *et al.* (1958) described abundant oospore formation *in vitro* in agar media and in potato leaves inoculated with isolates of opposite mating type. Germination of *in vitro* produced oospores and subsequent growth of sporulating mycelium was observed.

Outside central Mexico, no A2 mating type isolates were detected and *P. infestans* was found to reproduce asexually only. However, in the early 1980s, A2 mating type isolates were discovered in Switzerland (Hohl & Iselin, 1984) and subsequently in many other countries in Europe, Africa and Asia (Table 1). The discovery stimulated studies on the population genetics of *P. infestans*. Sexual crosses were performed *in vitro* (Shattock *et al.*, 1986a,b). Molecular markers were developed to provide tools for detailed genetic studies and unambiguous identification of *P. infestans* isolates (Tooley *et al.*, 1985; Goodwin *et al.*, 1992a, chapter 4; Drenth & Govers, 1993). Currently, several issues concerning the population genetics of *P. infestans* are intensively studied (Spielman *et al.*, 1991, chapter 3; Carter *et al.*, 1990, 1991; Goodwin *et al.*, 1991, 1992b; Goodwin *et al.*, 1992a, chapter 4; Fry *et al.*, 1992; Drenth *et al.*,

Table 1. Countries where the presence of the A2 mating type of *P. infestans* has been reported and the year in which an A2 mating type isolate was detected for the first time.

Country	Year	Reference
Mexico	1956	Niederhauser, 1956
East Germany	1980	Dagget *et al.*, 1993
Switzerland	1981	Hohl & Iselin, 1984
England\Wales	1981	Tantius *et al.*, 1986
Netherlands	1981	Frinking *et al.*, 1987
Scotland	1983	Malcolmson, 1985
Israel	1983	Grinberger *et al.*, 1989
Egypt	1984	Shaw *et al.*, 1985
Sweden	1985	Kadir & Umaerus, 1987
USSR	1985	Vorob'eva *et al.*, 1991
Japan	1985	Mosa *et al.*, 1989
West Germany	1985	Schöber & Rullich, 1986
Brazil	1986	Brommonschenkel, 1988
USA	1987	Deahl *et al.*, 1991
Poland	1988	Spielman *et al.*, 1991, chapter 3
Ireland	1988	O'Sullivan & Dowley, 1991
Canada	1989	Deahl *et al.*, 1991
Ecuador	1989	Fry *et al.*, 1993
Colombia	1990	Fry *et al.*, 1993
Bolivia	1990	S.B. Goodwin, unpublished
Korea	1991	Y.J. Koh, unpublished
China	?	Fry *et al.*, 1993

1993, chapter 6). For example, it is now well established that the A1 mating type population, which was present prior to the occurrence of the A2 mating type in Europe, has been replaced by new A1 and A2 mating type isolates (Spielman *et al.*, 1991, chapter 3; Drenth *et al.*, 1994). Knowledge on the occurrence of particular genotypes in different areas led to new insights in global spread of this fungus (Goodwin *et al.*, 1992b; Fry *et al.*, 1992, 1993; Drenth *et al.*, 1993, chapter 6). With both mating types present, the opportunity for sexual reproduction exists. When functional oospores are formed an increase in genotypic diversity can be expected. Furthermore, oospores are structures capable of persisting outside the host plant and can thus

act as an additional source of initial inoculum. Hence, drastic changes in the genetic structure of the population and the epidemiology of *P. infestans* were anticipated.

Here we will first present some taxonomical and biological features of *P. infestans* followed by a description of the disease cycle and the mode of reproduction and oospore formation. We will briefly mention the different methods of controlling potato late blight and then focus on the situation concerning *P. infestans* in central Mexico and the origin of the A2 mating type in Europe. Finally, the significance and consequences of the appearance of the A2 mating type on the population structure of *P. infestans* in The Netherlands are discussed.

THE PATHOGEN *PHYTOPHTHORA INFESTANS*

P. infestans belongs to the class Oomycetes, order Peronosporales, family Pythiaceae and genus *Phytophthora*. Characteristics for the Oomycetes are, the formation of oospores, a coenocytic mycelium, a diploid life cycle (Sansome & Brasier, 1973), cell walls which contain predominantly cellulose rather than chitin (Bartnicki-Garcia, 1968) and the inability to synthesize sterols (Hendrix, 1964). These features, in combination with the existence of typical tubular cristae in the mitochondria (Hemmes, 1983) and phylogenetic studies based on the small-subunit ribosomal DNA sequences, suggest that the Oomycetes are more closely related to the Chrysophytes (golden-brown algae) than to the higher fungi such as the Ascomycetes and Basidiomycetes (Gunderson *et al.*, 1987). Besides the genus *Phytophthora*, the order Peronosporales includes some other very destructive plant pathogens commonly known as the downy mildew fungi, e.g. *Bremia lactucae* and *Plasmopara viticola*, the downy mildew pathogens on lettuce and grapes, respectively. Another group of notorious plant pathogens in this order are the *Pythium* spp. which cause seedling damping-off, root rot and soft rot in a large variety of plants.

Characteristic for the genus *Phytophthora* is that several sporangia are subsequently formed at the terminal branches of tree-like sporangiophores. Sporangia can either germinate directly by forming a germtube or differentiate into three to eight or more biflagellate zoospores. The flagella enable the zoospores to move actively in water for short distances before they encyst and germinate to initiate infections. Zoospores contribute to the local spread of the disease.

All species of *Phytophthora* are plant pathogens and some cause root rots in a wide range of host plants. A well known example is *P. cinnamomi* which infects almost 1.000 different species of trees and shrubs (Zentmyer, 1980). Other *Phytophthora* species are restricted to one or a few hosts e.g. *P. fragariae* and *P. phaseoli* causing red stele root rot of strawberry and downy mildew on lima bean, respectively. Most *Phytophthora* diseases are favoured by rather low temperatures and high humidity in soil and atmosphere. These pathogens live and reproduce primarily in the soil and attack susceptible hosts at or below ground level. *P. infestans*, on the other hand is somewhat different from most of the other *Phytophthoras*. It is a typical foliar pathogen with air-borne sporangia. Nevertheless, it is also very well suited to infect and destroy

tubers in the soil. Although *P. infestans* is best known for causing late blight on potato and tomato it also infects quite a number of other Solanaceous species (Turkensteen, 1973, 1978).

DISEASE CYCLE OF *P. INFESTANS*

Asexual life cycle

In spring, when potato tubers are planted, diseased sprouts may arise from those tubers which are infested with mycelium of *P. infestans* (Fig. 1). Under favourable conditions, sporangiophores emerge from the stomata and release numerous airborne sporangia causing a rapid spread of the disease. At temperatures above 12 - 15 °C sporangia may germinate directly. Below 12 °C sporangia may differentiate into numerous motile zoospores which germinate after encystment. On leaf surfaces and stems, germinated sporangia and cysts form germtubes with appressoria from which penetration hyphae arise. Characteristically, a host cell next to stomatal guard cells is penetrated by the penetration hypha. Hyphal structures are formed in the epidermal cell from which the mycelium grows, initially intercellularly while intracellular haustoria are formed in the mesophyll cell layer (Pristou & Gallegly, 1954; Gees & Hohl, 1988). In the first stages of lesion development, a water-soaked area appears and here sporangiophores emerge predominantly from the stomata. Under favourable conditions such a rapidly expanding lesion carrying numerous sporangiophores with abundant sporangia is formed within four to five days after infection. Many asexual generations may be produced in one growing season which explains the tremendous potential for spread and epidemic development of the disease. The airborne sporangia can spread over distances up to several hundred kilometers (Aylor, 1986). In wet weather conditions, sporangia or zoospores are washed down from the leaves and carried into the soil. Here the spores germinate and the germtubes may penetrate the tubers at lenticells, wounds and eyes or at sites where the surface is not completely suberized. Most of the blighted tubers will rot in the soil or during storage. However, a few will survive the winter and they may be planted in the following season.

Van der Zaag (1956) and Hirst and Stedman (1960) gathered conclusive evidence to support the hypothesis stated by de Bary (1876) that *P. infestans* can indeed overwinter as a mycelium within infected tubers. They showed that only a small proportion of infected tubers give rise to infected sprouts. Nevertheless, infected sprouts will emerge and act as a source of inoculum. Van der Zaag (1956) estimated that one infected sprout per 20 to 600 square kilometer is sufficient to start the annual late blight epidemic. In this way, the disease is carried on from season to season. The hypothesis of Brefeld (1883) and de Bruyn (1926) that *P. infestans* can also overwinter as a saprophyte in the soil has not been substantiated. Zan (1962) and Lacey (1965) demonstrated that soil infectivity did not persist for more than 11 weeks in non-sterilized soils. This is by no means long enough to bridge the gap between successive potato crops in most potato growing areas and thus excludes a saprophytic phase in the disease cycle.

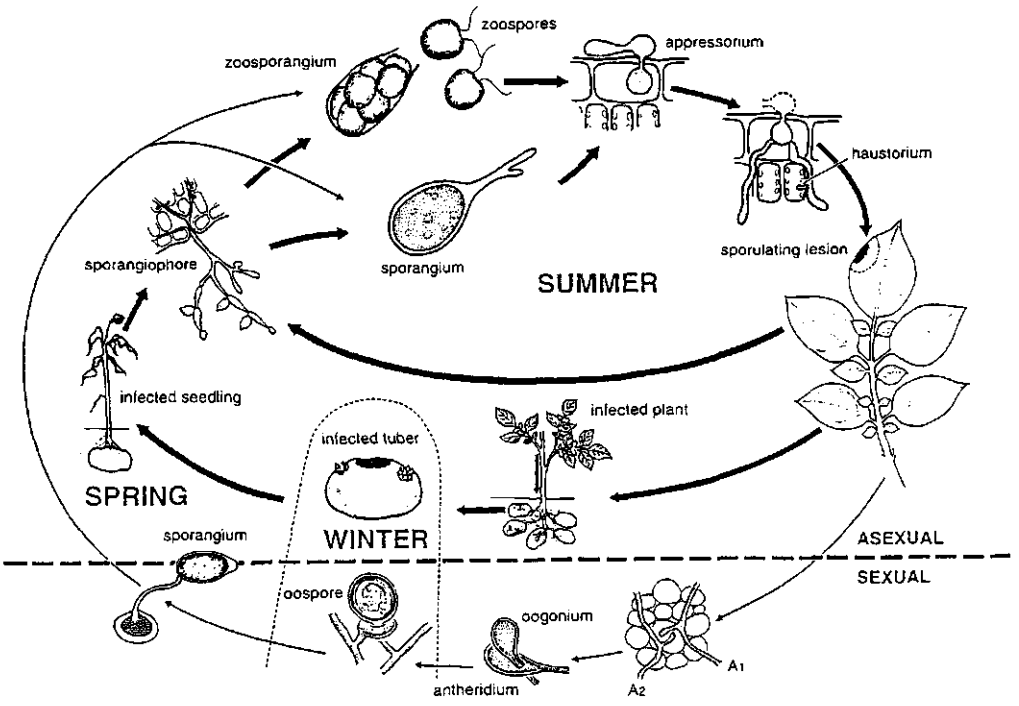

Figure 1. Disease cycle of potato late blight caused by *Phytophthora infestans*

Sexual life cycle

P. infestans is a heterothallic species and when hyphae of opposite mating type make contact, antheridia and oogonia are formed. The oogonium grows through the antheridium in an amphigynous configuration in which the antheridium surrounds the oogonial stalk (Fig. 1). There is no exchange of cytoplasm between antheridium and oogonium (Hemmes, 1983). The oogonium expands rapidly due to the flow of cytoplasm through the oogonial stalk from its own thallus. After this expansion phase the oogonial stalk is plugged. All nuclei in the oogonium except one migrate to the periphery where they disintegrate. Meiosis occurs in the multinucleate gametangium (Shaw, 1983b). A fertilization tube grows from the antheridium through the oogonial wall to deposit an antheridial nucleus in the oogonium. Subsequently lipid bodies and vacuoles are formed in the cytoplasm of the oogonium and they also migrate to the periphery. A thick oospore wall develops and the remaining cytoplasm is located in the centre of the ooplast. Blighted potato plants, containing oospores, remain in the field, decompose and oospores are liberated. Oospores can germinate and infect tubers and stolons of newly planted potatoes, as well as stems and leaves which come into contact with the soil (Schöber & Turkensteen, 1992). Oospore germination involves consumption of the lipid bodies in the

ooplast, dissolution of the oospore wall and the formation of one or more germtubes. These germtubes can either initiate mycelial growth directly or terminate in a sporangium which can germinate directly or produce zoospores.

As oospore formation was unknown from most potato growing areas of the world, there have been few studies of the conditions which favour sexual reproduction of *P. infestans* in nature. The appearance of A2 mating type isolates in Europe in the early 1980s (Table 1), implies the possibility of sexual reproduction which may seriously influence the epidemiological behaviour of the potato late blight fungus. Detailed information on production, survival, germination and infectiousness of oospores is essential.

Oospore formation and survival

In view of the presence of the A1 and A2 mating type isolates in The Netherlands, it is important to know whether oospore formation occurs in the field and whether the soil conditions and the climate are suitable for survival of oospores from one growing season to the next.

In general, oospores of oomycetous fungi are highly persistent structures. Oospores from e.g. *P. cactorum* can survive at least one year in soil (Malajczuk, 1983) and in mummified strawberries (Grove *et al.*, 1985). Oospores of *P. fragariae* can survive for at least three years in soil (Duncan, 1980). For the oomycete *Aphanomyces euteiches*, survival in soil for up to four years was reported (Kotova, 1979). To our knowledge only one study on the survival of *P. infestans* oospores was published. Perches and Galindo (1969) collected soil from a Mexican field two years after a severely blighted potato crop had been grown there. In greenhouse experiments, potatoes planted in this soil were infected on the lower part of the stems and on the leaves which were close to or in contact with the soil. Moreover, *P. infestans* could be isolated from this soil using selective media. They suggested that *P. infestans* oospores were responsible for the infection.

In order to prove that pairings of *P. infestans* isolates produce functional oospores which are able to survive the winter in the soil and to cause infections in the next growing season, it is of the utmost importance to determine whether sexually derived progeny caused infections. Therefore, one must be able to distinguish sexual and asexual progeny. For that purpose Goodwin *et al.* (1992a, chapter 4) employed DNA fingerprint probe RG-57 which is used to characterize *P. infestans* isolates. Two *P. infestans* isolates, collected in The Netherlands, were paired *in vitro* resulting in hybrid progeny as was shown after DNA fingerprint analyses (Goodwin *et al.*, 1992a, chapter 4). The same two isolates were used for the generation of oospores *in planta*. In floating potato leaf discs, inoculated with a mixture of sporangia from the parental isolates and incubated between 5 to 25 °C for several days, immense numbers of oospores were formed (chapter 8). Oospores extracted from the leaves germinated *in vitro* and the resulting isolates turned out to be pathogenic. DNA fingerprinting revealed that these isolates were hybrids from the two parental strains (Drenth *et al.*, 1994, chapter 7; chapter 8). To test oospore formation in the field, potato cultivars Bintje, Irene and Pimpernel and tomato cultivar Moneymaker, were inoculated with the same two parental *P. infestans* isolates. In the potato and

tomato leaves and the tomato fruits numerous oospores were detected which shows that oospore formation can easily occur under field conditions in The Netherlands (chapter 8). Moreover, in German and Dutch potato fields where infection occurred naturally, oospores were found (Götz, 1990; L.J. Turkensteen, unpublished). Currently, survival of oospores is being studied. Mixtures of leaves containing oospores and sandy soil are exposed to natural weather conditions in the open field and after fixed periods soil samples are tested for infectivity. Preliminary results indicate that after eight months, including the winter of 1992-1993, the soil still contains infectious material. DNA fingerprints of the isolates obtained revealed that the infections were caused by sexual progeny originating from oospores (chapter 8).

In conclusion, we have shown that oospore formation occurs under Dutch climatic conditions and that these oospores, after exposure to natural weather conditions for eight months in soil, gave rise to new pathogenic isolates. In order to quantify the epidemic impact of oospores as an inoculum source, the conditions for survival and germination have to be analyzed in more detail.

METHODS TO CONTROL POTATO LATE BLIGHT

When *P. infestans* first appeared in Europe in 1845, most potato cultivars turned out to be extremely susceptible. By the end of the 19th century, moderately resistant potato lines, generated through selection for resistance were available (Salaman, 1910). In the first half of 20th century, resistance governed by single resistance genes (R-genes) was regarded as a breakthrough. The R-genes were introduced by crossing *Solanum tuberosum* with species of *Solanum* that are highly resistant to late blight. In particular *Solanum demissum* (Lindl.) has been widely used as a source of R-genes against late blight. However, resistance based on R-genes was short-lived, as the fungus rapidly overcame resistance governed by the R-genes. This so-called breakdown of resistance is due to the appearance of virulent races which supposedly emerged either through mutation (Peterson & Mills, 1953; Toxopeus, 1956; Gallegly, 1968), sexual reproduction (Pristou & Gallegly, 1956), parasexuality (Leach & Rich, 1969) or somatic variation (Caten & Jinks, 1968).

In large parts of the world, late blight control is heavily dependent on the frequent application of protectant fungicides which are applied every 5-14 days. The frequency of application depends on the weather conditions and the susceptibility of the potato cultivar. Fungicides used to control *P. infestans* are Bordeaux Mixture, copperoxychloride, dithiocarbamates, triphenyltin compounds, cymoxanil and phenylamides. With the exception of the latter two, all these compounds function by killing spores and their germlings on the plant surface before infection occurs. They have little curative activity and are only effective when a complete cover of the foliage is maintained. Therefore, frequent applications are required for effective control. Once *P. infestans* has penetrated the host tissue, these fungicides can no longer affect the fungus. Once late blight becomes established in a field it is difficult to control. The

phenylamides, metalaxyl and cymoxanil, have systemic and eradicant properties. Metalaxyl has been used to control potato late blight in the early 1980s. However, metalaxyl resistant *P. infestans* isolates were found within one year after the introduction of this fungicide in The Netherlands in 1979 (Davidse *et al.*, 1981) and the use of metalaxyl is now discouraged and rather limited.

During the last four decades frequent and large scale applications of fungicides have been used to control potato late blight adequately. Hence, breeding for late blight resistant potato cultivars had low priority. Nowadays the growing environmental awareness of the general public forces growers to reduce fungicide applications drastically. In addition, the use of some effective fungicides will be forbidden in the near future. Moreover, the appearance of the A2 mating type and the possibility of sexual reproduction forming oospores, which act as additional inoculum, might initiate *P. infestans* epidemics earlier in the season, thus asking for a higher input of fungicides to control the disease. Therefore, late blight resistant cultivars are needed more than ever.

P. INFESTANS IN THE CENTRE OF ORIGIN, CENTRAL MEXICO

Several wild *Solanum* species native to Mexico have resistance against *P. infestans*. Using that information Reddick and Crosier (1933) were among the first to postulate that central Mexico might be the center of origin of *P. infestans*. Diversity observed in the host plant and in the fungus supported the hypothesis that the *Solanum-P. infestans* pathosystem had co-evolved there (Niederhauser & Mills, 1953). In 1956 the A2 mating type of *P. infestans* was identified in central Mexico (Niederhauser, 1956; Smoot *et al.*, 1958; Gallegly & Galindo, 1958). Besides oospore formation *in vitro* on different agar media, oospores were found *in planta* in leaves and stems of potato plants (Gallegly & Galindo, 1958; Smoot *et al.*, 1958; Estrada, 1967). These oospores were able to germinate and to produce pathogenic progeny (Smoot *et al.*, 1958; Romero & Erwin, 1967).

In central Mexico, A1 and A2 mating type isolates appear at equal frequency in the fungal population (Gallegly & Galindo, 1958). The Mexican isolates showed more virulence factors than isolates from asexually propagated populations collected in the United States (Tooley *et al.*, 1986). Five of the six known glucose phosphate isomerase (*Gpi*) allozyme alleles (83, 86, 90, 100, and 122) and all known peptidase (*Pep*) alleles (83, 92, 100) were found in Mexican *P. infestans* isolates collected in the 1980s (Goodwin *et al.*, 1992b). The frequency of allozyme alleles for *Gpi* and *Pep* does not deviate significantly from the Hardy-Weinberg equilibrium indicating random mating (Tooley *et al.*, 1985). DNA fingerprinting of isolates collected in central Mexico revealed that almost every isolate has a unique RG-57 genotype and all RG-57 hybridizing fragments known so far are represented (Goodwin *et al.*, 1992b). The high level of genetic diversity and the presence of allozyme alleles in Hardy-Weinberg equilibrium are consistent with the hypothesis that sexual reproduction occurs frequently in *P. infestans*

populations in central Mexico (Goodwin *et al.*, 1992b).

THE A2 MATING TYPE IN EUROPE

Ever since the causal agent of potato late blight was recognized as an oomycete, researchers have been eager to find oospores and the sexual cycle. The fact that two mating types exist in *P. infestans* (Gallegly & Galindo, 1958) and only one of them was present outside central Mexico can easily explain why earlier workers failed to find oospores. The discovery of the A2 mating type in Europe has revived the interest in oospores and the sexual cycle, mainly because of the relationship between the sexual cycle and the mode of survival of *P. infestans* during the winter.

In 1984 Hohl and Iselin reported the discovery of isolates with the A2 mating type among their *P. infestans* cultures which had been collected in Switzerland in 1981. By the mid 1980s, many other culture collections and field isolates had been screened for mating type and it turned out that in East Germany the first A2 mating type isolate was found in 1980 and in The Netherlands, Switzerland and the United Kingdom in 1981 (Table 1). We can conclude that in the early 1980s the A2 mating type isolates spread rapidly over Europe.

Between 1987 and 1990 large numbers of isolates were collected in The Netherlands. In those years the percentage A2 mating type isolates varied from 11 - 18% (Table 2). Similar percentages were found in neighbouring countries (Table 2). The majority of these isolates were collected in commercially grown potato crops. Isolates collected between 1988 and 1990 from potatoes and tomatoes in allotment gardens showed a much higher percentage of A2 mating types, on average 53%, and the genotypic diversity in these isolates, as determined by DNA fingerprinting, is much higher (Drenth *et al.*, 1993, chapter 6). The early occurrence of late blight, the equal frequency of A1 and A2 mating type isolates and the high level of genetic diversity suggests the occurrence of sexual reproduction in allotment gardens (Schöber & Turkensteen, 1992).

Ever since the discovery of A2 mating type isolates in Europe, hypotheses were brought forward to explain their appearance. One hypothesis states that the A2 mating type arose from the A1 mating type either through mutation or mitotic recombination (Shattock *et al.*, 1990). According to Sansome (1980) and Shaw (1983a,b) mating type is coded for by one gene with two alleles. Although *P. infestans* is heterothallic, selfing can occasionally occur (Ko, 1978; Shattock *et al.*, 1986a,b). Selfing of an A1 isolate results in offspring consisting of only A1 mating types. Selfing of an A2 isolate results in offspring with A1 and A2 mating types (Shaw, 1983a). In diploid isolates the genotype of A1 will be *aa* whereas the A2 mating type will be *Aa*. It seems quite unlikely that a mutation of an isolate with the A1 mating type, from recessive to dominant allele, gives rise to an A2 mating type isolate (Boccas, 1981; Shaw, 1983a,b).

Table 2. The distribution of A1 and A2 mating type isolates of *P. infestans* collected before and after 1980 in The Netherlands, East and West Germany and the United Kingdom.

	The Netherlands			West Germany[1]			East Germany[2]			United Kingdom[3,4]		
	A1	A2	%A2	A1	A2	%A2	A1	A2	%A2	A1	A2	%A2
Before 1980	5	0	0	1	0	0	9	0	0	6	0	0
After 1980												
1980	1	0	0									
1981	3	2	40							5	2	29
1982										110	24	18
1983										3	2	40
1984	9	0	0							6	0	0
1985	21	7	25	29	2	6				64	6	9
1986	15	6	29	12	0	0				107	19	15
1987	62	11	15	34	24	41	76	0	0	202	15	8
1988	75	16	18	63	27	30	29	1	3	139	10	7
1989	252	34	12	20	10	33	22	8	27			
1990	271	33	11	50	14	22	34	5	13			

Data obtained from:
[1] Schöber and Turkensteen, (1992).
[2] Götz, (1991).
[3] Data from 1981, 1982, 1983 and 1984 from Tantius *et al.* (1986).
[4] Data from 1985, 1986, 1987 and 1988 from Shattock *et al.* (1990).

A second hypothesis states that A2 mating type isolates were present before 1980 but in such a low frequency that they were never detected (Shaw, 1987). An increase in the frequency of certain genotypes can occur when the relative fitness of these genotypes changes due to e.g. a mutation or a change in the environment. Such a significant change in the environment for *P. infestans* might have been the introduction of the phenylamide fungicide metalaxyl in 1979 (Davidse *et al.*, 1981).

A third hypothesis explains the presence of A2 mating type isolates through a recent introduction from central Mexico. With the occurrence of the A2 mating type, the diversity for virulence factors has increased significantly (Schöber, 1983; Rullich & Schöber, 1988; Schöber & Turkensteen, 1992; Drenth *et al.*, 1994, chapter 7) The virulence diversity in European isolates is now similar to that in central Mexico where many different and complex races of *P. infestans* occur (Mills & Niederhauser, 1953; Rivera-Peña, 1990). European isolates, collected before and after 1980, differ in allozyme alleles. The A1 mating type isolates collected before 1980 had *Gpi* alleles 86 and 100 and *Pep* alleles 92 and 100. In A1 and A2 mating type isolates collected after 1980, the 86 *Gpi* and the 92 *Pep* alleles are replaced by a 90 and an 83 allele, respectively (Spielman *et al.*, 1991, chapter 3; Fry *et al.*, 1991, chapter 5). Both are present in Mexican isolates (Goodwin *et al.*, 1992b). These observations and additional DNA fingerprinting analysis of the Dutch *P. infestans* population (outlined in the next paragraph) all support the hypothesis of a recent introduction. Most likely the appearance of the A2 mating type can be explained by an introduction from Mexico by human activities such as trade of seed and ware potatoes and fresh products (tomatoes), or transport of soil containing oospores.

THE POPULATION DISPLACEMENT OF *P. INFESTANS* IN THE NETHERLANDS

In recent years, we determined several characteristics of a large collection of Dutch *P. infestans* isolates. The majority of the isolates was collected between 1980 and 1991. From isolates collected before 1980, only a limited number remained. Mating type, metalaxyl resistance, *Gpi* and *Pep* allozyme constitution, virulence, mitochondrial DNA type and RG-57 genotype of the isolates were determined. The virulence testing was done using a differential set of host plants containing 10 of the 11 known R-genes. The mitochondrial DNA type and the RG-57 genotype were determined by Southern blot hybridizations. Mitochondrial DNA is a very useful marker for migration events because it has maternal inheritance only. It will be passed intact from parent to progeny, regardless of recombination events that occur in the nuclear genome. DNA fingerprinting, using the genomic *P. infestans* probe RG-57, was employed to identify individual isolates (Goodwin *et al.*, 1992a, chapter 4).

Before 1980, the *P. infestans* population in The Netherlands consisted of only A1 mating types, all isolates were sensitive to metalaxyl and they possessed *Gpi* alleles 86 and 100 and *Pep* alleles 92 and 100 (Spielman *et al.*, 1991, chapter 3). Among 33 isolates only four different races were identified. The virulence factors 1, 3, 4, and 10, which correspond to R-genes R1,

R3, R4 and R10 introduced in commonly used potato cultivars, occurred frequently (Mooi, 1968, 1971; Drenth *et al.*, 1994, chapter 7). From the seven different mitochondrial DNA types that have been identified in *P. infestans*, type A was predominant. From the six other types type G was found once (Table 3). DNA fingerprint probe RG-57 hybridized to 15 fragments of genomic DNA in which no polymorphisms were detected (Drenth *et al.*, 1994, chapter 7). Hence, all these isolates possessed the same RG-57 genotype. We conclude that between 1966 and 1978 there was hardly any diversity in the *P. infestans* population in The Netherlands. The limited diversity found for virulence matched the R-genes present in commonly used potato cultivars.

Among isolates collected after 1980, the A2 mating type appeared along with the A1 mating type and new *Gpi* and *Pep* alleles appeared (Spielman *et al.*, 1991, chapter 3; Fry *et al.*, 1991, chapter 5). Diversity for virulence and complexity of races increased greatly (Spielman *et al.*,1991, chapter 3; Schöber & Turkensteen, 1992; Drenth *et al.*, 1994, chapter 7). Among 253 isolates, 73 different races were identified (Drenth *et al.*, 1994, chapter 7). These races contained many unnecessary virulence alleles (e.g. 5, 6, 7, 8 and 11) for which there are no corresponding resistance genes in the array of potato cultivars grown in The Netherlands. One isolate, collected in 1992, is virulent against all 11 known R-genes (L.J. Turkensteen, unpublished). In addition to mitochondrial DNA type A, type B was found for the first time among A1 mating type isolates collected in 1981 (Table 3). Mexican isolates also include type B mitochondrial DNA (Goodwin, 1991). Probe RG-57 hybridized to 26 genomic DNA fragments, 24 of which are polymorphic. Genotypic diversity within any one year was substantial; among 153 isolates, all collected in 1989, 35 different RG-57 genotypes were observed (Drenth *et al.*, 1993, chapter 6). Among 179 isolates collected between 1980 and 1991, 134 different RG-57 genotypes were found. From these, 121 were found only once (Drenth *et al.*, 1994, chapter 7). The 26 RG-57 fragments identified in the Dutch isolates appear to be a subset of the 27 fragments found in Mexican isolates collected in the 1980s (Goodwin *et al.*, 1992b; Drenth *et al.*, 1993, chapter 6). The appearance of different allozyme alleles, mitochondrial DNA types and RG-57 hybridizing fragments in the isolates collected during the eighties, compared to before 1980, all support the population displacement theory (Spielman *et al.*, 1991, chapter 3). The fact that the new characteristics were found before in Mexican isolates strongly suggests that the newly introduced isolates originated in Mexico.

In 1845, one year was sufficient for *P. infestans* to spread over vast parts of Europe (Bourke, 1964). At that time, no control measures were available and potato fields were heavily blighted, producing immense quantities of spores. In 1980 and 1981, genetically different A1 and A2 mating type isolates were found in a number of countries nearly simultaneously (Table 1), despite the fact that huge amounts of fungicides were applied. Therefore, it is reasonable to assume that the new isolates had been present for at least some time to facilitate spread over Europe. In 1976, a severe drought reduced the potato harvest in Europe and in the spring of 1977 vast quantities of potatoes were transported from Mexico to Europe. It is likely that this potato import was accompanied by *P. infestans* (Niederhauser, 1991); either in the form of

Table 3. Mitochondrial DNA types of *P. infestans* isolates collected in The Netherlands before and after 1980.

Year	# Isolates	Mitochondrial DNA type						
		A	B	C	D	E	F	G
Before 1980[1]								
1966	1	1	0	0	0	0	0	0
1968	1	1	0	0	0	0	0	0
1970	1	1	0	0	0	0	0	0
1974	1	1	0	0	0	0	0	0
1978	1	0	0	0	0	0	0	1
After 1980								
1980	1	1	0	0	0	0	0	0
1981	5	4	1	0	0	0	0	0
1984	4	2	2	0	0	0	0	0
1985	13	12	1	0	0	0	0	0
1986	9	9	0	0	0	0	0	0
1987	15	9	5	0	1	0	0	0
1988	30	24	5	0	0	1	0	0
1989	54	39	15	0	0	0	0	0
1990	30	20	5	0	2	3	0	0
1991	14	7	5	0	0	1	1	0

[1]In an additional 24 isolates from neighbouring countries collected before 1980 type A was predominant and type B was never found.

mycelium in potato tubers or as oospores attached to potato tubers. This renewed introduction of A1 and A2 mating type isolates will have resulted in a population displacement. The speed at which the population displacement took place indicates that the current *P. infestans* isolates possess a higher fitness than the isolates which were present before 1980 (Spielman *et al.*, 1991, chapter 3). Among the isolates collected after 1980 none have been found of the old genotype or with a combination of old and new *Gpi* and *Pep* alleles, 86 with 90 and 83 with 92, respectively. *In vitro* pairing of old A1 and new A2 isolates yields hybrids with combinations

of old and new allozyme alleles. DNA fingerprinting confirmed that the progeny consisted of sexual hybrids. However, the progeny had very low fitness and was not very pathogenic (A. Drenth, unpublished). Their reduced fitness may be explained by the build-up of a huge mutational load in the old A1 population. If one speculates that A1 mating type isolates, present before the introduction of the new population, originated from the initial introduction of *P. infestans* to Europe in 1845, they were condemned to asexual reproduction for about 130 years before their partner for sexual mating showed up. Assuming an average of 20 generations per year, 2600 asexual generations have passed. With a mutation rate of 11.2×10^{-6} per gene per generation (Schlager & Dickie, 1971) the probability of a mutation over 2600 generations will be 0.029 per gene. The unfavourable effects of many mutations in these genes will be masked as long as there are undamaged copies of the genes available. This is especially true for the old population which was predominantly tetraploid. However, the mutational load in the old A1 mating type isolates may have greatly reduced their suitability for sexual reproduction and it may be held responsible for the poor pathogenicity of *in vitro* produced sexual progeny involving old A1 mating type isolates.

Moreover, the fungal population size is greatly reduced during winter. Only a few isolates are able to survive the winter as mycelium in tubers and to start a new epidemic the following year. This results in a large genetic drift which seriously counteracts the natural selection against unfavourable mutations (Lynch & Gabriel, 1990). As a consequence of the heavy mutational load and the ensuing lack of reproductive potential, the old A1 mating type population might have been replaced by the new population to such an extent that no traces of the old population were found after 1980.

Another catalyst for a rapid population displacement by new *P. infestans* isolates might have been the introduction of metalaxyl in 1979. Very soon after its introduction, in 1980, resistance against this fungicide was found among *P. infestans* isolates (Davidse *et al.*, 1981). Resistance against metalaxyl might have been present in the newly introduced isolates from Mexico, or alternatively, the new isolates might have been able to acquire the resistance more rapidly. The large scale application of metalaxyl in 1979 and in 1980 provided a huge selection pressure for resistant isolates leading to a tremendous increase of their frequency in the fungal population (Davidse *et al.*, 1981). No metalaxyl resistant isolates were found in the old, tetraploid *P. infestans* isolates collected before 1980. In the new *P. infestans* population, resistance against metalaxyl was found in various A1 and A2 mating type isolates with different genetic backgrounds (Drenth *et al.*, 1993, chapter 6) suggesting many independent mutations leading to metalaxyl resistance and/or the inheritance of resistance through sexual reproduction.

CONCLUDING REMARKS

In this paper we have given an overview concerning survival over the seasons of the potato late blight fungus with special emphasis on the occurrence of sexual reproduction in The

Netherlands. The old A1 mating type isolates, present before 1980, were displaced rapidly by new A1 and A2 mating type isolates from Mexico. Genetic diversity in the new pathogen population is tremendous, strongly suggesting that sexual reproduction occurs in the field. Oospores, which can survive in soil between growing seasons, were found in the field. They can accumulate in the soil and infect emerging sprouts and leaves which are in contact with the soil. This new epidemiological process may lead to a more massive start of the epidemic earlier in the season. Sexual reproduction will lead to a more variable population with a higher level of adaptability than a purely asexually reproducing population (Maynard Smith, 1971). New pathogen genotypes will be generated all the time and particularly fit genotypes will dominate the pathogen population. It is obvious that the presence of both mating types, allowing sexual reproduction, makes late blight more difficult to control. To anticipate epidemic development, detailed studies on oospore production, survival, germination and infectivity are needed to determine the parameters of late blight epidemics caused by the new *P. infestans* population. These parameters are likely to be different from those in epidemics caused by old isolates. We expect that the current disease management strategies will have to be adjusted for adequate control of potato late blight.

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REFERENCES

Aylor, D.E., 1986. A framework for examining inter-regional aerial transport of fungal spores. Agricultural and Forestry Meteorology 38: 263-288.

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

Boccas, B.R., 1981. Interspecific crosses between closely related heterothallic *Phytophthora* species. Phytopathology 71: 60-65.

Bourke, P.M.A., 1964. Emergence of potato blight, 1843-46. Nature 203: 805-808.

Brefeld, O., 1883. Die Brandpilze. I. Botanische Untersuchungen über Hefenpilze, heft V. pp 25.

Brommonschenkel, S.H., 1988. Pathogenicity, compatibility, cytogenetics and isoenzyme patterns of Brazilian isolates of *Phytophthora infestans* (Mont.) de Bary. M.S. Thesis, Universidade Federal de Vicosa, Brazil. pp 82.

Bruyn, H.L.G. de, 1926. The overwintering of *Phytophthora infestans* (Mont) de Bary. Phytopathology 16: 121-140.

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

Carter, D.A., Archer, S.A., Buck, K.W., Shaw, D.S. & Shattock, R.C., 1991. DNA polymorphisms in *Phytophthora infestans*: the UK experience. p 272-294 in: Lucas, J.A., Shattock, R.C., Shaw, D.S. & Cooke, L.R. (eds.). *Phytophthora*. British Mycological Society, Cambridge University Press, Cambridge. pp 447.

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

Clinton, G.P., 1911. Oospores of potato blight. Science 33: 744-747.

Cox, A.E. & Large, E.C., 1960. Potato blight epidemics throughout the world. United States Department of Agriculture, Agriculture handbook 174. pp 230.

Dagget, S.S., Götz, E. & Therrien C.D., 1993. Phenotypic changes in populations of *Phytophthora infestans* from eastern Germany. Phytopathology 83: 319-323.

Davidse, L.C., Looijen, D., Turkensteen, L.J. & Van der Wal, D., 1981. Occurrence of metalaxyl-resistant strains of *Phytophthora infestans* in the Netherlands. European Plant Protection Organization. Bulletin 15: 403-409.

Deahl, K.L., Goth, R.W., Young, R., Sinden, S.L. & Gallegly, M. E., 1991. Occurrence of the A2 mating type of *Phytophthora infestans* in potato fields in the United States and Canada. American Potato Journal 68: 717-726.

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

Drenth, A., Goodwin, S.B., Fry, W.E. & Davidse, L.C., 1993. Genotypic diversity of *Phytophthora infestans* in The Netherlands revealed by DNA polymorphisms. Phytopathology 83: 1087-1092.

Drenth, A. & Govers, F., 1993. DNA fingerprinting of the potato late blight fungus, *Phytophthora infestans*. p 223-230 in: Schots, A., Dewey, F.M. & Oliver, R. (eds.). Modern assays for plant pathogenic fungi: Identification, detection and quantification. CAB international. pp 267.

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

Duncan, J.M., 1980. Persistence of mycelium of *Phytophthora fragariae* in soil. Transactions of the British Mycological Society 75: 383-387.

Estrada, S.P., 1967. Importancia de las oosporas de *Phytophthora infestans* (Mont.) de Bary como inoculo primario del "tizon tardio" de la papa. MsC thesis, Chapingo, Mexico. pp 43.

Frinking, H.D., Davidse, L.C. & Limburg, H., 1987. Oospore formation by *Phytophthora infestans* in host tissue after inoculation with isolates of opposite mating type found in the Netherlands. Netherlands Journal of Plant Pathology 93: 147-149.

Fry, W.E., Drenth, A., Spielman, L.J., Mantel, B.C., Davidse, L.C. & Goodwin, S.B., 1991. Population genetic structure of *Phytophthora infestans* in the Netherlands. Phytopathology 81: 1330-1336.

Fry, W.E., Goodwin, S.B., Matuszak, J.M., Spielman, L.J., Milgroom, M.G. & Drenth, A., 1992. Population genetics and intercontinental migrations of *Phytophthora infestans*. Annual Review of Phytopathology 30: 107-129.

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

Gallegly, M.E., 1968. Genetics of pathogenicity of *Phytophthora infestans*. Annual Review of Phytopathology 6: 375-396.

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

Gees, R. & Hohl, H.R., 1988. Cytological comparison of specific (R3) and general resistance to late blight in potato leaf tissue. Phytopathology 78: 350-357.

Goodwin, S.B., 1991. DNA polymorphisms in *Phytophthora infestans*: the Cornell Experience. p 256-271 in: Lucas, J.A., Shattock, R.C., Shaw, D.S. & Cooke, L.R. (eds.). *Phytophthora*. British Mycological Society, Cambridge University Press, Cambridge. pp 447.

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

Goodwin, S.B., Spielman, L.J., Matuszak, J.M., Bergeron, S.N. & Fry, W.E., 1992b. Clonal diversity and genetic differentiation among *Phytophthora infestans* populations in northern Mexico. Phytopathology 82: 955-961.

Götz, E., 1990. New aspects relating to *Phytophthora* control in potatoes. / Neue Aspekte bei der Bekampfung der Phytophthora in Kartoffeln. Kartoffelbau 6: 224-226.

Götz, E., 1991. Untersuchungen zum auftreten des A2-paarungstyps bei *Phytophthora infestans* (Mont.) de Bary in Ostdeutschland. Potato Research 34: 233-237.

Grinberger, M., Kadish D. & Cohen Y., 1989. Occurrence of the A2 mating type and oospores of *Phytophthora infestans* in potato crops in Israel. Phytoparasitica 17: 197-204.

Grove, G.G., Ellis, M.A., Madden, L.V. & Schmitthenner, A.F., 1985. Overwinter survival of *Phytophthora cactorum* in infected strawberry fruit. Plant Disease 69: 514-515.

Gunderson, J.H., Elwood, H., Ingold, A., Kindle, K. & Sogin, M.L., 1987. Phylogenetic relationships between *chlorophytes*, *chrysophytes* and *oomycetes*. Proceedings of the National Academy of Sciences, USA 84: 5823-5827.

Hemmes, D.E., 1983. Cytology of *Phytophthora*. p 9-40 in: Erwin, D.C., Bartnicki-Garcia, S. & Tsao, P.H. (eds.). *Phytophthora*: its biology, taxonomy, ecology and pathology. APS Press, USA. pp 392.

Hendrix, J.W., 1964. Sterol induction of reproduction and stimulation of growth of *Pythium* and *Phytophthora*. Science 144: 1028-1029.

Hirst, J.M. & Stedman, O.J., 1960. The epidemiology of *Phytophthora infestans*. II The source of inoculum. Annals of Applied Biology 48: 489-517.

Hohl, H.R. & Iselin, K., 1984. Strains of *Phytophthora infestans* with A2 mating type behaviour. Transactions of the British Mycological Society 83: 529-530.

Kadir, S. & Umaerus, U., 1987. *Phytophthora infestans* A2 compatibility type recorded in Sweden. p 223 in: Book of abstracts 10th triannual conf. EAPR. Aalborg, Denmark.

Ko, W.H., 1978. Heterothallic *Phytophthora*: Evidence for hormonal regulation of sexual reproduction. Journal of General Microbiology 107: 15-18.

Kotova, V.V., 1979. Distribution and pathogenic features of *Aphanomyces euteiches* Drechs. Rasprostranenie i osobennosti parazitizma griba *Aphanomyces euteiches* Drechs. Mikologyia i Fitopatologiya 13: 485-488.

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

Large, E.C., 1940. The advance of the fungi. Jonathan Cape Ltd., London. pp 488.

Leach, S.S. & Rich, A.E., 1969. The possible role of parasexuality and cytoplasmatic variation in race differentiation in *Phytophthora infestans*. Phytopathology 59: 1360-1365.

Lynch, M. & Gabriel, W., 1990. Mutation load and the survival of small populations. Evolution 44: 1725-1737.

Malajczuk, J., 1983. Microbial antagonism in *Phytophthora*. p 197-218 in: Erwin, D.C., Bartnicki-Garcia, S., Tsao, P.H. (eds). *Phytophthora*: its biology, taxonomy, ecology and pathology. APS Press, USA. pp 392.

Malcolmson, J.F., 1985. *Phytophthora infestans* A2 compatibility type recorded in Great Britain. Transactions of the British Mycological Society 85: 531.

Maynard-Smith, J., 1971. What use is sex ? Journal of Theoretical Biology 30: 319-335.

Mills, W.R. & Niederhauser, J.S., 1953. Observations on races of *Phytophthora infestans* in Mexico. Phytopathology 43: 454-455.

Mooi, J.C., 1968. Onderzoek naar het voorkomen van "hogere" fysio's van *Phytophthora infestans* in de Groninger Veenkolonien en aangrenzend gebied in 1968. Resultaten proefvelden fabrieksaardappelen 1968: 54-57.

Mooi, J.C., 1971. Het optreden van fysio's van *Phytophthora infestans* in de Groninger Veenkolonien en omgeven gebied in 1970 en 1971. Resultaten proefvelden fabrieksaardappelen 1971: 62-65.

Mosa, A.A., Kato, M., Sato, N., Kobayashi, K. & Ogoshi, A., 1989. Occurrence of the A2 mating type of *Phytophthora infestans* on potato in Japan. Annals of the Phytopathological Society of Japan 55: 615-620.

Niederhauser, J.S., 1956. The blight, the blighter, and the blighted. Transactions of the New York Academy of Science 19: 55-63.

Niederhauser, J.S., 1991. *Phytophthora infestans*: the Mexican connection. p 272-294 in: Lucas, J.A., Shattock, R.C., Shaw, D.S. & Cooke, L.R. (eds.). *Phytophthora*. British Mycological Society, Cambridge University Press, Cambridge. pp 447.

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

O'Sullivan, E. & Dowley, L.J., 1991. A note on the occurrence of the A2 mating type and self-fertile isolates of *Phytophthora infestans* in the Republic of Ireland. Irish Journal of Agricultural Research 30: 67-69.

Perches, E.S. & Galindo, A.J., 1969. Supervivencia del *Phytophthora infestans* (Mont) de Bary del Tizón tardio de la papa y jitomate. Agrociencia 5: 92-98.

Peterson, L.C. & Mills, W.R., 1953. Resistance of some American potato varieties to late blight of potatoes. American Potato Journal 30: 65-70.

Pethybridge, G.H. & Murphy, P.A., 1913. On pure cultures of *Phytophthora infestans* de Bary, and the development of oospores. Scientific Proceedings of the Royal Dublin Society 13: 566-588.

Pristou, R. & Gallegly, M.E., 1954. Leaf penetration by *Phytophthora infestans*. Phytopathology 44: 81-86.

Pristou, R. & Gallegly, M.E., 1956. Differential reaction of potato hosts to foreign and domestic potato physiological races of *Phytophthora infestans*. American Potato Journal 33: 287-295.

Reddick, D. & Crosier, W., 1933. Biological specialization in *Phytophthora infestans*. American Potato Journal 10: 129-134.

Rivera-Peña, A., 1990. 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.

Romero, S. & Erwin, D.C., 1967. Genetic recombination in germinated oospores of *Phytophthora infestans*. Nature 215: 1393-1394.

Rullich, G. & Schöber, B., 1988. Auftreten und Verbreitung der A2 Paarungstyps von *Phytophthora infestans* (Mont.) de Bary in der Bundesrepublik Deutschland. Kartoffelbau 39: 244-246.

Salaman, R.N., 1910. The inheritance of colour and other characteristics in the potato. Journal of Genetics 1: 7-46.

Sansome, E. & Brasier, C.M., 1973. Diploidy and chromosomal structural hybridity in *Phytophthora infestans*. Nature 241: 344-345.

Sansome E., 1980. Reciprocal translocation heterozygosity in heterothallic species of *Phytophthora* and its significance. Transactions of the British Mycological Society 74: 175-185.

Schlager, G. & Dickie, M.M., 1971. Natural mutation rates in the house mouse. Estimates for five specific loci and dominant mutations. Mutation Research 11: 89-96.

Schöber, B., 1983. Definition und Auftreten der Pathotypen von *Phytophthora infestans* (Mont.) de Bary. Kartoffelbau 34: 156-158.

Schöber, B. & Rullich G., 1986. Oosporenbildung von *Phytophthora infestans* (Mont) de bary. Potato Research 29: 395-398.

Schöber, R. & Turkensteen, L.J., 1992. Recent and future developments in potato fungal pathology. Netherlands Journal of Plant Pathology 98, Supplement 2: 73-83.

Shattock, R.C., Shaw, D.S., Fyfe, A.M., Dunn, J.R., Loney, K.H. & Shattock, J.A., 1990. Phenotypes of *Phytophthora infestans* collected in England and Wales from 1985 to 1988: mating type, response to metalaxyl and isoenzyme analysis. Plant Pathology 39: 242-248.

Shattock, R.C., Tooley, P.W. & Fry, W.E., 1986a. Genetics of *Phytophthora infestans*: characterization of single-oospore cultures from A1 isolates induced to self by intraspecific stimulation. Phytopathology 76: 407-410.

Shattock, R.C., Tooley, P.W. & Fry, W.E., 1986b. Genetics of *Phytophthora infestans*: Determination of recombination, segregation, and selfing by isozyme analysis. Phytopathology 76: 410-413.

Shaw, D.S., 1983a. The cytogenetics and genetics of *Phytophthora*. p 81-94 in: Erwin, D.C., Bartnicki-Garcia, S. & Tsao, P.H. (eds.). *Phytophthora*: its biology, taxonomy, ecology and pathology. APS Press, USA. pp 392.

Shaw, D.S., 1983b. The Peronosporales. A fungal geneticist's nightmare. p 85-121 in: Buczacki, S.T. (eds.). Oosporic plant pathogens a modern perspective. Academic Press, London. pp 352.

Shaw, D.S., 1987. The breeding system of *Phytophthora infestans*: the role of the A2 mating type. p 161-174 in: Day, P.R. & Jellis G.J. (eds.). Genetics and plant pathogenesis. Blackwell Scientific Publications, Oxford. pp 352.

Shaw, D.S., Fyfe, A.M., Hibberd, P.G. & Abdel-Sattar, M.A., 1985. Occurrence of the rare A2 mating type of *Phytophthora infestans* on imported Egyptian potatoes and the production of sexual progeny with A1 mating types. Plant Pathology 34: 552-556.

Smoot, A.J., Gough F.J., Lamey H.A., Eichenmuller, J.J. & Gallegly M.E., 1958. Production and germination of oospores of *Phytophthora infestans*. Phytopathology 48: 165-171.

Spielman, L.J., Drenth, A., Davidse, L.C., Sujkowski, L.J., Gu, W.K., Tooley, P.W. & Fry, W.E., 1991. A second world-wide migration and population displacement of *Phytophthora infestans* ? Plant Pathology 40: 422-430.

Tantius, P.H., Fyfe, A.M., Shaw, D.S. & Shattock, R.C., 1986. Occurrence of the A2 mating type and self fertile isolates of *Phytophthora infestans* in England and Wales. Plant Pathology 35: 578-581.

Tooley, P.W., Fry, W.E. & Villarreal Gonzalez, M.J., 1985. Isozyme characterization of sexual and asexual *Phytophthora infestans* populations. Journal of Heredity 76: 431-435.

Tooley, P.W., Sweigard, J.A. & Fry, W.E., 1986. Fitness and virulence of *Phytophthora infestans* isolates from sexual and asexual populations. Phytopathology 76: 1209-1212.

Toxopeus, H.J., 1956. Reflections on the origin of new physiological races in *Phytophthora infestans* and the breeding for resistance in potatoes. Euphytica 5: 221-237.

Turkensteen, L.J., 1973. Partial Resistance of tomatoes against *Phytophthora infestans*, the late blight fungus. Agricultural Research Report No. 810. Instituut voor Plantenziektenkundig Onderzoek, Wageningen, Nederland. Mededeling No. 633. pp 88.

Turkensteen, L.J. 1978. *Phytophthora infestans*: three new hosts and a specialized form causing a foliar blight of *Solanum muricatum* in Peru. Plant Disease Reporter 62: 829.

Vorob'eva, Y.V., Gridnev, V.V., Bashaeva, E.G., Pospelova, L.A., Kvasnyuk, N.Y., Kuznetsova, L.N., Shemyakina, V.P. Morozova, E.V. & Zherebtsova, L.N., 1991. Occurrence of A2 mating type isolates of *Phytophthora infestans* (Mont.) de Bary in the USSR. Mikologiya i Fitopatologiya 1: 62-67.

Woodham-Smith, C., 1962. The great hunger. Harper & Row, New York, USA. pp 508.

Zaag, D.E. van der, 1956. Overwintering en epidemiologie van *Phytophthora infestans*, tevens enige nieuwe bestrijdingsmogelijkheden. Tijdschrift over planteziekten 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.

Zentmyer, G.A., 1980. *Phytophthora cinnamomi* and the diseases it causes. Monogram 10. American Phytopathological Society, St. Paul, MN, USA. pp 96.

Chapter 3

A second world-wide migration and population displacement of *Phytophthora infestans*

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ABSTRACT

The appearance of the A2 mating type (previously restricted to central Mexico) in Europe during the 1980s prompted an investigation of the genetic make-up of European populations using allozyme loci as genetic markers. The investigation shows that major genetic changes have occurred in populations of *Phytophthora infestans* in The Netherlands, Poland, and the British Isles. It now appears that a new type of population has been introduced into several locations, and has displaced or is displacing the original populations in these locations. The new and old population types are characterized by unique allozyme alleles and genotypes. The mechanism for displacement of the "old" by the "new" population is not yet known.

INTRODUCTION

The first major migration of *Phytophthora infestans* became evident in the 1840s when it caused the Irish potato famine, as well as epidemics in the mid-Atlantic and north-eastern regions of the United States (Stevens, 1933; Large, 1940). These outbreaks probably came about through transport of the fungus from its ancestral home in the highlands of central Mexico to the United States and Europe. Since then, the late blight fungus has been distributed throughout the world (primarily in infected tubers used as seed), and is now the most important pathogen of potato on a worldwide basis (Hooker, 1981).

Following these initial migrations, populations of *P. infestans* appear to have remained generally stable until the late 1970s or early 1980s. Outside central Mexico, only the A1 mating type was detected, while in the highlands of central Mexico, each of the two mating types (A1 and A2) occurred in approximately equal frequency (Gallegly & Galindo, 1958; Tooley *et al.*, 1985). This distribution of mating types had important implications for genetic structure: *Phytophthora infestans* reproduces both sexually and asexually, but sexual recombination occurs only between opposite mating types. (Although mating type segregates in crosses, the genetic basis is not known: Shaw, 1983; Spielman *et al.*, 1990.) Consistent with these facts, Tooley *et al.* (1985) found that genotype frequencies for two allozyme loci in an asexual collection did not conform to Hardy-Weinberg expectations, while genotype frequencies in a central Mexican collection were similar to those expected of a randomly mating sexual population.

In the early 1980s, however, reports of A2 mating types from several European countries (Hohl & Iselin, 1984; Malcolmson, 1985; Tantius *et al.*, 1986) indicated that the situation had changed. Since then the A2 mating type has been found in numerous other locations in Europe, the Middle East, Asia and South America (Spielman, 1991; H. Hohl, University of Zurich, unpublished data). Several hypotheses have been circulated to explain the widespread appearance of A2 mating types: (i) A2 strains could have migrated from a region already occupied by A2 mating types; (ii) A1 isolates could have undergone mutation to produce A2 isolates and then migrated from their place of origin; and (iii) A2 isolates could have been present at very low

levels in places such as Europe, and merely increased to detectable frequencies in the late 1970s. Knowledge of the genetic make-up of *P. infestans* populations would enable evaluation of these hypotheses, but until now there has been insufficient data to discriminate among these hypotheses. In addition to the work of Tooley *et al.* (1985) cited above, there are two other relevant publications. Tooley *et al.* (1989) found that 34 isolates from Peru were A1 mating type, and identical, with one exception, at the allozyme loci glucose phosphate isomerase (*Gpi*) and peptidase (*Pep*). Shattock *et al.* (1990) reported that English and Welsh isolates collected between 1985 and 1988 were almost totally monomorphic for *Gpi*.

The goal of the present work was to characterize additional populations of *P. infestans* not included in the study by Tooley *et al.* (1985), especially in regions in which the A2 mating type had recently appeared. During this study, it became apparent that major changes in genotype frequencies were occurring, or had recently occurred, in Europe. Therefore the documentation of these changes was added as a second goal. Data are reported which indicate that a new population of *P. infestans* has displaced an old population in several locations in Europe.

MATERIALS AND METHODS

Collections

Collections were obtained from The Netherlands, the earliest known location outside Mexico for A2 strains, and from Japan (kindly provided by N. Sato), where recent studies established the presence of A2 strains (Mosa *et al.*, 1989). Collections were also obtained from Poland where A2 strains had not previously been found. Small samples predating the detection of A2 mating types were obtained from Switzerland (courtesy of H. Hohl) and the United Kingdom (courtesy of R.C. Shattock). The United States (known only to contain A1 strains when the samples were obtained) is represented by the 39 isolates analyzed by Tooley *et al.* (1985) and 21 additional isolates obtained between 1986 and 1989.

Where possible, random sampling methods were employed. The central, southern and north-eastern regions of Poland are represented in each of the Polish collections. Sites were selected randomly from these regions, and each site is represented by only one isolate. The 1989 collection from The Netherlands came from randomly selected sites in the northern, central and south-western regions. Each site is represented by between 2 and 27 isolates, obtained by sampling along transects (if disease was abundant) or by sampling every lesion found (if disease was rare). However, the earlier collections from The Netherlands and the Japanese collection are not random samples but simply represent those cultures which were available. The United States/Canada collection came primarily from the north-eastern United States and eastern Canada. The wide range in dates is due to the fact that outbreaks of late blight have not been common recently in the United States and Canada, and those which do occur are rapidly controlled with fungicides, making large-scale collection of isolates difficult.

Characterization of isolates

For each isolate, the mating type was determined by pairing with A1 and A2 testers on 10% V8 agar at 18°C in the dark (Spielman *et al.*, 1989). Genotypes at two polymorphic allozyme loci, *Gpi* (glucose phosphate isomerase, E.C.5.3.1.9) and *Pep* (peptidase, E.C.3.4.3.1), were determined using procedures of Spielman *et al.* (1990). (For convenience these loci will be designated *Gpi* and *Pep*.) In the early stages of this work it was not possible to distinguish between some alleles with similar mobilities. For resolving the *Gpi* 90 and 100 alleles, the histidine-HCl, pH 6 system described by Spielman *et al.* (1990), or a morpholine-citrate, pH 6 system (electrode: 0.037 molar citric acid titrated to pH 6 with *N*-(3-aminopropyl)morpholine gel: electrode buffer diluted 1 : 9), was used. Run times were between 6 and 8 h at 150 V and 40-60 mA. There are also two *Pep* alleles, 83 and 92, which can be distinguished on the Tris-borate-EDTA, pH 8.7 system of Spielman *et al.* (1990), with a run time of 6-7 h at 225 V and approximately 30 mA. Work done before these conditions were developed was published in a preliminary report (Spielman, 1991); some isolates listed there as *Pep* 92/100 are now known to be 83/100. The correct genotypes are presented here. The allelic relationships of *Gpi* 90 and *Pep* 83 were determined from a genetic cross, which was performed following the procedures in Spielman *et al.* (1989).

Analysis

The allozyme data were used to calculate Hedrick's genotypic identity (Hedrick, 1971). In order to compare early and recent collections, identity values were calculated for all of the Polish and Dutch collections, despite the small size of the 1951-78 and 1980-82 Dutch samples.

RESULTS

Characterization of populations

The collections studied fell into two categories, each characterized by different sets of alleles: (i) those which were strictly of A1 mating type and (ii) those which included both A1 and A2 mating types. In the strictly A1 collections, we found only two alleles at *Gpi* (86 and 100) and two at *Pep* (92 and 100). In collections containing both A1 and A2 mating types (except the Japanese collection), *Gpi* 86 and *Pep* 92 were less frequent or absent, and there were two additional alleles, *Gpi* 90 and *Pep* 83 (Table 1). All the collections contained a small fraction of the potential numbers of dilocus genotypes, and these genotypes were found repeatedly in different collections (Tables 2-4), suggesting that reproduction was predominantly asexual in these populations. This supposition is supported by a more detailed analysis of the 1989 collection from The Netherlands (Fry *et al.*, 1991), which showed that the structure was clonal at both the subpopulation and overall population levels. It is therefore most appropriate to describe the collections in terms of genotypes.

Table 1. Allele frequencies in collections of *Phytophthora infestans*.

Allele	United States	Peru[a]	Japan	The Netherlands				Poland		
				1951-78	1980-82	1984-85	1989[b]	1985-87	1988	1989
Gpi										
86	0.42	0.50	0.25	0.50	0.10	0	0	0.50	0.24	0
90	0	0	0	0	0.10	0.40	0.43	0	0.15	0.35
100	0.57	0.50	0.75	0.50	0.80	0.60	0.56	0.50	0.63	0.65
Pep										
83	0	0	0	0	0.15	0.29	0.28	0	0.06	0.05
92	0.45	0.49	0.17	0.36	0.05	0	0	0.45	0.24	0
100	0.54	0.52	0.84	0.64	0.80	0.71	0.72	0.55	0.70	0.95
Sample size	58	34	30	7	10	24	180	21	42	41

[a]Data of Tooley *et al.* (1989).
[b]Data of Fry *et al.* (1991).

The most common dilocus genotype in the strictly A1 collections was *Gpi* 86/100 *Pep* 92/100, but isolates homozygous at either one (but not both) of the two loci also occurred (Tables 2-4). The United States/Canada collection, and early collections from The Netherlands (1951-78) and Poland (1985-87) are included in this group, as is the Peruvian collection analyzed by Tooley *et al.* (1989). Two Swiss isolates from 1981 were also *Gpi* 86/100 *Pep* 92/100 and A1 mating type. Seven UK isolates collected between 1980 and 1982 also belong in this group: all were A1 mating type, and five were *Gpi* 86/100 *Pep* 92/100, one was *Gpi* 100/100 *Pep* 92/100 and one was *Gpi* 86/100 *Pep* 96/100. (The *Pep* 96 allele is so far unknown from any other isolate, including recent UK samples [Shattock *et al.* 1990; P.W. Tooley, unpublished data]). The genotypes *Gpi* 86/100 *Pep* 92/100, *Gpi* 86/100 *Pep* 100/100, and *Gpi* 100/100 *Pep* 92/-- are designated old genotypes (Tables 2-4), as explained in the discussion.

In collections containing both A1 and A2 mating types (except Japan), the genotypes *Gpi* 90/100 or 100/100 and *Pep* 83/100 or 100/100 occurred, either in combination with genotypes from the first group (The Netherlands 1980-82 and Poland 1988), or exclusively (The Netherlands 1984-89 and Poland 1989). The Japanese collection differed from the other collections of mixed mating type in the absence of the alleles *Gpi* 90 and *Pep* 83. Each mating type was, nevertheless, strongly associated with a different genotype, the A1 with *Gpi* 86/100 and the A2 with *Gpi* 100/100, and the most common A2 genotype was *Gpi* 100/100 *Pep* 100/100.

Table 2. Genotype frequencies in collections of *Phytophthora infestans* from The Netherlands.

Gpi	*Pep*	Mating type	Collection dates			
			1951-78	1980-82	1984-85	1989[a]
Old genotypes						
86/100	92/100	A1	0.71(2)[b]	0.10	0	0
86/100	100/100	A1	0.29(1)[b]	0.10	0	0
New genotypes						
90/100	83/100[c]	A1	0	0.10	0.33	0.53
		A2	0	0.10	0.17	0
90/100	100/100	A1	0	0	0.13	0.33
		A2	0	0	0.17	0.01
100/100	83/100[c]	A1	0	0	0.04	0.02
		A2	0	0.10	0.04	0.01
100/100	100/100	A1	0	0.40	0.08	0.01
		A2	0	0.10	0.04	0.09
Sample size			7	10	24	180

[a]Data of Fry *et al.* (1991).

[b]Some of these isolates (in parentheses) appeared to have lost the ability to produce oospores, so that mating type could not be definitely determined. However, no A2 mating types were reported from The Netherlands during this period.

[c]Because, initially, *Pep* 83 and 92 could not be distinguished, these genotypes were listed as *Pep* 92/100 in a preliminary report (Spielman, 1991).

The genotypic frequencies in this collection do not represent the actual frequencies in Japan, because the sample is not representative of the population: only about one third of randomly collected isolates are A1 mating type there (Mosa *et al.*, 1989). The allozyme genotypes *Gpi* 90/100 *Pep* 83/100, *Gpi* 100/100 *Pep* 83/100, *Gpi* 90/100 *Pep* 100/100, and *Gpi* 100/100 *Pep* 100/100 are designated new genotypes (Tables 2-4), as explained in the discussion. All A2 mating type isolates had one of the new genotypes.

Genetic tests

The allelic relationships of *Gpi* 90 were determined by analyzing the F1 progeny of a sexual cross. The parents were: 1115, collected from The Netherlands in 1985; A1 mating type, *Gpi* 90/100, *Pep* 83/100, and 618, collected from Toluca, Mexico, in 1987; A2 mating type, *Gpi* 86/122 *Pep* 100/100. Twenty-one progeny were obtained from this cross. The *Gpi* genotypes

Table 3. Genotype frequencies in *Phytophthora infestans* collections from Poland.

Gpi	*Pep*	Mating type	Collection dates		
			1985-87	1988	1989
Old genotypes					
86/100	92/100	A1	0.91	0.48	0
86/100	100/100	A1	0.10	0	0
New genotypes					
90/100	83/100[a]	A1	0	0.12	0.12
90/100	100/100	A1	0	0.17	0.29
		A2	0	0	0.32
100/100	100/100	A1	0	0.19	0.06
		A2	0	0.05	0.21
Sample size			21	42	41

[a]Because, initially *Pep* 83 and 92 could not be distinguished, these genotypes were listed as *Pep* 92/100 in a preliminary report (Spielman, 1991).

(with numbers of progeny in parentheses) were: 86/86 (1), 86/100 (7), 86/122 (1), 90/100 (1), 90/122 (5), 100/100 (1), 100/122 (4), and 86/100/122 (1). The *Pep* genotypes were: 83/100 (11) and 100/100 (10).

Genotypic identity

Hedrick's genotypic identity indicated that early and late collections were very dissimilar (Table 5). The most recent collections from The Netherlands and Poland had a high level of identity, and both were dissimilar to the United States collection. Early collections from The Netherlands and Poland were similar to each other and to the United States collection.

DISCUSSION

The observations indicate that two different types of *P. infestans* populations occur in the European collections examined: pre-A2 or "old" populations (with only the A1 mating type) and post-A2 or "new" populations (with both A1 and A2 mating types). The two types of populations are distinguished by unique allozyme alleles and genotypes which serve as indicators. This conclusion is supported by genetic data spanning the periods during which the changes took place for three different regions of Europe: covering periods ranging from 1951-89 for The Netherlands

Table 4. Genotype frequencies in *Phytophthora infestans* collections from the United States, Peru and Japan.

Gpi	*Pep*	Mating type	Collections		
			United States (1979-89)	Peru[a] (1984-86)	Japan (1988)
Old genotypes					
86/100	92/100	A1	0.72	0.97	0.33
86/100	100/100	A1	0.12	0.03	0.17
100/100	92/92	A1	0.03	0	0
100/100	92/100	A1	0.12	0	0
New genotypes					
100/100	100/100	A2	0	0	0.50
Sample size			58	34	30

[a]Data of Tooley *et al.* (1989).

Table 5. Genotypic identities (Hedrick, 1971) among collections from The Netherlands, Poland and the United States.

	Poland			The Netherlands			
	1985-87	1988	1989	1951-78	1980-82	1984-85	1989
Poland							
1988	0.73						
1989	0.05	0.60					
The Netherlands							
1951-78	0.97						
1980-82				0.37			
1984-85				0.11	0.69		
1989			0.82	0.12	0.65	0.99	
United States							
1979-89	0.98		0.12	0.97			0.07

1985-89 for Poland, and 1980-82 for the UK, and recent data for the British Isles are available from two additional sources (see below). The earliest samples from all three regions belonged to the old population type (Tables 2 and 3), as did two early isolates from Switzerland (for which, unfortunately, no recent isolates were obtained). Admittedly, a limited number of living cultures from the pre-A2 European populations were obtained, but the consistency with which old genotypes were found in all the available samples indicates that old population types were widespread and common.

Other regions of the world which contain the old population type include the United States and Peru (Table 4). Collections from the United States (isolated in 1979-89) and Peru (isolated in 1984-86) were made up entirely of representatives of the "old" population. Both of these regions contained only A1 mating types at the time of sampling, although there is a recent report of an A2 isolate in the United States (Deahl *et al.*, 1990).

The new population type is found in recent collections from The Netherlands, Poland (Tables 2 and 3), and the British Isles. The information for the British Isles comes from two sources. P.W. Tooley (unpublished data) found that 24 Irish isolates collected in 1989 belonged to the new population type; their genotypes were *Gpi* 90/100 or 100/100 and *Pep* 83/100 or 100/100. Shattock *et al.* (1990) also document the disappearance of the old genotype *Gpi* 86/100 in British collections from 1987 and 1988. Unfortunately, the assay conditions they used did not discriminate between *Gpi* 90 and 100, or between *Pep* 83 and 92, so the exact composition of these samples is not known.

Although the Japanese sample does not reflect the real incidence of Al and A2 strains in Japan, it does provide a preliminary picture of the genotypic composition of *P. infestans* there at the time of sampling. The Japanese sample appears to contain members of both old and new population types, although none of the new-type isolates carried the *Gpi* 90 and *Pep* 83 alleles. These indicator alleles may have actually been present but were not detected in the small sample obtained, or there could be a real difference between the situation in Japan and that in Europe. Additional genetic markers would be useful in resolving this uncertainty, as well as in further explorations of our conclusions; especially useful would be the kind of information offered by DNA polymorphisms. The genotypic identity values in Table 5 indicate that collections from different countries containing new genotypes are closely related to each other (identity values close to one), and are distantly related (identity values close to zero) to collections containing old genotypes. These relationships could be tested further by characterizing isolates for an array of random DNA restriction fragment length polymorphism and such a study is now underway.

It is noteworthy that the patterns of genotypic distribution found in Europe are not at all typical of samples from the Toluca region of central Mexico, which historically has contained both mating types, and is believed to be within the center of origin of the pathosystem. Diversity is much higher in the Toluca region, and the distribution of genotypes and mating types appears to be the result of random mating (Tooley *et al.*, 1985; Spielman, 1991; J.M. Matuszak, unpublished data). This contrasts with the European collections, in which the genotypes found are consistent with clonal reproduction. The failure to find recombinants between the new and

Table 6. Virulence complexity of *Phytophthora infestans* isolates from The Netherlands in 1970, 1971 and 1989.

Year of collection	Sample number	Number of pathotypes	Maximum number of virulence factors	Source
1989	39	20	7	A.Drenth, unpublished data
1971	20	4	3	Mooi, 1971
1970	13	4	3	Mooi, 1971
1970+1971	33	4	3	Mooi, 1971

old genotypes, and the absence of many potential genotypic classes, are strong indicators of clonal structure.

These findings lead to the conclusion that large parts of the range of *P. infestans* outside Mexico may have been dominated by the old population type before the 1970s. The association between the presence of both mating types and new allozyme alleles indicates that the appearance of A2 mating types outside central Mexico was due to migration, and that along with both A1 and A2 mating types, the migrating population carried characteristic genetic markers. Mexico is the most likely source of the new population, since historically it has harboured both mating types, and the *Gpi* 90 and *Pep* 83 alleles occur there in low to moderate frequencies (Spielman, 1991; J.M. Matuszak, unpublished data).

Neither of the two other hypotheses described earlier explains our findings as well as migration. Mutation from one or more A1 strains would have been unlikely to produce the array of distinctive traits associated with the new population type. The possibility of A2 strains existing in low numbers for many years and then expanding to produce the present situation is equally untenable. This would have required a change in relative fitness, either through mutation or through a change in conditions. We rule out mutation to higher fitness for the reason given above. The only change in conditions which seems relevant is the use of the fungicide metalaxyl in the early 1980s. If a rare A2 strain (which could have carried several unique markers) also carried metalaxyl resistance, it would have had a selective advantage in the presence of the fungicide. However, if this were the case, metalaxyl resistance would have been restricted to A2 mating types until sexual recombination distributed it to A1 strains, and there was no such association between the two traits in the collections examined (A. Drenth, L.J. Spielman & L.J. Sujkowski, unpublished data). If several metalaxyl resistant strains were acted upon in this manner, it would be unlikely that none carried the most common allozyme genotype present in the sensitive population.

Four *Gpi* genotypes were expected from the cross between isolates 1115 and 618 (86/90, 86/100, 90/122 and 100/122), but only three of these appeared. In addition, five other genotypes were found, four of which (86/86, 86/122, 90/100 and 100/100) could have been the result of self mating. The fifth (86/100/122) designates a five-banded phenotype which appears to be due

to the superimposed patterns of three alleles. This genotype could have been produced by a combination of hybridization and non-disjunction. Such patterns have occasionally been observed in other crosses and in field isolates (Spielman, 1991). The allelic nature of *Pep* 83 has been determined in a previous cross (Spielman *et al.*, 1990) and is supported by these results. The irregularities associated with *Gpi* genotypes in this cross may be due to the fact that the parents had different relative DNA contents and therefore probably were of different ploidy (W. Gu, unpublished data). Nevertheless, the occurrence of progeny with three of the four expected genotypes demonstrate that *Gpi* 90 is allelic with *Gpi* 100 and 122 and therefore can be assigned to the same locus. The absence of progeny with the *Gpi* 86/90 genotype may have been due to the small number of progeny obtained, or to lower fitness associated with that genotype.

Preliminary data also suggest that individuals in the new population have more virulence factors (specific genetic determinants which allow the fungus to overcome specific resistance genes in the host). Table 6 presents the virulence phenotypes of isolates from 1970, 1971 and 1989 in The Netherlands (Mooi, 1971; A. Drenth, unpublished data). In 1970 and 1971, no isolate had more than three virulence factors, and there were only four different combinations, all of which contained virulence factors which matched those in commercial cultivars (Mooi, 1971). In contrast, analysis of 39 isolates collected in 1989 (using the same set of host differentials carrying single resistance genes) indicated that some isolates carried as many as seven specific virulence factors, in 20 different unique combinations (pathotypes) (A. Drenth, unpublished data). The specific resistances common in Dutch cultivars have been the same (R1, R3, R4 and R10) for the last 20 years. A similar picture emerges from two studies of *P. infestans* isolates from western Germany. Schöber (1983) reports that the maximum number of virulence factors per isolate remained at four between 1972 and 1980, but rose to eight in 1982. The number of different pathotypes went from seven to eleven over the same period. In 1987, isolates were found which carried nine virulence factors, and a sample of 49 yielded 22 different pathotypes (Rullich & Schöber, 1988). Again, the same set of differentials was used throughout these studies, although sample sizes were not given for the earlier years.

In addition, it appears that the new genotypes are fitter than the old in Europe, since they rapidly increased in frequency after introduction. However, a mechanism explaining the greater fitness is currently unknown and should be investigated. In this regard, the situation we describe for *P. infestans* resembles that for *Ophiostoma ulmi*, the cause of Dutch Elm Disease, in the United Kingdom and western Europe, where aggressive strains are displacing the non-aggressive strain (Brasier, 1987). In some locations, the non-aggressive strain is no longer detectable and may be headed for extinction. In other cases, changes in host plant populations can be linked to changes in plant-pathogen populations. For example, major changes in the genetic composition of populations of wheat rust (*Puccinia graminus* f. sp. *tritici*), detected through analysis of allozyme and virulence genotypes, appear to be associated with widespread adoption of new host cultivars carrying different resistance genes (Burdon *et al.*, 1982). However, there has been no obvious change in the potato and tomato host populations during the hypothesized displacement of the resident European population of *P. infestans*, so we believe that directional selection in

a single resident population is not the explanation for the phenomenon observed.

The detection of a new, and probably more fit, population of *P. infestans* raises the possibility of an increased threat to world-wide agriculture. In order to reduce this threat, the geographical distribution of the new population on a world-wide basis needs to be determined; the possible effects on agriculture need to be assessed; and strategies for suppressing late blight of potato and tomato need to be re-evaluated. The population displacement phenomenon also presents a unique opportunity for the study of micro-evolution in process.

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REFERENCES

Brasier, C.M., 1987. Recent genetic changes in the *Ophiostoma ulmi* population: the threat to the future of the elm. p 213-226 in: M.S. Wolfe, M.S. & Caten, C.E. (eds.). Populations of Plant Pathogens: Their Dynamics and Genetics. Blackwell Scientific Publications, Oxford. pp 280.

Burdon, J.J., Marshall, D.R., Luig, N.H. & Gow, D.J.S., 1982. Isozyme studies on the origin and evolution of *Puccinia graminis* f. sp. *tritici* in Australia. Australian Journal Biological Sciences 35: 231-238.

Deahl, K.L., Goth, R.W., Young, R., Sinden, S.L. & Gallegly, M.E., 1990. Occurrence of the A2 mating type of *Phytophthora infestans* in potato fields in the United States and Canada. Phytopathology 80: 670.

Fry, W.E., Drenth, A., Spielman, L.J., Mantel, B.C., Davidse, L.C. & Goodwin, S.B., 1991. Population genetic structure of *Phytophthora infestans* in The Netherlands. Phytopathology 81: 1330-1336.

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

Hedrick, P.W., 1971. A new approach to measuring genetic similarity. Evolution 25: 276-280.

Hohl, H.R. & Iselin, K., 1984. Strains of *Phytophthora infestans* from Switzerland with A2 mating type behaviour. Transactions of the British Mycological Society 83: 529-530.

Hooker, W.J., 1981. (ed.) Compendium of Potato Diseases. American Phytopathological Society, St. Paul, MN. pp 123.

Large, E.C., 1940. The Advance of the Fungi. Jonathan Cape Ltd. London. pp 488.

Malcolmson, J.F., 1985. *Phytophthora infestans* A2 compatibility type recorded in Great Britain. Transactions of the British Mycological Society 85: 531.

Mooi, J.C., 1971. Het optreden van fysio's van *Phytophthora infestans* in de Groninger Veenkolonien en omgeven gebied in 1970 en 1971. Resultaten proefvelden fabrieksaardappelen 1971: 62-65.

Mosa, A.A., Kato, M., Sato, N., Kobayashi, K. & Ogoshi, A., 1989. Occurrence of the A2 mating type of *Phytophthora infestans* on potato in Japan. Annals of the Phytopathological Society of Japan 55: 615-620.

Rullich, G. & Schöber, B., 1988. Auftreten und Verbreitung der A2 Paarungstyps von *Phytophthora infestans* (Mont.) de Bary in der Bundesrepublik Deutschland. Kartoffelbau 39: 244-246.

Schöber, B., 1983. Definition und Auftreten der Pathotypen von *Phytophthora infestans* (Mont.) de Bary. Kartoffelbau 34: 156-158.

Shattock, R.C., Shaw, D.S., Fyfe, A.M., Dunn, J.R., Loney, K.H. & Shattock, J.A., 1990. Phenotypes of *Phytophthora infestans* collected in England and Wales from 1985 to 1988: mating type, response to metalaxyl and isoenzyme analysis. Plant Pathology 39: 242-248.

Shaw, D.S., 1983. The Peronosporales. A fungal geneticist's nightmare. p 85-121 in: Buczacki, S.T. (eds.). Oosporic plant pathogens a modern perspective. Academic Press, London. pp 352.

Spielman, L.J., 1991. Isozymes and the population genetics of *Phytophthora infestans*. p 231-241 in: Lucas, J.A., Shattock, R.C., Shaw, D.S. & Cooke, L.R. (eds.). *Phytophthora*. British Mycological Society, Cambridge University Press, Cambridge. pp 447.

Spielman, L.J., McMaster, B.J. & Fry, W.E., 1989. Dominance and recessiveness at loci for virulence against potato and tomato in *Phytophthora infestans*. Theoretical and Applied Genetics 77: 832-838.

Spielman, L.J., Sweigard, J.A., Shattock, R.C. & Fry, W.E., 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.

Stevens, N.E., 1933. The dark ages in plant pathology in America: 1830-1870. Journal of the Washington Academy of Sciences 23: 435-446.

Tantius, P.H., Fyfe, A.M., Shaw, D.S. & Shattock, R.C., 1986. Occurrence of the A2 mating type and self-fertile isolates of *Phytophthora infestans* in England and Wales. Plant Pathology 35: 578-581.

Tooley, P.W., Fry, W.E. & Villarreal Gonzalez, M.J., 1985. Isozyme characterization of sexual and asexual *Phytophthora infestans* populations. Journal of Heredity 76: 431-435.

Tooley, P.W., Therrien, C.D. & Ritch, D.L., 1989. Mating type, race composition, nuclear DNA content, and isozyme analysis of Peruvian isolates of *Phytophthora infestans*. Phytopathology 79: 478-481.

Chapter 4

Cloning and genetic analyses of two highly polymorphic, moderately repetitive nuclear DNAs from *Phytophthora infestans*

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ABSTRACT

Randomly selected clones from a *Phytophthora infestans* partial genomic library were characterized by hybridizing individual clones to Southern blots of total genomic DNA digested with the restriction enzyme *Eco*RI. Among 59 clones that were screened on seven different central-Mexican isolates, five revealed a unique fragment pattern for each isolate tested. Two of these clones were tested further; the fragment patterns produced by both were somatically stable when probed to DNA from 63 single-zoospore (asexual) progeny from five different "parent" isolates. For one probe, RG-57, each fragment appeared to represent a unique genetic locus in three different crosses, and each locus segregated for the presence or absence of a fragment. No fragments were found to be allelic, but two pairs of co-segregating loci were identified. Genetic analyses of the other probe (RG-7) revealed many more pairs of cosegregating fragments and some fragments which were allelic. When these probes were hybridized to DNA from the other five species in *Phytophthora* group IV, probe RG-57 hybridized strongly to DNA from *P. colocasiae*, *P. phaseoli* and *P. mirabilis*, but weakly or not at all to that of *P. hibernalis* and *P. ilicis*. Probe RG-7 hybridized fairly strongly to DNA from all six species. Because the sequence recognized by probe RG-57 appears to be evolutionarily conserved, and is dispersed, moderately repetitive and highly polymorphic, it could be very useful in additional studies on the genetics and population biology of *P. infestans*.

INTRODUCTION

Phytophthora infestans (Mont.) de Bary, the causal organism of the late blight disease of potato and tomato, is the most important worldwide factor limiting the production of potatoes in the absence of fungicides (Hooker, 1981). In spite of the great cost to agriculture imposed by this disease, surprisingly little is known about the genetics and population biology of *P. infestans*. In the past, progress on its genetics was constrained by the inability to germinate sufficient numbers of oospores for meaningful analyses, and by a paucity of well-characterized genetic markers. The problem of oospore germination has been largely overcome (Shattock *et al.*, 1986), so that the primary limiting factor is now a lack of suitable markers. Although a number of markers are potentially available, few have been studied in detail (reviewed in Shaw, 1991). Most progress has come from two allozyme loci, glucose phosphate isomerase and peptidase (reviewed in Spielman, 1991). However, the limited number of loci and the low level of variability limits the usefulness of allozymes, and few other markers are available (Shaw, 1991). Thus, there is a pressing need for additional unambiguous, easily scored genetic markers, if progress in our understanding of the genetics and population biology of *P. infestans* is to continue.

In recent years, the development of restriction fragment length polymorphisms (RFLPs) has considerably advanced the study of genetics, and complete linkage maps are being developed

based on RFLP markers. In addition, highly polymorphic, moderately repetitive probes have been developed that provide DNA fingerprints in mammals (Jeffreys *et al.*, 1985b; Jeffreys & Morton, 1987), birds (Wetton *et al.*, 1987; Burke & Bruford, 1987), plants (Dallas, 1988) and some fungi (Scherer & Stevens, 1988; Hamer *et al.*, 1989). Such probes would be particularly useful for elucidating the population biology and genetics of genetically difficult organisms such as *P. infestans*. Because this organism can reproduce both sexually and asexually, it is particularly desirable to have markers that can distinguish sexual from asexual progeny, and that can be used to identify various clonal lineages in areas where the fungus relies primarily on asexual reproduction. In this chapter we report the cloning and genetic characterization of two highly polymorphic, moderately repetitive nuclear DNAs from *P. infestans*.

MATERIALS AND METHODS

Fungal isolates

Seven *P. infestans* isolates, 510, 543, 562, 568, 575, 580 and 618, were used for screening probes. All were originally isolated in central Mexico and have been successful as parents in genetic crosses. These isolates were chosen in part because they represent much of the diversity found in central Mexico for allozyme and virulence markers. The isolates were grown on Rye A agar (Caten & Jinks, 1968) at 18°C. Replicate cultures of all isolates were stored under oil and cryogenically at -135°C.

Isolates for genetic analyses were 30 progeny from cross 64 (isolate 568 × isolate 575), and 35 progeny from cross 68 (isolate 580 × isolate 618), kindly supplied by L.J. Spielman. An additional 62 progeny were obtained from a cross between Dutch isolates 80029 and 88133 (cross 71). Single-zoospore (uninucleate) isolates were made from five different "parent" isolates as described in Caten & Jinks (1968), except that the zoospores were initially plated onto water agar rather than Rye B medium. Five isolates, 662, 663, 1176, 1177 and 1178, were chosen for single-zoospore analysis because they were believed to be of elevated or odd ploidy levels, and therefore the most likely to be unstable. Most of the single-zoospore progeny came from two isolates, 662 and 663, from northwestern Mexico (near Los Mochis). Isolate 662 was included because it has a ploidy level between diploid and triploid (W. Gu, personal communication), and has a three-allele, five-banded phenotype for glucose phosphate isomerase (*Gpi*) that is probably due to trisomy for the chromosome containing the *Gpi* locus. Isolate 663 is probably a diploid (W. Gu, personal communication). Isolates 1176, 1177 and 1178 were isolated in Poland by L.S. Sujkowski. Two of these, 1176 and 1177, are probably triploids or aneuploids (C.D. Therrien, personal communication). The ploidy level of isolate 1178 has not been determined.

To examine the evolutionary conservation of the repetitive DNAs, isolates of the other five species in Waterhouse's (1963) *Phytophthora* group IV (the group to which *P. infestans* belongs) were also studied. Isolates of *P. mirabilis* (n=5), *P. colocasiae* (n=2), *P. ilicis* (n=4) and *P. hibernalis* (n=2) were kindly supplied by M.D. Coffey from the *Phytophthora* culture

collection at the University of California, Riverside. One isolate of *P. phaseoli* was supplied by P.W. Tooley, Plant Disease Laboratory, USDA-ARS, Fort Detrick, MD.

DNA manipulations

Tissue was grown in 20 ml of V-8 broth (100 ml V-8 juice, 1 g $CaCO_3$ and 0.05 g β-sitosterol per liter) or pea broth (filtrate from 120 g autoclaved frozen peas per liter) in 9 cm disposable Petri dishes at 18°C. Each Petri dish was inoculated with several small mycelial plugs cut from the border of an actively growing colony and incubated for 1-3 weeks without shaking. Mycelia from two to three broth plates were placed on 9 cm Whatman No. 1 filter paper discs in a Büchner funnel and the extra broth removed by vacuum filtration. Following the removal of large pieces of agar, the partially dried mycelia were frozen at -80°C and lyophilized overnight.

For DNA extractions the lyophilized tissue was ground in liquid nitrogen, suspended in 4 ml of prewarmed (65°C) extraction buffer (50 mM Tris, pH 8.0; 150 mM EDTA; 1% sarkosyl) by vortexing for 30-60 s, and incubated 20 min at 65°C. Subsequently, 4 ml of cold 5 M NH_4OAc were added to each tube, mixed by inversion, placed on ice for 20 min, and centrifuged for 10 min at 8240 g. The supernatant was transferred to a fresh tube, precipitated with a two-thirds volume (about 5 ml) of isopropanol, and centrifuged for 5 minutes at 8240 g. The pellets were dried, and resuspended in 0.5 ml of TE (10 mM Tris, pH 8.0; 1 mM EDTA). RNase A was added to a final concentration of 0.1 mg/ml, the tubes were incubated for 20 min at 37°C, then extracted twice with 25:24:1 phenol:chloroform:isoamyl alcohol. The DNA was precipitated with a one-tenth volume of 3 M NaOAc and two volumes of absolute ethanol. The tubes were centrifuged for 2 min to collect the DNA, the pellets were dried, washed in 70% ethanol, and resuspended in 100-300 μl of TE.

Larger quantities of highly purified DNA were obtained using a modification of the above method. DNA was prepared from tissue grown in 40 broth-plates as described above. Following the isopropanol precipitation, the DNA was resuspended in TE and CsCl was added to give a buoyant density of 1.57 g/ml. Mitochondrial and nuclear DNAs were separated using bisbenzimide as described by Garber & Yoder (1983).

Restriction enzymes were purchased from various sources and used according to the manufacturer's directions, except that spermidine was added to each reaction buffer to a final concentration of 4 mM. Gel electrophoresis in 0.9% agarose gels, alkaline blotting to nylon membranes, hybridization with ^{32}P random-primed probes (Feinberg & Vogelstein, 1983), and autoradiography, were all according to standard protocols (Maniatis *et al.*, 1982; Ausubel *et al.*, 1987). Two μg of DNA per lane was generally sufficient to obtain good exposures in 1-8 days. All blots were hybridized at 65°C and washed three times for 10 min each in: 2 × SSC, 0.1% SDS; 1 × SSC, 0.05% SDS; and 0.5 × SSC, 0.025% SDS. Following autoradiography, the blots were stripped by washing at 42°C for 10 min each in: 0.1 N NaOH; 100 mM Tris, pH 7.7, 0.1 × SSC, 0.1% SDS; and 0.1 × SSC, 0.1% SDS.

Densitometry was performed with an LKB Ultroscan XL Laser Densitometer using a 633 nm helium-neon laser. Integrations and other manipulations of the scanned data were done with

the Gelscan XL laser densitometer software package.

A *P. infestans* partial genomic library was made from isolate 627 (obtained near Saltillo in northeastern Mexico) according to standard protocols (Maniatis *et al.*, 1982; Ausubel *et al.*, 1987). Ten μg of CsCl-purified nuclear DNA were digested to completion with *Eco*RI, and size fractionated on a 0.9% agarose gel. Fragments from 0.5 to 2.5 kb were excised from the gel and purified with GeneClean (Bio 101 Inc., La Jolla, Calif.) before being ligated into Blue Script (Stratagene, San Diego, Calif.) and transformed into *E. coli* strain DH5α. Approximately 150 bacterial colonies were selected at random for analysis and plasmid DNA was prepared using the alkaline lysis protocol of Ausubel *et al.* (1987). Insert sizes ranged from 0.8 to 2.4 kb. Approximately 100 ng of DNA from each plasmid was labeled with ^{32}P using the random primer method (Feinberg & Vogelstein, 1983) and hybridized to blots containing 4 μg per lane of CsCl-purified DNA from each of the seven different central-Mexican *P. infestans* isolates digested with *Eco*RI.

RESULTS

Characterization of cloned DNAs

The hybridization patterns produced by 59 genomic clones were divided into three classes: (1) those with a broad indistinct smear of hybridization with or without distinct fragments, or those that had more than 20 fragments, were classified as highly repetitive (Fig. 1A, B); (2) those with from five to 20 fragments were considered moderately repetitive (Fig. 1C, D); while (3) hybridization patterns with only one to four fragments were classified as single- or low-copy (Fig. 1E, F). Hybridization patterns that had from one to four heavy fragments with additional faint fragments, were also considered to be low-copy. Among all probes tested, 35 (59%) gave moderately or highly repetitive fragment patterns (Table 1). Single-copy DNAs made up the largest single class when those with additional faint fragments were included. These faint fragments were both larger and smaller than the heavy fragments and so could not be due to partial digestion. Over 50% of all probes tested revealed polymorphic fragments (Table 1). All of the moderately repetitive DNAs and about one-half of the single-copy DNAs were polymorphic; most of the single-copy polymorphisms were due to faintly hybridizing fragments.

Five probes gave unique hybridization patterns for each of the seven isolates tested. The two probes RG-7 and RG-57, that revealed the largest numbers of highly polymorphic fragments, were selected for use as potential DNA fingerprinting probes. Two additional probes gave the same fragment pattern as RG-7. Probe RG-7 was 1.6 kb and probe RG-57 was 1.2 kb. A total of 24 fragments was identified for probe RG-7, and 25 fragments for RG-57. Individual isolates had from nine to 15 fragments for probe RG-7 and from 11 to 16 for RG-57. The sizes of the fragments ranged from 1.6 to 18 kb for probe RG-7 and from 1.2 to 18 kb for RG-57. A highly polymorphic, moderately repetitive fragment pattern was also produced when total genomic DNA of four isolates (568, 575, 580, 618) was digested with the restriction enzymes

*Pst*I, *Hin*dIII, *Dra*I and *Hae*III, blotted and hybridized with probe RG-57 as described above. However, the best resolution of all fragments was obtained with the *Eco*RI digests.

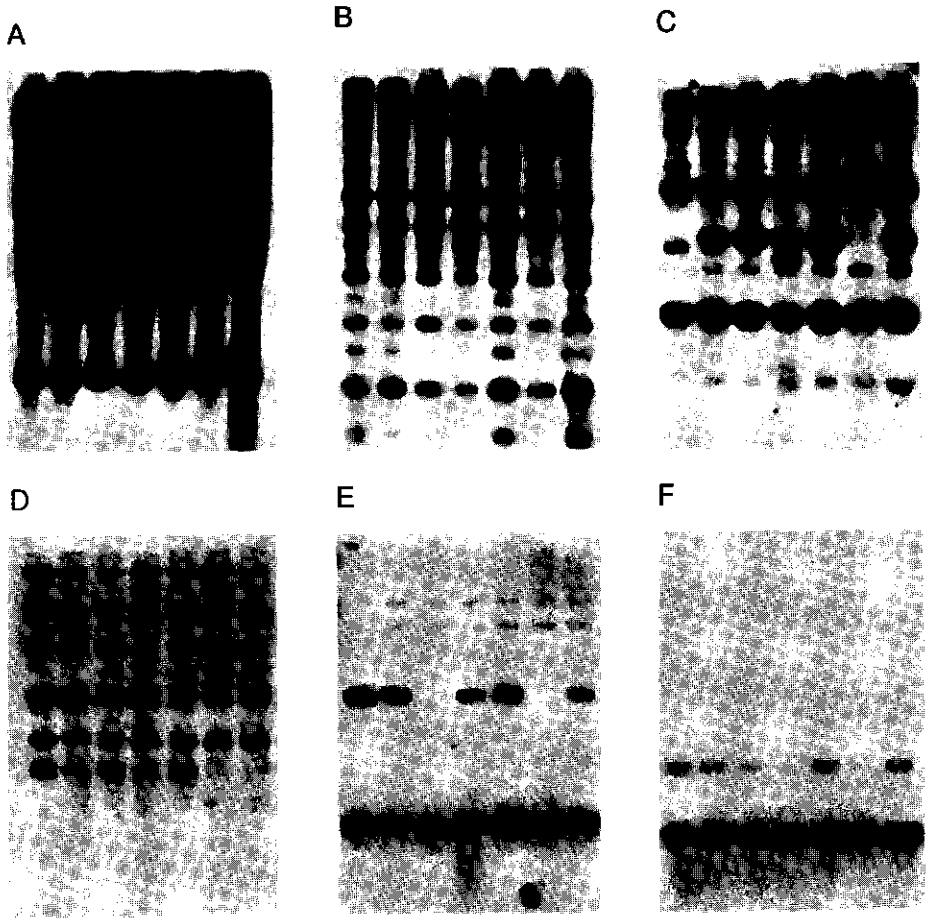

Figure 1. Typical hybridization patterns: **A, B** highly repetitive DNA; **C, D** moderately repetitive DNA; **E,F** single-copy DNA. Each lane contains 4 μg of CsCl-purified genomic DNA from one of seven different central-Mexican *P. infestans* isolates (from left to right): 510, 543, 562, 568, 575, 580, 618.

Table 1. The proportion of clones that gave each type of hybridization pattern when hybridized to total digested DNA of the seven central-Mexican *P. infestans* isolates 510, 543, 562, 568, 575, 580, 618 and the percent of fragment patterns that were polymorphic.

Hybridization pattern	No. of probes	Percent of all probes	Percent polymorphic
Highly repetitive	18	30	17
Moderately repetitive	17	29	100
Single-copy	24	41	50

Somatic stability of repetitive DNAs

To verify the somatic stability of the fragment patterns produced by the two DNA fingerprinting probes, individual nuclei were isolated via single zoospores into known homokaryotic isolates. Sixty-three single-zoospore isolates were tested from the five different "parent" isolates. The number of single-zoospore progeny from each "parent" ranged from five to 25. In all cases the patterns of the single-zoospore progeny were identical to their "parent" (Fig. 2); thus, the coenocytic mycelium of each "parent" isolate was homokaryotic, and the DNA fragment patterns produced by both probes were mitotically stable. Seventeen out of the 25 fragments produced by probe RG-57, and 14 of the 24 fragments for RG-7, were tested in this analysis.

Genetic analyses of repetitive DNAs

Based on previous genetic analyses using DNA fingerprinting probes (Jeffreys *et al.*, 1985a, 1986; Jeffreys & Morton, 1987), it was expected that most fragments would represent heterozygotes, and would segregate 1:1 in a cross in which one parent had the fragment and the second parent did not. However, this was not always true; sometimes all of the progeny received a fragment that one of the parents lacked. Fragments that were transmitted to all of the progeny were approximately twice as intense as those that were only transmitted to half the progeny (e.g., Fig. 3A, fragments 3 and 7). Thus, the original hypothesis that most fragments would be heterozygous was incorrect, and the more intensely hybridizing fragments were in fact homozygous: it was possible to determine the genotype of each isolate at each locus by visual inspection of the autoradiograms. The results of the genetic analyses of probe RG-57 in crosses 64, 68 and 71 are shown in Table 2. All of the fragments in the progeny were present in at least one of the parents, and all parental fragments were transmitted to at least some of the progeny. No mutations were observed in the 127 progeny from the three crosses. In all cases, the progeny appeared to segregate for the presence or absence of the fragment, and each fragment appeared to represent a unique genetic locus. Three types of segregation were noted for the fragments produced by probe RG-57: (1) when one parent was homozygous for a fragment that the other parent lacked, all of the progeny inherited the fragment; (2) when one parent was heterozygous for a fragment that the other parent lacked, about 50% of the progeny inherited the fragment;

and (3) when both parents were heterozygous for a fragment, about three-fourths of the progeny inherited the fragment. In all cases, progeny segregations were consistent with what was expected based on the fragment intensities in the parents. For example, fragment 5 in cross 68 gave eight progeny that appeared to be homozygous based on fragment intensities, with an 8:17:8 ratio for homozygous +/+:heterozygous +/—:homozygous —/— (1:2:1 expected).

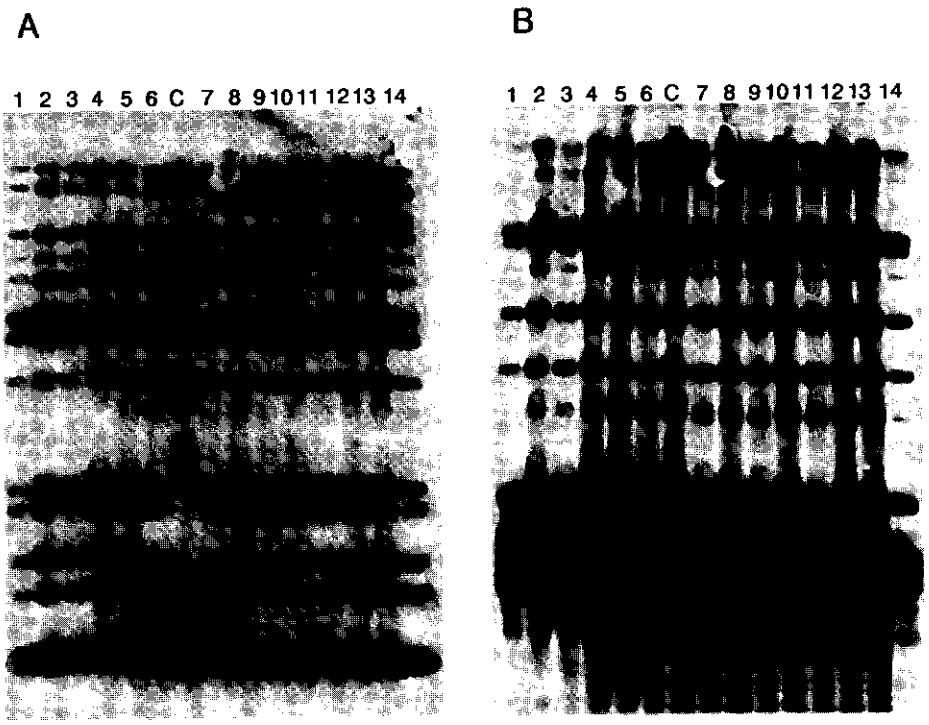

Figure 2. Somatic stability of moderately repetitive DNA fragment patterns. **A** DNA from 14 single-zoospore "progeny" from "parent" isolate 663 probed with RG-57; **B** the same blot probed with RG-7. Lane C is a control isolate (isolate 575).

Two fragments in cross 68 had segregation patterns that differed from what was expected (Table 2). There was a slight deficiency of transmission for fragment 23, and fragment 10 segregated 34:1 (presence:absence) when a 3:1 ratio was expected. With the possible exception of fragments 23 and 21, no fragments were allelic. In cross 68, one parent (isolate 580) appeared heterozygous for fragments 21 and 23, while the second parent (isolate 618), was heterozygous for fragment 21 only; all eight progeny that lacked fragment 21 inherited fragment 23, and two progeny inherited both fragments.

Table 2. Fragments identified by probe RG-57 that segregated in the progeny of crosses 64, 68 and 71.

Fragment	Cross 64				Cross 68				Cross 71			
	Parents		Ratio[a]	χ^2	Parents		Ratio[a]	χ^2	Parents		Ratio[a]	χ^2
	568	575			580	618			80029	88133		
23	–	+	16:10[b] (1:1)[c]	1.38	+	–	10:21 (1:1)	3.90**				
22									+	–	58:0 (1:0)	0.00
21					+	+	23:8 (3:1)	0.01	–	+	58:0 (1:0)	0.00
20	+	–	26:0 (1:0)	0.00								
19	–	+	7:14 (1:1)	2.33					–	+	34:24 (1:1)	1.72
18									–	+	36:22 (1:1)	3.38
16	–	+	12:14 (1:1)	0.15					+	–	39:19 (1:1)	6.90**
15[d]	+	–	14:14 (1:1)	0.00								
12	+	–	11:19 (1:1)	2.13								
10	–	+	30:0 (1:0)	0.00	+	+	34:1 (3:1)	9.15**				

8	+	—	15:15 (1:1)	0.00	+	—	14:16 (1:1)	0.13	—	+	31:27 (1:1)	0.28
7[e]					+	—	32:0 (1:0)	0.00	—	+	36:22 (1:1)	3.38
6[d]	+	—	16:14 (1:1)	0.13								
5	+	—	30:0 (1:0)	0.00	+	+	25:8 (3:1)	0.01				
3[e]					+	—	32:0 (1:0)	0.00	—	+	36:22 (1:1)	3.38
2									+	—	58:0 (1:0)	0.00
1									—	+	58:0 (1:0)	0.00

Note: due to slight degradation of some DNA samples and the high background on one blot, it was not possible to score all progeny for all fragments, thus there is some variation in the number of progeny scored for each fragment in crosses 64 and 68. The total number of hybrid progeny was 30 for cross 64, 35 for cross 68 and 58 for cross 71.

[a] Presence:absence of fragment (+:—).

[b] Observed.

[c] Expected based on fragment intensities.

[d] Fragments 6 and 15 cosegregated in all progeny tested, but two of the progeny that could be scored for fragment 6 could not be scored for fragment 15.

[e] Fragments 3 and 7 cosegregated in cross 71.

[**] Significantly different from the expected ratio, $P<0.05$.

These data are consistent with the hypothesis that fragments 23 and 21 represent different alleles at the same locus, with isolate 580 being a fragment 23/21 heterozygote, and isolate 618 a fragment 21/no fragment heterozygote. However, this result is also consistent with the hypothesis of two loci tightly linked in repulsion; larger progeny sizes and additional crosses will be required to resolve this issue.

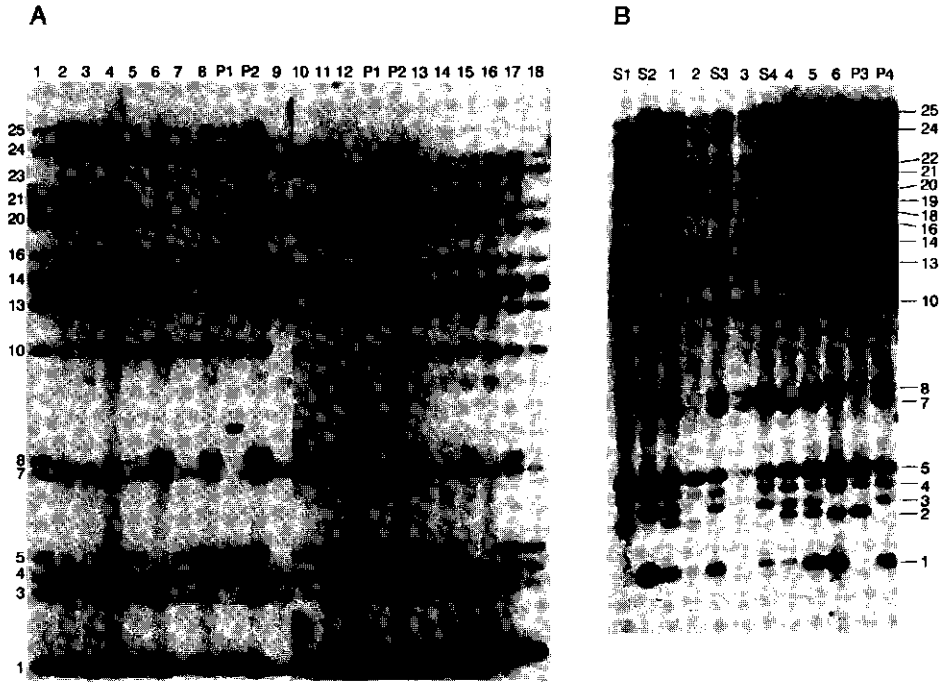

Figure 3. Genetic segregations of fragments revealed by probe RG-57. **A** 18 progeny from cross 68; **B** six hybrid progeny and four selfs or non-recombinant parental types from cross 71. *P1*, parent isolate 618; *P2*, parent isolate 580; *P3*, parent isolate 80029; *P4*, parent isolate 88133; *S1*, non-recombinant progeny or self of isolate 80029; *S3*, non-recombinant progeny or self from isolate 88133; *S2* and *S4*, two selfs of parent isolate 88133. All other lanes contain DNA from hybrid progeny. The *fragment numbers* indicated to the left and right correspond to those in the text and Table 2.

Isolate 568 was heterozygous for fragments 6 and 15, and both fragments cosegregated in cross 64 (no recombinants among 30 progeny). These fragments probably represent two loci that are closely linked in coupling. Although an alternative hypothesis is that these two fragments are part of the same phenotype at one locus due to an internal restriction site, this seems less likely because both fragments are found separately in other isolates (S.B. Goodwin, unpublished).

Table 3. Results of densitometry; areas under the curve for fragments 1-8 for representative isolates from cross 71. The relative intensities of the fragments vary from row to row depending on the amount of DNA loaded in each lane of the gel.

Isolate	Fragment						
	1	2	3	4	5	7	8
P3[a]	—	1.39	—	0.36	3.55	—	—
P4	1.47	—	0.71	0.60	2.41	2.02	2.40
5	2.26	0.72	0.75	0.42	3.27	1.84	2.21
S2	6.90	—	3.64	1.89	6.24	4.00	1.72

[a]These isolate designations correspond to those above the lanes in Fig. 3B.

Ten fragments were different between the parents of cross 71 (Table 2). Four of these were homozygous and segregated 1:0. The remaining six fragments were heterozygous and segregated approximately 1:1. There was an excess of transmission for all six fragments, but the ratios did not differ significantly from 1:1 except for fragment 16 from parent 80029 which segregated approximately 2:1. This fragment did show the expected 1:1 segregation in cross 64; the cause of the anomalous segregation for this fragment in cross 71 is unknown. Fragments 3 and 7 cosegregated in cross 71 (cotransmitted to 36 out of 58 hybrid progeny), and therefore probably represent two very tightly linked loci. All of the other loci appeared to be unlinked.

Two of the 62 progeny from cross 71 were clearly selfs (Fig. 3B, lanes S2 and S4), while two other progeny were either selfs or non-recombinant parental types (Fig. 3B, lanes S1 and S3). Both of the confirmed selfs were derived from parent isolate 88133 and show changes in fragment intensities corresponding to increased homozygosity. Isolate 88133 (P4 in Fig. 3B) was heterozygous for fragments 3, 7, 8, 18 and 19. Self S2 (Fig. 3B) went from heterozygous to homozygous for fragments 3 and 7, and this can be seen as changes in the intensities of these fragments (note particularly the relative intensities of fragments 7 and 8 in parent P4 and selfs S2 and S4). Self S4 became homozygous for fragments 8 and 18, and homozygous for the absence of fragment 19.

The differences in fragment intensities were quantified by densitometry, and the areas under the curve for a few representative isolates are given in Table 3. Fragment intensities varied, and some fragments were consistently darker than others, regardless of genotype. Fragments 2, 3 and particularly 4 were usually fainter than the others, even when homozygous. However, some patterns do emerge. Progeny isolate 5 was heterozygous for fragments 1, 2, 3, 4, 7 and 8 and the intensities of the fragments correspond to those that were expected based on the genotypes of the parents (Fig. 3B, Table 3). The change to increased homozygosity for fragments 1, 3 and 7 in self S2 relative to parent P4 is quantified

in Table 3. Fragments 7 and 8 have approximately equal areas where they are both heterozygous in parent P4 and progeny 5 (with fragment 7 consistently a little fainter), but in self S2 fragment 7 has more than twice the area of fragment 8. A similar pattern was observed for the other isolates in this cross and for those in the other crosses (data not shown).

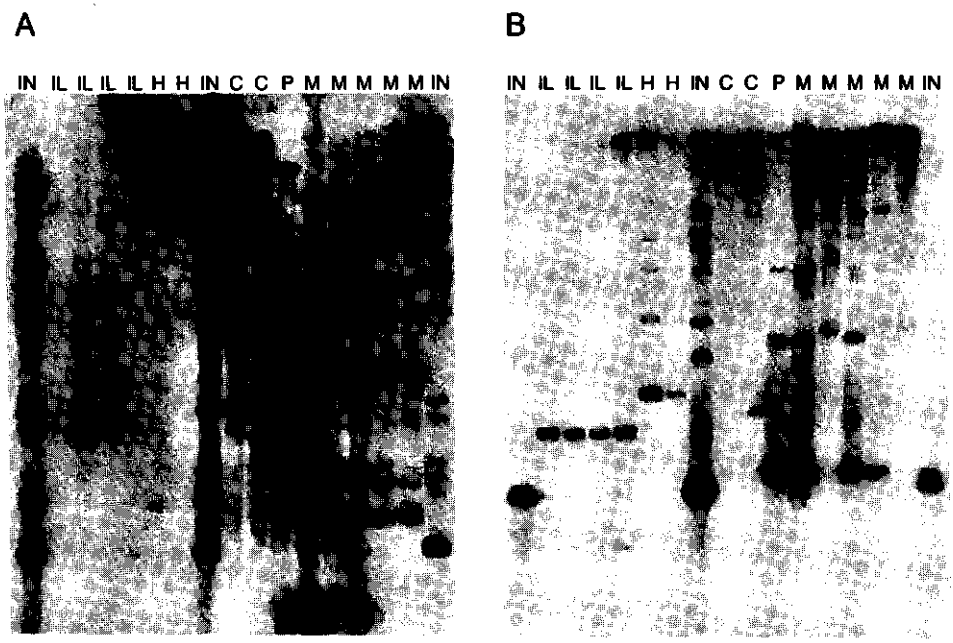

Figure 4. Evolutionary conservation of the moderately repetitive nuclear DNAs revealed by probes RG-57 and RG-7. **A** probe RG-57; **B** the same blot probed with RG-7. Approximately 4 μg of *Eco*R1 digested DNA were loaded in each lane. *IN, P. infestans; IL, P. Ilicis; H, P. hibernalis; C, P. colocasiae; P, P. phaseoli;* and *M, P. mirabilis*. Each lane contains DNA of a different field isolate. Isolate numbers (from left to right) are as follows: 580, P3939, P6098, P6099, P6100, P0647, P3822, 175, P1179, P1696, 330, P3008, P3005, P3007, P3009, P3010, 185.

For probe RG-7 the results were somewhat different. One of the parents of cross 64 had three fragments that the other parent did not have. All three fragments cosegregated and were transmitted to 12 out of 26 progeny. There were eight fragments for probe RG-7 that differed

between the parents of cross 68. Two fragments were homozygous in isolate 618 and were transmitted to all of the progeny. Two other fragments in isolate 618 cosegregated 15:21 (+:—) and appeared to be allelic to two other cosegregating fragments that segregated 21:15. Two fragments in isolate 580 were cotransmitted to 19 of the 36 progeny tested. Thus, for probe RG-7 there was a much higher incidence of cosegregating, allelic or closely linked fragments. Although this probe also revealed a highly polymorphic, easily scored fragment pattern, the large number of cosegregating fragments renders genetic interpretation of the fragment patterns of field isolates almost impossible, and limits the number of independent genotypes that can occur. Although this probe can still be useful for distinguishing clonal genotypes, its utility for analyzing the genetic structure of *P. infestans* populations is much more limited than that of probe RG-57.

Evolutionary conservation of repetitive DNAs

Probe RG-57 hybridized strongly to DNA from *P. infestans*, *P. mirabilis*, *P. colocasiae*, and *P. phaseoli*, but only faintly or not at all to that from most isolates of *P. ilicis* and *P. hibernalis* (Fig. 4A). Two isolates of *P. ilicis* did have two or three moderately hybridizing fragments. Each species seems to possess a unique set of fragments. This probe appeared to provide a useful polymorphic fingerprint for *P. colocasiae* (15 different fragments among two isolates tested) and *P. mirabilis* (12 fragments among five isolates tested). It may also be useful with *P. phaseoli* (five strongly hybridizing fragments), but the degree of polymorphism for these fragments could not be evaluated because only one isolate of this species was available for study. There were four different phenotypes among the five *P. mirabilis* isolates studied. Faintly hybridizing fragments were visible in some isolates of *P. ilicis* and *P. hibernalis*; therefore, it is likely that these species have different families of related repetitive sequences.

Probe RG-7 gives one or two strongly hybridizing, monomorphic fragments in *P. infestans*, with a number of fainter, polymorphic fragments, depending on exposure (Fig. 2B and 4B). Each of the other species had a unique set of fragments for probe RG-7 (Fig. 4B). The pattern produced by this probe on DNA from *P. phaseoli* and three of the five *P. mirabilis* isolates was similar to that for *P. infestans*; one intensely hybridizing fragment with a number of fainter polymorphic fragments. Two of the *P. mirabilis* isolates, and both representatives of *P. colocasiae*, had a number of faintly hybridizing high molecular weight fragments, without a single intensely hybridizing fragment. All fragments in *P. ilicis* and *P. hibernalis* were monomorphic, and hybridized less intensely than did those in *P. infestans*, *P. mirabilis* or *P. phaseoli*. Analysis of the ethidium bromide-stained gels, and probing the same blots with mitochondrial and ribosomal DNA probes (data not shown), revealed that all DNA samples were digested to completion. Therefore, the faintly hybridizing fragments are due to differences in sequence similarity and not to partial digestion. The rDNA probe revealed a monomorphic fragment (approximately 11 kb) in *P. infestans*. This fragment did not correspond to any of those produced by probes RG-7 and RG-57, so neither of these clones contains ribosomal DNA.

DISCUSSION

To be useful for DNA fingerprinting, a cloned DNA sequence should reveal fragments at a large number of highly polymorphic, unlinked genetic loci. For a fungus, it is also important to show that the fragment patterns are stable through asexual reproduction. These conditions have been met for *P. infestans* probe RG-57. However, the large number of cosegregating fragments for probe RG-7 impedes accurate genetic interpretation of the fragment patterns in field isolates, and this seriously limits the utility of this probe except to identify different clonal lines. These results emphasize the need for thorough genetic analyses of moderately repetitive DNA fragment patterns before they can be used as "DNA fingerprints".

The mitotic and meiotic stability of the fragments revealed by the *P. infestans* probes RG-7 and RG-57 differs from the variable number of tandem repeats (VNTR) loci (Nakamura *et al.*, 1987) that have been so useful for DNA fingerprinting in other organisms, and in which spontaneous mutations to new length alleles are observed fairly often (Jeffreys *et al.*, 1988; Westneat, 1990). An additional difference with the VNTR loci is that the loci identified by probes RG-7 and RG-57 are not "hypervariable". There are only two alleles at each locus, based on the presence or absence of the variable sequence. For probes RG-7 and RG-57, all of the fragments are retained on the gel and are clearly resolved, so it is unlikely that they are segregating with unknown or unresolvable fragments. This contrasts to other DNA fingerprinting probes, where allelic pairs are common and, when not identified, are assumed to be in a large complex of unresolvable smaller fragments (Jeffreys *et al.*, 1986). The genetic behavior of the moderately repetitive DNA detected by probe RG-57 is similar to that of the MGR sequence in *Magnaporthe grisea* (Hamer *et al.*, 1989; Hamer & Givan, 1990).

A large proportion of the *P. infestans* genome may be repetitive DNA; almost 60% of the genomic probes tested in this study contained repetitive sequences, and almost a third revealed highly repetitive fragment patterns. This observation is consistent with the repetitive DNA contents reported for other oomycetes. For example, using C_0t curve analyses, the proportion of repetitive DNA in *P. megasperma* has been estimated to be about 50% (B.M. Tyler & Y. Mao, personal communication), while that for *Bremia lactucae* was 65% (Francis *et al.*, 1990). The highly repetitive DNAs occur so many times throughout the genome that a smear of hybridization is produced by Southern analysis of total digested DNA. Initially, these patterns looked like they might be caused by degraded DNA. However, testing the same blots with other probes revealed sharp fragments, proving that the DNA samples were not degraded. None of the clones contained ribosomal DNA, and although these probes were not tested for cross hybridization, because each gave a unique hybridization pattern, there appear to be many different series of highly repetitive sequences that occur throughout the *P. infestans* genome.

The evolutionary conservation of the *P. infestans* repetitive probe RG-57 is intermediate between that for Jeffrey's probe, which hybridizes to most eukaryotic genomes including mammals (Jeffreys *et al.*, 1985b; Jeffreys & Morton, 1987), birds (Wetton *et al.*,

1987; Burke & Bruford, 1987), plants (Dallas, 1988) and even some fungi (Braithwaite & Manners, 1989), and probes from some true fungi, such as the MGR repeat from *M. grisea*, which appear to be species-specific, and do not hybridize to closely related species or even to strains isolated from different hosts (Hamer *et al.*, 1989). The plus/minus mode of inheritance for probe RG-57 could mean that this sequence corresponds to some sort of transposable element. If so, transposition events must not occur often because the same suite of fragments was found in most isolates studied. This may explain the rather limited repertoire of alleles compared to VNTR loci, in which new length variants can be generated presumably by unequal crossovers at the tandem repeats (Jeffreys *et al.*, 1988).

If probe RG-57 is a transposable element, it must have been introduced prior to the divergence of the lines leading to *P. infestans*, *P. mirabilis*, *P. phaseoli* and *P. colocasiae*. Although interspecific transfer of transposable elements has been documented in some organisms (see Syvanen, 1987; Houck *et al.*, 1991), it would be unlikely in these *Phytophthora* species because their strong host specificities preclude contact even where their ranges overlap. *Phytophthora* group IV contains species with predominantly amphigynous antheridia, caducous sporangia, and strong host-specificities (Waterhouse, 1963). The low level of hybridization of probe RG-57 to DNA from *P. ilicis* and *P. hibernalis* may provide evidence that this is not a natural grouping. Although much more data are needed before firm conclusions can be made, these preliminary results might indicate that group IV should be split into two, one of which would be an "infestans" group that includes those species hybridizing most strongly to probe RG-57. *Phytophthora ilicis* and *P. hibernalis* may belong in a separate division within group IV, or could be more closely related to the species in one of the other groups. The hypothesis that group IV may be polyphyletic is supported by the results with probe RG-7, which gave very similar patterns in *P. infestans*, *P. mirabilis* and *P. phaseoli*. DNA from *P. hibernalis* and *P. ilicis* hybridized less strongly to this probe. The position of *P. colocasiae* is unclear with probe RG-7. The lack of overlap between any of the fragments found in isolates of *P. infestans* with those in *P. mirabilis* provides additional justification for Galindo & Hohl's (1985) separation of these taxa into two separate species.

The moderately repetitive DNA revealed by probe RG-57 provides an evolutionarily conserved, highly polymorphic, dispersed, moderately repetitive fragment pattern. One obvious application for this probe will be in RFLP mapping. With the exception of the two pairs of closely linked loci, and the possible case of allelism or tight linkage for fragments 23 and 21, all other fragments appeared to represent separate unlinked loci. Unfortunately, it was not possible to determine the linkage relationships of all fragments because they did not all segregate in the same cross. However, if the additional fragments that were not studied in these crosses are also at separate loci, this probe may hybridize to at least 25 different loci in *P. infestans*, most of which are probably unlinked. Because *P. infestans* contains only nine or ten chromosomes (Sansome & Brasier, 1973), it is likely that most chromosomes can be marked genetically with this one probe. Selfing occurs to varying degrees in crosses between *P. infestans* isolates and, as shown above, probe RG-57 provides a sensitive tool for detecting selfing and for distinguishing hybrids from selfs. This will be

particularly valuable where allozyme variation is insufficient for this purpose.

Another potential use for this probe is in sorting out heterokaryosis, parasexuality and ploidy variation. The lack of segregation among single-zoospore isolates in this study showed that the fragments were mitotically stable, and that the five "parent" isolates were homokaryotic. If heterokaryosis does occur in *P. infestans*, these data indicate that it is probably rare. Although this probe does not have the same resolving power as the Jeffrey's probe in humans, it does provide data on a large number of loci simultaneously, and there is thus a low probability of unrelated individuals having the same multilocus genotype. Therefore, this probe will be very useful for elucidating the clonal structure of populations, and for studying the fate of particular clonal lines during the course of an epidemic or from year to year. Furthermore, because it is possible to determine the genotype of an individual at each locus by visual inspection, the data are amenable to traditional methods of population genetic analysis. This probe will also be useful for studying the evolution of the species in *Phytophthora* group IV. For example, because the sets of fragments in *P. infestans* and *P. mirabilis* are different, this probe provides a powerful tool that can be used to test for introgression from one species to the other where they occur sympatrically. Thus, probe RG-57 may provide the means for addressing many of the previously intractable questions about the basic biology, ecology and evolution of *P. infestans*.

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REFERENCES

Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. & Struhl, K., 1987. Current Protocols in Molecular Biology. Greene Publishing Associates/Wiley Interscience, New York. pp 400.

Braithwaite, K.S. & Manners, J.M., 1989. Human hypervariable minisatellite probes detect DNA polymorphisms in the fungus *Colletotrichum gloeosporioides*. Current Genetics 16: 473-475.

Burke, T. & Bruford, M.W., 1987. DNA fingerprinting in birds. Nature 327: 149-152.

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

Dallas, J.F., 1988. Detection of DNA "fingerprints" of cultivated rice by hybridization with a human minisatellite DNA probe. Proceedings of the National Academy of Sciences USA 85: 6831-6835.

Feinberg, A.P. & Vogelstein, B., 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Analytical Biochemistry 132: 6-13.

Francis, D.M., Hulbert, S.H. & Michelmore, R.W., 1990. Genome size and complexity of the obligate fungal pathogen, *Bremia lactucae*. Experimental Mycology 14: 299-309.

Galindo, J. & Hohl, H.R., 1985. *Phytophthora mirabilis*, a new species of *Phytophthora*. Sydowia, Annales Mycologici Ser II 38: 87-96.

Garber, R.C. & Yoder, O.C., 1983. Isolation of DNA from filamentous fungi and separation into nuclear, mitochondrial, ribosomal, and plasmid components. Analytical Biochemistry 135: 416-422.

Hamer, J.E., Farrall, L., Orbach, M.J., Valent, B. & Chumley, F.G., 1989. Host species-specific conservation of a family of repeated DNA sequences in the genome of a fungal plant pathogen. Proceedings of the National Academy of Sciences USA 86: 9981-9985.

Hamer J.E. & Givan S., 1990. Genetic mapping with dispersed repeated sequences in the rice blast fungus: Mapping the *SMO* locus. Molecular and General Genetics 223: 487-495.

Hooker, W.J., 1981. (ed.) Compendium of potato diseases. American Phytopathological Society, St. Paul, MN. pp 123.

Houck, M.A., Clark, J.B., Peterson, K.R. & Kidwell, M.G., 1991. Possible horizontal transfer of *Drosophila* genes by the mite *Proctolaelaps regalis*. Science 253: 1125-1129.

Jeffreys, A.J. & Morton, D.B., 1987. DNA fingerprints of dogs and cats. Animal Genetics 18: 1-15.

Jeffreys, A.J., Wilson, V. & Thein, S.L., 1985a. Hypervariable "minisatellite" regions in human DNA. Nature 314: 67-73.

Jeffreys, A.J., Wilson, V. & Thein, S.L., 1985b. Individual-specific "fingerprints" of human DNA. Nature 316: 76-79.

Jeffreys, A.J., Wilson, V., Thein, S.L., Weatherall, D.J. & Ponder, B.A.J., 1986. DNA "fingerprints" and segregation analysis of multiple markers in human pedigrees. American Journal of Human Genetics 39: 11-24.

Jeffreys, A.J., Royle, N.J., Wilson, V. & Wong, Z., 1988. Spontaneous mutation rates to new length alleles at tandem-repetitive hypervariable loci in human DNA. Nature 332: 278-281.

Maniatis, T., Fritsch, E.F. & Sambrook, J., 1982. Molecular Cloning: a Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. pp 545.

Nakamura, Y., Leppert, M., O'Connell, P., Wolff, R., Holm, T., Culver, M., Martin, C., Fulimoto, E., Hoff, M., Kumlin, E. & White, R., 1987. Variable number of tandem repeats (VNTR) markers for human gene mapping. Science 235: 1616-1622.

Sansome, E. & Brasier, C.M., 1973. Diploidy and chromosomal structural hybridity in *Phytophthora infestans*. Nature 241: 344-345.

Scherer, S. & Stevens, D.A., 1988. A *Candida albicans* dispersed, repeated gene family and its epidemiologic applications. Proceedings of the National Academy of Sciences, USA 85: 1452-1456.

Shattock, R.C., Tooley, P.W. & Fry, W.E., 1986. Genetics of *Phytophthora infestans*: Determination of recombination, segregation, and selfing by isozyme analysis. Phytopathology 76: 410-413.

Shaw, D.S., 1991. Genetics. p 131-170 in: Ingram D.J. & Williams, P.H. (eds.). *Phytophthora infestans*, the cause of late blight of potato. Advances in Plant Pathology. Vol. 7. Academic Press, London. pp 273.

Spielman, L.J., 1991. Isozymes and the population genetics of *Phytophthora infestans*. p 231-241 in: Lucas, J.A., Shattock, R.C., Shaw, D.S. & Cooke, L.R. (eds.). *Phytophthora*. British Mycological Society, Cambridge University Press, Cambridge. pp 447.

Syvanen, M., 1987. Molecular clocks and evolutionary relationships possible distortions due to horizontal gene flow. Journal of Molecular Evolution 26: 16-23.

Waterhouse, G.M., 1963. Key to the species of *Phytophthora* de Bary. Mycological Papers, no. 92. Commonwealth Mycological Institute, Kew.

Westneat, D.F., 1990. Genetic parentage in the indigo bunting: a study using DNA fingerprinting. Behavioral Ecology and Sociobiology 27: 67-76.

Wetton, J.H., Carter, R.E., Parkin, D.T. & Walters, D., 1987. Demographic study of a wild house sparrow population by DNA fingerprinting. Nature 327: 147-149.

Chapter 5

Population genetic structure of *Phytophthora infestans* in The Netherlands

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ABSTRACT

Isolates of *Phytophthora infestans* were collected from six different regions in The Netherlands in September-October 1989 and subsequently characterized. Regions contained one to four sampling sites and yielded 186 isolates. Additionally, 19 isolates from an ongoing metalaxyl-resistance monitoring project were characterized. In total, 205 isolates were characterized in terms of allozymes (glucose phosphate isomerase [*Gpi*] and peptidase [*Pep*], mating type and metalaxyl resistance. The analysis revealed 17 different genotypes. Samples from some sites were highly heterogeneous, whereas samples from other sites appeared homogeneous. Three genotypes each were detected in five of the six regions and together accounted for 61% of all isolates. Metalaxyl-resistant isolates accounted for 35% of the total sample and 45% of the samples from commercial fields. Chi-square contingency analysis indicated significant differences in genotype frequencies among subpopulations from different regions of the country, between A1 and A2 individuals, and between potato and tomato isolates. In most locations the frequency of allozyme alleles differed significantly from frequencies expected according to Hardy-Weinberg equilibrium. The results were consistent with asexual reproduction, although the occurrence of a low level of sexual reproduction cannot be excluded.

INTRODUCTION

Variability in virulence and fungicide resistance in populations of fungal plant pathogens contributes to failures in established disease management procedures. Recently, variation in sensitivity to fungicides has been especially troublesome, and for decades, diversity in specific virulence has severely limited the usefulness of cultivars with specific resistances. Certainly, *Phytophthora infestans* (Mont.) de Bary fits this general scenario (Davidse *et al.*, 1981, 1989; Umaerus *et al.*, 1983). Resistance to the specific fungicide, metalaxyl, has limited the usefulness of that fungicide in various locations around the world, and the occurrence of many specific virulence factors in populations of *P. infestans* has eliminated the use of genes for specific resistance as an important disease management strategy. On the premise that knowledge of the population genetics of fungal plant pathogens may eventually contribute to the development of more durable disease management strategies, we have initiated such a study for *P. infestans*.

Biochemical and molecular markers contribute significantly to the abilities of scientists to gain understanding of the population biology of fungi. Spieth's study of the saprophytic fungus, *Neurospora intermedia* (Spieth, 1975), indicated that levels of diversity are comparable to those of *Drosophila*, with most of the diversity occurring within local populations. Comparisons among collections of *Puccinia graminis* f. sp. *tritici* (causal agent of stem rust of wheat) suggested that global diversity in that pathogen population was relatively low, although levels of diversity within populations were variable (Burdon & Roelfs, 1985a, 1985b, 1986).

Leung and Williams (1986) found little variation in allozyme genotypes within a geographically diverse collection of isolates of *Magnaporthe grisea* (causal agent of rice blast) two electrophoretic types accounted for 90% of the samples. Diversity was higher from non-rice hosts. The recent availability of various molecular markers provides a potentially powerful tool for analyzing phylogenic relationships and population genetic structure (Brown *et al.*, 1990; McDonald & Martinez, 1990).

Information concerning the population genetics of *P. infestans* is still minimal, but is beginning to develop (Fry & Spielman, 1991; Spielman, 1991; Spielman *et al.*, 1990a, 1991). There was much greater diversity for allozyme genotypes in a population from central Mexico than in populations from other locations in the world. Preliminary tests indicated that alleles for glucose phosphate isomerase (E.C.5.3.1.9) (*Gpi*) in the population from central Mexico fit Hardy-Weinberg equilibrium, but such alleles from a population of isolates from the United States and Canada did not (Tooley *et al.*, 1985). The number of allozyme alleles useful in population studies now number at least 10 (six for *Gpi*, four for peptidase [E.C.3.4.3.1] [*Pep*]) (Spielman *et al.*, 1989, 1990b). Each of the *Gpi* and *Pep* alleles has been shown to function according to Mendelian expectation in classical genetics studies (Spielman *et al.*, 1990b).

Discovery in the mid-1980s of the A2 mating type in Europe (Hohl & Iselin, 1984) was the first indication of significant changes in the populations of *P. infestans* there and subsequently throughout the world. Before the 1980s the A2 mating type had been found only in central Mexico (Fry & Spielman, 1991). Further analysis of the allozymes in individuals from various locations around the world stimulated the hypothesis that a new population (apparent during the 1980s) of *P. infestans* was displacing an older (pre-1980s) population in Europe (Spielman, 1991; Spielman *et al.*, 1990a).

The major goal of our study was to determine the distribution of genetic diversity in populations of *P. infestans* at several geographical levels (within a country, among regions in a country, and among sites within a region). Obviously, such a question needed to be tested in a location with numerous potato fields and where late blight could be easily found. The Netherlands fit these criteria. Results of this analysis would be helpful in several respects, but we were particularly interested in estimating the contribution of sexual reproduction in a location where both mating types had existed for a relatively short period.

MATERIALS AND METHODS

Collection of isolates

Isolates were obtained in two ways. First, several collecting trips to commercial potato fields and community gardens in various locations in The Netherlands were conducted during September and October 1989 (Table 1). All tomato and potato plants in community gardens were inspected and plants along a series of transects in commercial fields were inspected. Each field

Table 1. Sites sampled randomly for *Phytophthora infestans* in The Netherlands in 1989.

Region	Site	Nearest town	Code number	Host	Field size	Disease severity[a]	Number of isolates	Collection date
NE	2	Ter Wisch	89140	Potato	< 2 ha	10-20% defoliation	27	7 Sept.
NE	3	Ter Wisch	89141	Potato	< 1 ha	Very mild	2	7 Sept.
NW	1	Middenmeer	89142	Potato (cv. Bintje)	10 ha	Very mild	17	7 Sept.
NW	2	Middenmeer	89143	Potato (cv. Bintje)	10 ha	Very mild	18	7 Sept.
C	1(t)	Wageningen	89147	Tomato	Community garden	Very mild	20	11 Sept.
C	1(p)	Wageningen	89148	Potato	Community garden	Moderate	16	11 Sept.
W	1	Leiden	89154	Potato	Community garden	1-10% defoliation	12	15 Sept.
W	2(p)	Leiden	89155	Potato	Community garden	Very mild	6	15 Sept.
W	2(t)	Leiden	89156	Tomato	Community garden	Very mild	7	15 Sept.
SW	1	Middelburg	89153	Potato	Volunteers	Moderate	17	19 Oct.

[a] Disease severity was not measured quantitatively, so only general description are given here: Very mild = one lesion per plant and infected plants are widely separated; Moderate = one to several lesions per plant; more severe disease is indicated via an estimate of defoliation.

Figure 1. Collections sites of *Phytophthora infestans* in The Netherlands in 1989. One or a few sites were sampled in each of six regions (NE, N, NW, W, C, SW) to yield 186 isolates. All sites in a region were within 25 km of a central point, and regions were separated by 75-300 km.

or contiguous group of community gardens was regarded as a single site, and sites within 40 km were regarded to be from the same region. The minimum distance between collecting regions was approximately 75 km, and the maximum distance was approximately 300 km (between Middelburg in the southwest and Ter Wisch in the northeast) (Fig. 1). Regions represented the northeast, north, northwest, west, central and southwest (NE, N, NW, W, C and SW) parts of The Netherlands (Fig. 1). Fields were scouted and single lesion samples were collected randomly. Where there was sufficient late blight, at least 30 single-lesion samples were obtained from a site by random collecting. Unfortunately, most sites did not enable collection of 30 samples. Individual isolates were obtained from leaves with single lesions, or from single tubers, using standard isolation techniques.

Isolates were also obtained from infected tubers and leaves sent to the Agricultural University in Wageningen during the summer and fall 1989 as part of an ongoing analysis of the frequency of metalaxyl resistance in The Netherlands. Usually only one or a few isolates were obtained from each sample.

Culture of isolates

After isolation into pure culture, isolates were kept on Rye A medium (Caten & Jinks, 1968), at 18°C in the dark. For analysis of allozymes, isolates were cultured on liquid Rye A medium (without agar) in 9-cm petri plates for approximately 14 days at 18°C in the dark. For assessment of mating type, isolates were grown on clarified Rye A agar medium in proximity to a strain of known mating type (A1 or A2). Each isolate was tested against a known A1 strain and against a known A2 strain. Oospores usually appeared within 5-10 days.

Metalaxyl resistance

The floating leaf disk test was used as described by Davidse *et al.* (1989), except that only three test concentrations of metalaxyl were used: 0.0, 1.0, and 10 μg/ml. Each of five leaf disks was inoculated with a suspension of sporangia obtained from a sporulating tuber slice or from a culture growing on Rye A medium for each metalaxyl concentration. Inoculated floating leaf disks were incubated in the light (16 h per day, intensity 75,000 lx) at 15°C for 5-7 days. Sporulation within 5-6 days on leaves floating on 1.0 or on 1.0 and 10.0 μg/ml indicated metalaxyl resistance.

Allozyme analysis

Mycelium from a single 9-cm petri plate was harvested from liquid culture by filtration and then stored at —80°C until further use. The mycelium (approximately 0.12 g wet weight) was ground to a powder in a mortar and pestle cooled with liquid nitrogen. The ground mycelium was transferred to a microcentrifuge tube and 0.4-0.6 ml Tris-citrate gel buffer, pH 7 (Shaw & Prasad, 1970: buffer system I), was added to each tube. The homogenate was not centrifuged but was stored at 4°C for use within a few days or stored at —80°C for later use.

Electrophoresis was done using standard techniques (Shields *et al.*, 1983). For *Gpi*, we used a gel buffer of 0.01 M histidine-HCl, pH 6.0, and an electrode buffer of 0.135 M Trizma base, 0.04 M citric acid, pH 6.0. These gels were run for 6-8 hours at approximately 150 V and approximately 50-60 mA at 4°C for resolution of the 90/100 genotypes. (Allozyme genotypes are described in terms of the relative mobilities of their bands of activity in an electric field in starch gels [Spielman *et al.*, 1990b]. The most common allele is assigned a mobility of 100 and other alleles are assigned numbers based on their relative mobility. Thus 90/100 refers to a heterozygous genotype with two alleles, one allele being the most common type, and the other producing an enzyme that migrates 90% as far as the most common type.) For *Pep* we used buffer 10 of Soltis *et al.*(1983); the gel buffer was a 1:4 dilution of the electrode buffer (0.18 M Trizma base, 0.10 M boric acid, 0.001 M Na-EDTA, pH 8.7). These gels were run for 6-8 h at 290 V and approximately 40 mA at 4°C. All gels were 12% hydrolyzed potato starch (for electrophoresis, Sigma, St. Louis, MO). Enzymes were detected using agar overlays and the techniques of Micales *et al.* (1986), Shaw and Prasad (1970), and Siciliano and Shaw (1976). Controls to assess the relative

mobilities of allozymes were from the culture collection at Cornell University.

Iso-electric focusing proved to be a useful technique for *Gpi* isozyme analysis. Samples were loaded on the cathode side of a 5% polyacrylamide gel (LKB Ampholine 1804-102, Piscataway, NJ), pH range 4.0-6.5, with a 3% degree of cross-linkage and ampholine concentration of 2.2% (w/v). The gel was run for 1.5 h at 4°C at a constant power of 25 W. *Gpi* was detected using the agar overlay as described above.

Statistical analyses

Contingency chi-square analyses were conducted to detect differences in frequencies among subpopulations from the six regions, between A1 and A2 individuals, and between isolates obtained from potato and tomato, and to compare observed allozyme genotype frequencies with those expected at Hardy-Weinberg equilibrium. Genotypic analyses were emphasized because of the importance of asexual reproduction in this fungus. Analyses based on allele frequencies are most appropriate for sexually reproducing organisms. Groupings were done on traits thought to be neutral, no distinction was made between metalaxyl-resistant and -sensitive isolates. Groupings were constructed to achieve expected values of 5 or more, because expected values of < 5 can lead to unreliable chi-square values. When only a few expected values were below 5 (and above approximately 2.5), chi-square values were calculated, but clearly identified.

The extent of population subdivision also was assessed using Nei's gene diversity analysis (Nei, 1973). Gene diversity within each region or subpopulation (H_s) was calculated for each allozyme locus in each location. The mean proportion of the total gene diversity (H_t) that was due to differences among subpopulations (G_{st}) was calculated according to the methods of Nei and Chesser (1983) for obtaining unbiased estimates of H_s and H_t assuming a mixed mating model.

Table 2. Locations of *Phytophthora infestans* samples submitted from the advisory service during fall 1989.

Region	Site	Nearest town	Code number	Host	Number of isolates	Acquisition date
NE	1	Erica	89102	Potato (cv. Ehud, leaves)	7	7 Sept.
NE	4	Ter Wisch	89150	Potato (cv. Astarte, leaves)	20	26 Sept.
N	1	Appelscha	89157	Potato (cv. Astarte, tubers)	8	10 Nov.
N	2	Appelscha	89158	Potato (cv. Van Gogh, tubers)	9	10 Nov.

Table 3. Genotypes of *Phytophthora infestans* isolates collected in The Netherlands in 1989.

Genotype				Number of isolates in various 1989 collections[a]														
Gpi	*Pep*	MT[b]	MR[c]	NE-1	NE-2	NE-3	NE-4	N-1	N-2	NW-1	NW-2	C-1(t)	C-1(p)	W-1	W-2(p)	W-2(t)	SW-1	NL	TOTAL
100/100	100/100	1	S						1			1	2					2	6
100/100	100/100	2	S									18	1					6	25
100/100	83/100	1	R										1						1
100/100	83/100	1	S										1		2				3
100/100	83/100	2	R		1														1
100/100	83/100	2	S															1	1
90/100	100/100	1	R		2				1	1	1		1					2	8
90/100	100/100	1	S		6			5	4	12	17		2				7	3	56
90/100	100/100	2	S										1						1
90/100	83/100	1	R	7	15	1	19	1	2	4			2					1	52
90/100	83/100	1	S		1			2	1				1	12			9	1	27
90/100a[d]	100/100	1	R		2	1							3				1		7
90/100a	100/100	1	S										1		4	7			12
90/100a	100/100	2	R															1	1
90/100a	100/100	2	S									1						1	2
90/100a	83/100	1	R				1												1
90/100a	83/100	2	S															1	1
TOTALS (individuals)				7	27	2	20	8	9	17	18	20	16	12	6	7	17	19	205
(genotypes)				1	6	2	2	3	5	3	2	3	11	1	2	1	3	10	17

[a]Locations of collections (except NL) are indicated in Tables 1 and 2 and pictured in Figure 1. NL indicates the 19 isolates selected from the metalaxyl monitoring project. All A2 isolates from that project were analyzed and geographically close A1 isolates were selected for comparison.

[b]MT = mating type (A1 or A2).

[c]MR indicates metalaxyl resistance (resistant R, or sensitive S).

[d]90/100a differs from 90/100 in the relative staining intensity of various electromorphs of *Gpi* as described in the text.

RESULTS

Collection of isolates

A total of 205 isolates was collected (Tables 1-3). Most isolates (142) were obtained by random sampling of single lesions in commercial potato fields or community gardens during September -October 1989 (Table 1). Of the many fields and garden sites inspected by the authors, five commercial fields and three sets of gardens had late blight. Additionally 44 isolates were obtained from samples submitted from the advisory service during fall 1989 (Table 2). These 44 isolates came from four sites, but the details of sampling (random or nonrandom) are unknown and thus are identified separately (Table 2). Finally, 19 additional isolates (ten A2 and nine A1 isolates) were selected (not randomly sampled) from those received during summer 1989 for a metalaxyl-resistance monitoring project. The ten A2 isolates represented all of the A2's received during the summer.

The sites had widely diverse characteristics (Tables 1 and 2). Host density in community gardens was dramatically less than in commercial fields. Both host genotypes and field size varied. In most sites sampled, disease was very light (widely scattered lesions), apparently caused by the unusually warm and dry 1989 growing season. However, one commercial field (NE-2) and potatoes in one set of community gardens (W-1) had disease so severe that some defoliation had occurred.

Allozyme analysis

Two alleles for *Gpi* and two alleles for *Pep* were detected. For *Gpi* the 90/100 genotype occurred in 84% of the isolates, whereas the 100/100 genotype occurred in 16% of the isolates. However, there were two forms of the 90/100 genotype, which differed in the relative staining intensity of the 100 homodimer. The most common type of 90/100 genotype was one in which the 100 homodimer stained with expected intensity, but in the other genotype (labeled 90/100a), the 100 band was extremely faint. Iso-electric focusing of selected samples confirmed the 90/100 designations. Controls for *Gpi* included isolates 575, 579, 618, 619, 807, 821 and 1118, from the collection at Cornell University with genotypes of 100/122, 100/100, 86/122, 86/100, 86/100, 86/100 and 90/100, respectively. The 86 allele for *Gpi* was not detected in any of the 205 isolates.

For *Pep*, two genotypes were detected: 83/100 and 100/100, with frequencies of 42 and 58%, respectively. Detection of the 83 allele for *Pep* was unexpected, and to our knowledge it is the first report of this allele in Europe. Comparisons with the peptidase patterns of control isolates confirmed the presence of the allele. The control isolates were isolates 562, 575, 579 and 807 from the culture collection at Cornell University, with *Pep* patterns of 83/100, 100/100, 92/92 and 92/100, respectively. None of the 1989 isolates collected in The Netherlands had the *Pep* 92 allele.

Characterization of isolates

Analysis of the 205 isolates revealed 17 different genotypes (Table 3). When we eliminated metalaxyl resistance as a distinguishing character, the most commonly occurring genotype (79 individuals) was A1, 90/100 for *Gpi* and 83/100 for *Pep*. Except for the community gardens in Wageningen (C-1[p] C-1[t]), the A2 type was found only rarely.

The *Gpi* and *Pep* markers identified some collections as considerably heterogeneous, but others as apparently homogeneous. Collections NE-1, W-1 and W-2(t) were completely homogeneous, and collections NW-2 and NE-4 were dominated by a single genotype. In contrast, collections NE-2, C-1(p), N-1 and N-2 appeared heterogeneous (Table 3).

The distribution of *Gpi* and *Pep* alleles was tested via chi-square analysis for conformation to Hardy-Weinberg equilibrium. The analysis was done by grouping all 90/100 genotypes together. In general the distribution of *Gpi* alleles was more divergent from that expected than was the distribution of *Pep* alleles. In all comparisons for which the chi-square value was reliable, the observed frequency of alleles was significantly different from that expected (Table 4).

Metalaxyl resistance

The fact that fungicide resistance is a selectable marker, strongly affected by fungicide use, was clear in this collection. In the collection as a whole, metalaxyl resistance occurred in approximately 35% of the isolates (Table 3). However, the proportion of metalaxyl-resistant isolates was much higher from commercial fields (about 45%), where metalaxyl has been widely used, than from community gardens (11%), where metalaxyl is rarely used. Metalaxyl resistance was detected in all genotypes found in commercial potato fields. Interestingly, no isolate obtained from infected tomato (all obtained in community gardens) was resistant to metalaxyl.

Analysis for genetic substructure

Several types of genetic substructuring were tested. Fungicide resistance was not used as a character in these analyses because the genetic base was unclear, and because it is a strongly selected phenotype. Approximately 6% of the overall gene diversity (Nei & Chesser, 1983) was due to differences among regions (G_{st} = 0.061). The unbiased mean values for total (H_t) and within region (H_s) gene diversity were 0.412 and 0.387, respectively. This indicates a low level of population subdivision.

Other substructuring was detected from analysis of genotypic frequencies. Subpopulations from different regions were significantly different from each other -- whether the comparison was based on all isolates from six regions, potato isolates from six regions, or potato isolates from commercial fields (Table 5). The A1 isolates appeared to represent a different subpopulation than did the A2 isolates in most comparisons. The A1 and A2 samples were significantly different when all 186 isolates were examined or when only the central region isolates (C-1[t], C-1[p]) were examined (Table 5). Finally, isolates from potato

Table 4. Contingency chi-square analysis[a]: Conformation to Hardy-Weinberg equilibrium.

Region or group	*Gpi*				*Pep*			
	Genotype	Obs.	Exp.	χ^2	Genotype	Obs.	Exp.	χ^2
NE[b]	90/90	0	13.4	13.4	83/83	0	20.2	20.2
	90/100	55	28.0	26.0	83/100	45	26.8	12.3
	100/100	1	14.6	12.7	100/100	11	9.0	0.4
				52.1**[c]				32.9**
N	90/90	0	3.7	3.7	83/83	0	0.6	0.6
	90/100	16	8.5	6.6	83/100	6	5.0	0.2
	100/100	1	4.8	3.1	100/100	11	11.4	0.0
				{13.4**}[d]				...[e]
NW	90/90	0	8.8	8.8	83/83	0	1.0	1.0
	90/100	35	17.4	17.8	83/100	4	3.8	0.1
	100/100	0	8.8	8.8	100/100	31	31.1	0.0
				35.4**				...[e]
C	90/90	0	1.2	1.2	83/83	0	0.2	0.2
	90/100	13	10.6	0.5	83/100	6	5.3	0.1
	100/100	23	24.2	0.1	100/100	30	30.5	0.0
				...[e]				...[e]
W	90/90	0	5.3	5.3	83/83	0	2.0	2.0
	90/100	23	12.4	9.1	83/100	14	10.1	1.5
	100/100	2	7.3	3.8	100/100	11	13.0	0.3
				18.2**				...[e]

SW	90/90	0	4.3	4.30	83/83	0	1.1	1.1
	90/100	17	8.4	8.80	83/100	9	6.5	1.0
	100/100	0	4.3	4.30	100/100	8	9.3	0.2
				{17.40**}[d]				...[e]
Total collection	90/90	0	34.4	34.4	83/83	0	9.5	9.5
	90/100	159	91.1	50.6	83/100	84	65	5.6
	100/100	27	60.4	18.4	100/100	102	111	0.7
				103.4**				15.8**
C-1(t)	90/90	0	0	0	83/83	0	0	0
	90/100	1	1	0	83/100	0	0	0
	100/100	19	19	0	100/100	20	20	0
				...[e]				na[f]
C-1(p)	90/90	0	2.3	2.3	83/83	0	0.5	0.5
	90/100	12	7.5	2.7	83/100	6	4.9	0.2
	100/100	4	6.3	0.8	100/100	10	10.6	0.0
				{5.8*}[d]				...[e]

[a]Each analysis had one degree of freedom.

[b]Regions are identified in Figure 1.

[c]Probabilities of a greater chi-square due to chance were: *= <0.05; **=0.005.

[d]The chi-square may be unreliable because one expected value was between 5.0 and 3.0.

[e]The chi-square value was not calculated because expected values were too low.

[f]The analysis was not applicable because there was no diversity for either *Gpi* or *Pep*.

Table 5. Contingency chi-square analysis for nonrandom frequencies.

Groupings	Chi-square	
	Gpi	*Pep*
Regional comparisons[a]		
All isolates (six regions)	{90.5***[b](5)[c]}[d]	58.5***(5)
Potato isolates (six regions)	...[e]	49.9***(5)
Potato isolates from commercial fields (NE,N,NW,SW)	...[e]	47.0***(3)
A1:A2 comparisons		
All isolates six regions	...[e]	16.9***(5)
19 summer isolates	{4.3*(1)}[f]	...[e]
Central region isolates {C-1(t) and C-1(p)}	15.4***(1)	...[e]
Potato:tomato host comparisons		
Central and western isolates	17.2***(1)	26.1***(1)
Central isolates	18.7***(1)	...[e]

[a]The six regions are identified in Figure 1.

[b]The probability of a great chi-square value is indicated by numbers of asterisks:* = < 0.05; *** = < 0.005.

[c]The number in parentheses indicates the degrees of freedom.

[d]This chi-square value may be unreliable because three of 12 expected values were < 5.0 (2.5, 3.6 and 2.5).

[e]The chi-square was not calculated because expected values were too low.

[f]This chi-square value may be unreliable because three of four expected values were between 4.2 and 5.0.

appeared to represent a different subpopulation than did the isolates from tomato (Table 5). This result held for comparison of all potato and tomato isolates or only the potato and tomato isolates from the central region (Table 5). However, at one site (W-2) the tomato isolates had the same genotype as the majority of potato isolates (Table 3). Small sample size at this site precluded assessment of statistical significance.

Additional A1:A2 comparison

In an additional effort to assess the similarity of A1 and A2 isolates in The Netherlands, all of the A2 isolates (n=10) and nine additional A1 isolates selected from those submitted to the metalaxyl-resistance monitoring project in 1989 were characterized. The A1 isolates were chosen from the same sites as the A2 isolates if possible, but if not, from a geographically close location. The vast majority of A1 isolates were 90/100 for *Gpi*, whereas most A2

isolates were 100/100 or 90/100a for *Gpi* (Table 3). Even though these isolates were submitted from all over The Netherlands, the frequencies of *Gpi* alleles in A1 isolates were not significantly different from the frequencies in A2 isolates. The expected number of *Pep* alleles was too low in some categories to enable assessment of statistical significance (Table 5).

DISCUSSION

The present study confirmed previous studies in identifying limited diversity in *P. infestans* in The Netherlands, but differed from other studies in terms of the specific genotypes detected. The absence of the 86/100 genotype for *Gpi* in any of the 205 isolates was surprising because of the prevalence of this genotype in early collections (pre-1980s) from The Netherlands (Spielman *et al.*, 1991). However, these data are consistent with a recent report from the United Kingdom indicating no individuals with *Gpi* genotype of 86/100 in recent collections (Shattock *et al.*, 1990). The common occurrence of individuals with 90/100 genotype for *Gpi* in The Netherlands is not necessarily inconsistent with recent reports from the United Kingdom where only 100/100 genotypes for *Gpi* were detected. The 90/100 genotype is only detectable with the lower pH gels done in this study, which were not done when the United Kingdom population was characterized. Had we used pH 7.0 gels, we also would not have been able to distinguish the 90/100 from the 100/100 genotype. The 90 allele is legitimate because it segregated according to Mendelian expectation in the two crosses that we have done (L.J. Spielman, unpublished).

The discovery of the 83/100 genotype for *Pep* was unexpected but very interesting, because this is the first report of the *P. infestans* allele from western Europe. In earlier reports this allele had been erroneously recorded as 92/100 (Spielman, 1991). The 83 allele was first detected in the late 1980s (Spielman *et al.*, 1990a). However, the 83/100 genotype is sometimes difficult to distinguish from the 92/100 genotype because of poor resolution of *Pep* bands on gels. Many controls are needed. Therefore some isolates previously characterized as 92/100 had been misidentified. For example, isolate 1117 collected from The Netherlands in the mid-1980s was initially characterized by us as 92/100 for *Pep*, and only after documentation of the 83/100 genotype (Spielman *et al.*, 1990a) was this isolate correctly characterized as 83/100. We have reevaluated our isolates characterized before the discovery of the 83/100 genotype and have found that all isolates (previously labeled as 92/100) collected in Europe after 1982 were actually 83/100 (L.J. Spielman, unpublished). However, isolates collected before 1980 and characterized as 92/100 were correctly identified as 92/100 (Spielman *et al.*, 1991). The 83 allele is apparently characteristic of the "new" (early 1980s and later) population of *P. infestans* in Europe, which has apparently displaced the "old" (pre-1980s) population (Spielman *et al.*, 1991).

The present study indicated that some genotypes are widely distributed, whereas others may be unique to a single site. Individuals that were mating type A1 and 90/100 for

Gpi and 83/100 for *Pep* were found in all regions of the country. Each of six genotypes was found in only one site (Table 3). Some sites appeared to contain only individuals of a single genotype, whereas other sites contained individuals with different genotypes. Thus our results for *P. infestans* are similar to those for *Puccinia graminis f. sp. tritici* (Burdon & Roelfs, 1986) in that diversity was variable among subpopulations.

An important question in The Netherlands and in other locations where both A1 and A2 mating types occur is whether or not sexual reproduction is contributing to the epidemiology of late blight. Although data from the present study do not conclusively resolve this issue, the infrequent occurrence of A2 isolates, and the discoveries that the A2 mating type is associated with certain allozyme genotypes and that A1 mating types are associated with other allozyme genotypes, is more consistent with the notion of asexual reproduction than with significant sexual reproduction. Associations between mating type and allozyme genotypes have not been detected in central Mexico (Tooley *et al.*, 1985). If sexual reproduction were common and contributing to the diversity of The Netherlands population, one would expect A1 and A2 individuals to have similar allozyme genotypes and both to be represented in all regions of the country. Conformation or lack of conformation of allele frequencies to those expected at Hardy-Weinberg equilibrium is not at all conclusive in this issue because of the limited diversity and many generations of asexual reproduction during epidemics.

The occurrence of metalaxyl resistance in several different genotypes supports the hypothesis that this trait has developed many times in a variety of genetic backgrounds (especially if sexual recombination is rare). The influence of selection is reflected in collections from commercial fields relative to collections from community gardens. The incidence of metalaxyl resistance in community gardens was 11% whereas the incidence of metalaxyl resistance in commercial potato fields was approximately 45%. Metalaxyl has been used rarely if at all in community gardens, but it has been used for years in commercial potato production (Davidse *et al.*, 1989).

This study also sheds some light on the question of host-plant specialization in *P. infestans*. Some reports suggest that isolates from tomato are different from those on potato (Giddings & Berg, 1919; Turkensteen, 1973), and some of our data support this suggestion. Tomato isolates from the Wageningen community gardens (C-1[t]) appeared to be quite distinct from the potato isolates (C-1[p]) collected there. The absence of metalaxyl resistance in isolates from tomato hosts may support the hypothesis of host-plant specialization. Presumably, metalaxyl has been used much less on tomatoes (community gardens) than on potatoes (commercial production). However, tomato isolates from Leiden community gardens (W-2[t]) had a genotype identical to that of the majority of potato isolates (W-2[p]) from Leiden. Thus our data support the concept of patchy population substructuring of *P. infestans* according to host plants.

Several results of this study support the hypothesis that a new population of *P. infestans* is replacing an older population in Europe (Spielman, 1991; Spielman *et al.*, 1991). The 86/100 genotype for *Gpi* and the 92/100 genotype for *Pep* are characteristic of the older

(pre-1980s) population but not of the new population. The frequency of the 86/100 genotype for *Gpi* continues to decrease in The Netherlands (not detectable in the 205 isolates of this study). Similarly, it was not detected in recent collections in the United Kingdom. However, collections of *P. infestans* made in the early 1980s and late 1970s in The Netherlands and United Kingdom were dominated by individuals with this genotype (R.C. Shattock, personal communication; Spielman, 1991). In addition, the absence of *Pep* 92/100 in this collection provides further evidence of population change in western Europe. Studies of earlier collections identified this genotype in western Europe (Spielman, 1991). The discovery of the 83/100 genotype for *Pep* in this study provides an additional difference between the current and previous (pre-1980s) isolates of *P. infestans*. The 83/100 genotype has been found in central Mexico, but not in the United States and Canada (Spielman, 1991).

The present study provides preliminary results concerning the population structure of *P. infestans* in The Netherlands. Analysis of gene diversity (G_{st}) was not very helpful in interpreting population substructure. The relatively low value for G_{st} (= 0.06) indicated little substructure among geographic regions. However, chi-square analysis of genotypic diversity indicated significant differences among populations in different regions (Table 5). Furthermore, conclusions are limited because of the limited number of markers and small sample sizes. Unfortunately, the number of isolates collected was limited because the unusually dry and sunny 1989 growing season limited the occurrence of late blight, even in The Netherlands. Many fields and gardens had no detectable late blight. More precise conclusions also will be available with the application of a greater number of markers. We expect to provide these results by application of restriction fragment length polymorphism analysis to this collection. With a larger number of polymorphic markers it will be possible to identify with greater certainty the degree of geographic substructuring, and to determine with greater confidence the contribution (if any) of sexual reproduction to population diversity in The Netherlands.

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REFERENCES

Brown, J.K.M., O'Dell, M., Simpson, C.G. & Wolfe, M.S., 1990. The use of DNA polymorphisms to test hypotheses about a population of *Erysiphe graminis f. sp. hordei*. Plant Pathology 39: 376-390.

Burdon, J.J. & Roelfs, A.P., 1985a. Isozyme and virulence variation in asexually reproducing populations of *Puccinia graminis* and *Puccinia recondita* on wheat. Phytopathology 75: 907-913.

Burdon, J.J. & Roelfs, A.P., 1985b. The effect of sexual and asexual reproduction on the isozyme structure of populations of *Puccinia graminis*. Phytopathology 75: 1068-1073.

Burdon, J.J. & Roelfs, A.P., 1986. Isozymic variation in *Puccinia graminis f. sp. tritici* detected by starch-gel electrophoresis. Plant Disease 70: 1139-1141.

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

Davidse, L.C., Henken, J., Dalen, A. van, Jespers, A.B.K. & Mantel, B.C., 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.

Davidse, L.C., Looijen, D., Turkensteen, L.J. & Van der Wal, D., 1981. Occurrence of metalaxyl-resistant strains of *Phytophthora infestans* in The Netherlands. European Plant Protection Organization Bulletin 15: 403-409.

Fry, W.E. & Spielman, L.J., 1991. Population Biology. p 171-192 in: Ingram D.J. & Williams, P.H. (eds.). *Phytophthora infestans*, the cause of late blight of potato. Advances in Plant Pathology. Vol. 7. Academic Press, London. pp 273.

Giddings, N.J. & Berg, A., 1919. A comparison of the late blight of tomato and potato: A preliminary report. Phytopathology 9: 209-211.

Hohl, H.R. & Iselin, K., 1984. Strains of *Phytophthora infestans* with A2 mating type behavior. Transactions of the British Mycological Society 83: 529-530.

Leung, H. & Williams, P.H., 1986. Enzyme polymorphism and genetic differentiation among geographic isolates of the rice blast fungus. Phytopathology 76: 778-783.

McDonald, B.A. & Martinez, J.P., 1990. DNA restriction fragment length polymorphisms among *Mycosphaerella graminicola* (anamorph *Septoria tritici*) isolates collected from a single wheat field. Phytopathology 80: 1368-1373.

Micales, J.A., Bonde, M.R. & Peterson, G.L., 1986. The use of isozyme analysis in fungal taxonomy and genetics. Mycotaxon 27: 405-449.

Nei, M., 1973. Analysis of gene diversity in subdivided populations. Proceedings of National Acadamy of Sciences, USA 70: 3321-3323.

Nei, M. & Chesser, R.K., 1983. Estimation of fixation indices and gene diversities. Annals of Human Genetics 47: 253-259.

Shattock, R.C., Shaw, D.S., Fyfe, A.M., Dunn, J.R., Loney, K.H. & Shattock, J.A., 1990. Phenotypes of *Phytophthora infestans* collected in England and Wales from 1985 to 1988: Mating type, response to metalaxyl and isozyme analysis. Plant Pathology 39: 242-248.

Shaw, C.R. & Prasad, R., 1970. Starch gel electrophoresis of enzymes - A compilation of recipes. Biochemical Genetics 4: 297-320.

Shields, C.R., Orton, T.J. & Stuber, C.W., 1983. An outline of general resource needs and procedures for the electrophoretic separation of active enzymes from plant tissue. p 443-486 in: Tanksley, S.D. & Orton, T.J. (eds.). Isozymes in Plant Genetics and Breeding. Part A. Elsevier Science, Amsterdam.

Siciliano, J.J. & Shaw, C.R., 1976. Separation and visualization of enzymes on gels. p 185-209 in: Smith, I. (eds.). Chromatographic and Electrophoretic Techniques. Vol. 2. Heinemann, London.

Soltis, D.E., Haufler, C.H., Darrow, D.C. & Gastomy, G.J., 1983. Starch gel electrophoresis of ferns: A compilation of grinding buffers, gel and electrode buffers, and staining schedules. American Ferns Journal 73: 9-27.

Spielman, L.J., 1991. Isozymes and the population genetics of *Phytophthora infestans*. p 231-241 in: Lucas, J.A., Shattock, R.C., Shaw, D.S. & Cooke, L.R. (eds.). *Phytophthora*. British Mycological Society, Cambridge University Press, Cambridge. pp 447.

Spielman, L.J., Drenth, A., Davidse, L.C., Sujkowski, L.J., Gu, W.K., Tooley, P.W. & Fry, W.E., 1991. A second world-wide migration and population displacement of *Phytophthora infestans*? Plant Pathology 40: 422-430.

Spielman, L.J., McMaster, B.J. & Fry, W.E., 1989. Dominance and recessiveness at loci for virulence against potato and tomato in *Phytophthora infestans*. Theoretical and Applied Genetics 77: 832-838.

Spielman, L.J., Gu, W.K. & Fry, W.E., 1990a. Genetic relationships among *Phytophthora infestans* populations from Europe, North America, and Japan. Phytopathology 80: 1006 (Abstract).

Spielman, L.J., Sweigard, J.A., Shattock, R.C. & Fry, W.E., 1990b. The genetics of *Phytophthora infestans*: Segregation of allozyme markers in F2 and backcross progeny and the inheritance of virulence against potato resistance R2 and R4 in F1 progeny. Experimental Mycology 14: 57-69.

Spieth, P.T., 1975. Population genetics of allozyme variation in *Neurospora intermedia*. Genetics 80: 785-805.

Tooley, P.W., Fry, W.E. & Villareal Gonzalez, M.J., 1985. Isozyme characterization of sexual and asexual *Phytophthora infestans* populations. Journal of Heredity 76: 431-435.

Turkensteen, L.J., 1973. Partial resistance of tomatoes against *Phytophthora infestans*, the late blight fungus. Agricultural Research Report No. 810. Instituut voor Plantenziektenkundig Onderzoek, Wageningen, The Netherlands. Mededeling No 633. pp 88.

Umaerus, V., Umaerus, M., Erjefält, L. & Nilsson, B.A., 1983. Control of *Phytophthora infestans* by host resistance: Problems and Progress. p 315-326 in: Erwin, D.C., Bartnicki-Garcia, S. & Tsao, P.H. (eds.). *Phytophthora*: its biology, taxonomy, ecology and pathology. APS Press, USA. pp 392.

Chapter 6

Genotypic diversity of *Phytophthora infestans* in The Netherlands revealed by DNA polymorphisms

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ABSTRACT

We used DNA fingerprinting to estimate genotypic diversity among 153 isolates of *Phytophthora infestans* collected from potato and tomato plants in 14 fields distributed over six regions in The Netherlands. The DNA fingerprint probe, RG-57, hybridized to 21 fragments of genomic DNA, 16 of which were polymorphic. Thirty-five RG-57 genotypes were identified among the 153 isolates. Half of the isolates had the most widely distributed genotype, which was found in 10 fields in five of the six regions sampled. However, 89% of the genotypes were detected in only one field, and 60% occurred only once. Two mitochondrial DNA types, designated A and B, were found. Type A occurred in 143 isolates and was found in all fields in every region. Type B, in contrast, was found in only 10 isolates, all collected in community gardens. Partitioning of the genotypic diversity into components with the Shannon diversity index revealed that 52% of the diversity was associated with differences occurring within fields, 8% was due to differences among fields within regions, and 40% was accounted for by differences among regions. Genotypic differentiation was observed between isolates collected in community gardens and in commercial potato fields. Canonical variate analysis grouped isolates from commercial potato fields together, regardless of the geographic distance between the fields. Isolates from community gardens differed among regions and differed from isolates collected in commercial potato fields.

INTRODUCTION

Phytophthora infestans (Mont.) de Bary, the fungus that causes late blight of potato (*Solanum tuberosum* L.) and tomato (*Lycopersicon esculentum* Mill.), is considered the most damaging pathogen of potatoes worldwide (Hooker, 1981). This oomycete is heterothallic, with two mating types designated A1 and A2. Pairing between isolates of opposite mating type results in the production of oospores.

Until the early 1980s, only the A1 mating type was distributed throughout the world; the A2 mating type was detected only in central Mexico. The appearance of the A2 mating type in Europe in 1981 (Hohl & Iselin, 1984) was the first indication that *P. infestans* populations in Europe were changing. Subsequently, the A2 mating type was detected in many countries in Europe (Malcolmson, 1985; Tantius *et al.*, 1986), Egypt (Shaw *et al.*, 1985), Japan (Mosa *et al.*, 1989) and the United States (Deahl *et al.*, 1991).

New allozyme genotypes were associated with the introduction of the A2 mating type into Europe, providing additional evidence for a major change in these populations (Spielman *et al.*, 1991). Because both mating types have been found within this "new" population, sexual reproduction of the pathogen is possible and can generate new genotypes with greater adaptability than in a strictly asexually reproducing population. Furthermore, functional oospores in soil can be an additional source of inoculum.

In a previous study (Fry *et al.*, 1991), we used allozymes and mating types to characterize isolates of *P. infestans* collected in The Netherlands. Significant differences in genotype frequencies were detected not only among isolates collected in different regions of the country, but also between isolates of different mating types and between isolates collected from potato and tomato (Fry *et al.*, 1991). Because we analyzed the diversity in only two allozymes, glucose phosphate isomerase (*Gpi*) and peptidase (*Pep*), and because the diversity for these allozymes is limited to two alleles, similarity of allozyme genotypes does not necessarily indicate a lack of genetic diversity among the isolates. The allozyme markers were simply not numerous enough to define an isolate-specific genotype. Hence, numerous polymorphic markers are necessary to distinguish *P. infestans* isolates unambiguously and to assess the level of genetic diversity accurately.

The availability of DNA fingerprint probes that detect numerous restriction fragment length polymorphisms within the genome of *P. infestans* (Goodwin *et al.*, 1992b) enables more detailed analysis of *P. infestans* isolates. Probe RG-57, which hybridizes to 25 different nuclear DNA fragments, is derived from a *P. infestans* genomic library and represents a moderately repetitive nuclear DNA sequence (Goodwin *et al.*, 1992b). Of the hybridizing fragments, 13 are known to segregate independently (Goodwin *et al.*, 1992b). The fragment patterns are somatically stable and are transmitted to sexual progeny in a Mendelian fashion. Therefore, probe RG-57 provides genetic markers that probably span a large part of the *P. infestans* genome (Goodwin *et al.*, 1992b), and RG-57 DNA fingerprinting is a powerful tool for analyzing the genetic structure of *P. infestans* populations (Goodwin *et al.*, 1992c). For example, RG-57 DNA fingerprinting of *P. infestans* isolates from northern Mexico revealed much greater diversity than was detected with *Gpi* and *Pep* allozyme markers (Goodwin *et al.*, 1992c).

In addition to nuclear DNA polymorphisms, the mitochondrial DNA of *P. infestans*, which is a circular DNA molecule of 36.2 kb (Klimzack & Prell, 1984), is polymorphic. Analysis of 14 *P. infestans* isolates from The Netherlands identified two mitochondrial DNA types, A and B (Goodwin, 1991). Type A is widely distributed throughout the world, whereas type B is restricted to places where the A2 mating type is found (Goodwin, 1991). Mitochondrial DNA is a useful marker for migration events because it has maternal inheritance only (Goodwin, 1991).

In the experiments described here, we attempted to determine the genotypic diversity within *P. infestans* populations in The Netherlands as revealed by DNA fingerprint probe RG-57, the number of *P. infestans* genotypes, the geographic distribution of these genotypes, and the types of mitochondrial DNA present in Dutch *P. infestans* isolates. These data will enhance our understanding of the population genetics of *P. infestans* and may be useful for disease management strategies in the future.

MATERIALS AND METHODS

Collection and culture of *P. infestans*

Isolates of *P. infestans* were collected in The Netherlands during the summer of 1989 as described by Fry *et al.* (1991) (Fig. 1). A two-level hierarchical sampling scheme (fourteen fields within six regions) was used to allow genetic diversity to be partitioned within and among regions (Table 1). Single-lesion samples were collected randomly from potato and tomato plants in community gardens and commercial potato fields. Community garden isolates from potato and tomato were collected from contiguous gardens. Fields within 40 km of each other were considered to be in the same region. The minimum and maximum distances between regions were 75 and 300 km, respectively.

Southern analysis

Mycelium for DNA extraction was grown in liquid Rye A medium (Caten & Jinks, 1968) in the dark at 18°C for about 14 days. Mycelium was ground to a fine powder in liquid nitrogen and mixed for 4 min at 55°C with 2.5 ml of extraction buffer containing three parts of water-saturated phenol, two parts of triisopropylnaphthalene sulfonic acid (20 mg/ml), two parts of 4-aminosalicylic acid (120 mg/ml), and one part of 5x RNB (1 M Tris/HCL, 1.25 M NaCl, 0.25 M ethylene glycol-bis-(2-aminoethyl ether) N,N,N',N'-tetraacetic acid). The mixture was shaken vigorously for 2 min, and 0.25 vol of chloroform-isoamyl alcohol (24:1, v/v) was added. The aqueous phase was then extracted once with an equal volume of phenol-chloroform-isoamyl alcohol (25:24:1, v/v/v) and twice with an equal volume of chloroform-isoamyl alcohol (24:1, v/v). Nucleic acids were precipitated with 0.6 vol of 2-propanol, dissolved in $T_{10}E_1$ (10 mM Tris-HCl [pH 8], 1 mM EDTA), and treated for 30 min with RNase A (20 μg/ml) at 37°C. This method yields 250-500 μg of DNA per gram (wet weight) of mycelium. For Southern blot analysis, approximately 5 μg of genomic DNA was digested with the restriction enzyme *Eco*RI according to the manufacturer's instructions and size-fractionated on a 0.8% agarose gel for 14-16 h at 40 V (600 Vh) before alkaline transfer to Hybond N^+ (Amersham) hybridization membrane.

Membranes were prehybridized for 2 h at 65°C in 10 ml of hybridization solution (5x SSC, 5x Denhardt's solution, 0.5% sodium dodecyl sulfate [SDS], 100 μg of salmon sperm DNA per milliliter). Probes were labelled with [α-^{32}P]dATP according to the random-primer labelling method of Feinberg and Vogelstein (1983) and allowed to hybridize overnight. Filters were then washed three times for 20 min each at 65°C in 2x SSC, 0.5% SDS, and three times for 20 min each in 0.5x SSC, 0.1% SDS, and exposed for 1-3 days at −80°C to Kodak Xomat S film backed with an intensifier screen. Probe RG-57 was removed from the membrane by incubation for 30 min in 0.4 N NaOH at 42°C. Membranes were then hybridized with 25 ng of total purified mitochondrial DNA (Goodwin, 1991) to reveal polymorphisms in the mitochondrial DNA.

Figure 1. The six regions in The Netherlands where isolates of *Phytophthora infestans* were collected in 1989: northeast (NE), north (N), northwest (NW), west (W), central (C) and southwest (SW). One to four fields were sampled in each region.

Table 1. Regions sampled for *Phytophthora infestans* in The Netherlands in 1989 and the occurrence of RG-57 genotypes by field.

Region[a]	Field	No. isolates	RG-57 genotypes
Commercial potato fields			
NE	1	7	28
NE	2	24	3,10,13,28,29,32
NE	3	2	22,28
NE	4	2	28
N	5	8	28
N	6	8	2,28,32
NW	7	15	28
NW	8	18	28
SW	14	14	9,20,24-30,33-35
Community potato gardens			
C	10[b]	15	6,8,12,15,16,18,19,23,28
W	11	12	22,31
W	12[c]	6	17,21
Community tomato gardens			
C	9[b]	15	1,4-7,11,14
W	13[c]	7	17

[a] NE = northeast, N = north, NW = northwest, C = central, W = west, SW = southwest.

[b] Fields 9 and 10 were contiguous.

[c] Fields 12 and 13 were contiguous.

Data analysis

A multicharacter genotype was derived for each isolate based on the DNA fingerprint pattern. The RG-57 genotypes were classified on the basis of the presence or absence of fragments, and each fragment was assumed to represent a single genetic locus (Goodwin *et al.*, 1992b). Isolates with the same RG-57 fingerprint pattern were considered to be identical genotypes.

The Shannon diversity index (Bowman *et al.*, 1971) was used to measure genotypic diversity as described previously (Goodwin *et al.*, 1992a). Genotypic diversity in each field was calculated as

$$h_o = - \sum_{i=1}^{k} p_i \ln pi,$$

where p_i is the frequency of isolates with the i^{th} genotype in the field and k is the number of genotypes in the field.

Because a two-level hierarchical sampling scheme (fields within regions) was used when isolates were collected, the total genotypic diversity can be partitioned into components based on the amount of diversity within and among subpopulations. The relative magnitude of each component was assessed following methods developed by Lewontin (1972), Zhang *et al.* (1987), and Goodwin *et al.* (1992a). For each region, h_{field} was calculated as the mean of h_o for all fields in the region, and h_{region} was calculated as the mean frequency of all genotypes within the region. The total diversity, h_{total}, was determined from the mean frequencies of all genotypes in the entire sample. The mean within-field and among-field within-region diversity values, $\bar{h}_{field}$ and $\bar{h}_{region}$, were the average h_{field} and h_{region} values, weighted by the number of fields in each region. The total diversity was allocated to hierarchical components as follows: $\bar{h}_{field} / h_{total}$ is the proportion of total diversity that is due to differences within fields; $(\bar{h}_{region} - \bar{h}_{field}) / (h_{total})$ is the proportion of total diversity due to differences among-fields within-regions; and $(h_{total} - \bar{h}_{region}) / h_{total}$ is the proportion of total diversity due to differences among-regions.

The data obtained with probe RG-57 (presence or absence of fragments) and mitochondrial DNA types (A or B) were also analyzed by canonical variate analysis (CVA) with the GENSTAT statistical package (Genstat 5, release 2.1; Rothamsted Experimental Station, Harpenden, United Kingdom). CVA is a multivariate technique that does not require any underlying distributional assumptions (Gittins, 1985). This analysis allows the detection of genotypic differentiation among groups of isolates, for example, isolates from different fields, rather than among the individual isolates. In the multidimensional space of the original variables, CVA selects directions such that the ratio of among-field diversity to within-field diversity exhibited in each direction is maximized, before reducing the number of dimensions. Thus, a large part of the diversity can be represented by the first two latent vectors, which can be plotted on two axes. In this way, genotypic differentiation among preassigned groups can be visualized. Groups were formed by fields (14 groups) to gain insight into the genotypic differentiation among fields.

RESULTS

Identification and geographic distribution of genotypes

DNA fingerprint patterns of 153 isolates collected in six different geographic regions in The Netherlands (Table 1) showed that probe RG-57 hybridized to 21 fragments of nuclear genomic DNA, 16 of which were polymorphic (Table 2). Fragments 1, 13, 20, 21 and 25 were invariant (Fig. 2). Thirty-five genotypes were identified among the 153 isolates (Table 3). Most of the genotypes (60%) were unique.

Fragment frequencies among isolates collected in the northeast and the north were fairly similar (Table 2). Of the fragments found in isolates collected in the north, only fragment 17, which was found at a low frequency in the north, was not found in isolates collected in the northeast. The isolates collected in the northwest did not show any diversity.

Isolates from the central region contained all the fragments found in the 1989 population (Table 2). Polymorphism of fragments 5, 10, 14, 18 and 24 was found only among these isolates, and they were the only isolates that contained fragment 18. Fragments 6, 9, 14a, 16 and 19 occurred at a much lower frequency in the isolates collected in the central region than in isolates collected from commercial potato fields in the northeast, north, northwest and southwest.

The isolates collected in the west lacked fragment 6 and had a relatively low frequency of fragments 9 and 16 compared to the isolates collected in commercial potato fields. Polymorphism for fragment 4 was restricted to isolates collected in the southwest, and these isolates had fragment 17 at a higher frequency than all other isolates.

Genotypic diversity varied among regions. In the central region and southwest regions, genotypic diversity was high: of the 21 RG-57 fragments identified in isolates collected in the central region, 15 were polymorphic, resulting in 15 different genotypes (Table 2); in the southwest, 12 genotypes were found. In the north and west, genotypic diversity was limited to three and four genotypes,respectively. The northwest had no diversity at all; all isolates had the same genotype.

Most of the genotypic diversity in the northeast was in field 2, in which six genotypes were found (Table 1). Genotype 28 was present in all four fields in the northeast. Genotype 28 was prevalent in the north; only two other genotypes, 2 and 32, were found there. In the northwest, all of the isolates from both of the fields sampled were genotype 28. In contrast, the isolates collected in the central region were very diverse. Field 9 contained seven genotypes, six of which were unique; the seventh (genotype 6) was also found in contiguous field 10. Seven of the nine genotypes found in field 10 were unique to this field. Field 11 in the west contained two genotypes; one (genotype 31) was unique, and the other (genotype 22) was also found in field 3 in the northeast. Fields 12 and 13 contained genotypes that were only found in the west. Field 14 in the southwest was infested by 12 genotypes, 10 of them unique to this field. The other two were the prevalent genotype 28 and genotype 29, which was also identified in field 2 in the northeast.

Table 2. Number of RG-57 genotypes, RG-57 hybridizing fragments and polymorphic fragments and their relative frequencies in *Phytophthora infestans* isolates collected in six regions in The Netherlands.

	Region[a]					
	NE	N	NW	C	W	SW
Different RG-57 genotypes	7	3	1	15	4	12
RG-57 hybridizing fragments	19	20	18	21	18	20
RG-57 polymorphic fragments	8	10	0	15	4	10
Fragment No.						
1	1	1	1	1	1	1
2	0.11	0.25	0	0.27	0.56	0.36
3	0.97	0.94	1	0.73	1	0.93
4	1	1	1	1	1	0.50
5	1	1	1	0.83	1	1
6	0.86	0.94	1	0.13	0	0.93
7	0.97	0.94	1	0.73	1	0.93
8	0.91	0.94	1	0.93	0.92	0.64
9	0.86	0.94	1	0.40	0.44	0.79
10	1	1	1	0.83	1	1
13	1	1	1	1	1	1
14	1	1	1	0.97	1	1
14a	1	0.94	1	0.40	1	1
16	0.97	0.94	1	0.20	0.56	0.86
17	0	0.06	0	0.30	0	0.64
18	0	0	0	0.03	0	0
19	0.91	0.94	1	0.50	1	0.93
20	1	1	1	1	1	1
21	1	1	1	1	1	1
24	1	1	1	0.97	1	1
25	1	1	1	1	1	1

[a] NE = northeast, N = north, NW = northwest, C = central, W = west, SW = southwest.

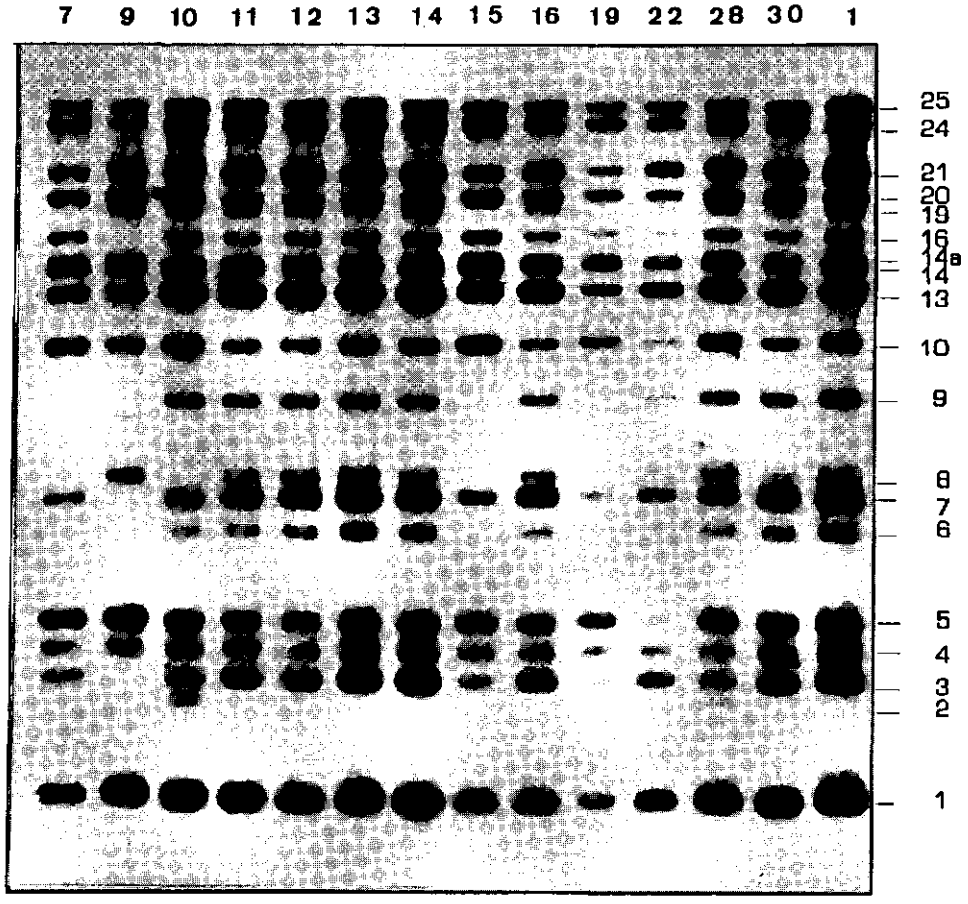

Figure 2. Autoradiograph of a Southern blot after hybridization with probe RG-57, showing DNA fingerprint patterns of representative isolates of *Phytophthora infestans* from the northeast region in The Netherlands. The numbers along the top are isolate numbers. Isolate 1 is from field 3; the other isolates shown are from field 2. The fragment numbers indicated at the right correspond to those used by Goodwin *et al.* (1992b,c). Six genotypes can be observed on this autoradiograph (see Table 1): isolate 9 is genotype 3, isolate 7 is genotype 10, isolate 10 is genotype 29, isolate 28 is genotype 32, isolates 15 and 19 are genotype 13, and isolates 1, 11-14, 16, 22 and 30 are genotype 28 (see Table 3 for genotypes).

Table 3. DNA fingerprint patterns (1 = presence, 0 = absence of each fragment) revealed by probe RG-57, the mitochondrial DNA types identified in each RG-57 genotype and the frequency of each genotype among 153 isolates of *Phytophthora infestans* from The Netherlands.

RG-57 genotype	RG-57 fragment[a]																					Mitochondrial DNA type	Frequency
	1	2	3	4	5	6	7	8	9	10	13	14	14a	16	17	18	19	20	21	24	25		
1	1	0	1	1	0	1	1	0	0	1	1	1	0	0	0	0	0	1	1	1	1	A	1
2	1	1	0	1	1	0	0	0	0	1	1	1	0	0	1	0	0	1	1	1	1	A	1
3	1	0	0	1	1	0	0	1	0	1	1	1	1	0	0	0	1	1	1	1	1	A	1
4	1	0	0	1	1	0	0	1	0	1	1	1	0	0	1	0	1	1	1	1	1	B	1
5	1	0	1	1	1	0	1	1	0	0	1	1	0	0	0	0	1	1	1	1	1	A	4
6	1	0	1	1	1	0	1	1	0	1	1	1	0	0	0	0	0	1	1	1	1	B	5
7	1	1	0	1	1	0	0	1	0	1	1	1	0	1	0	0	0	1	1	1	1	A	1
8	1	1	0	1	0	0	0	1	1	1	1	1	1	0	0	0	0	1	1	1	1	A	4
9	1	1	0	1	1	0	0	0	0	1	1	1	1	0	1	0	0	1	1	1	1	A	1
10	1	0	1	1	1	0	1	0	0	1	1	1	1	1	0	0	0	1	1	1	1	A	2
11	1	0	1	1	1	0	1	1	0	1	1	1	0	0	1	0	0	1	1	1	1	A	3
12	1	0	0	1	1	0	0	1	1	1	1	1	1	0	1	0	1	1	1	0	1	B	1
13	1	0	1	1	1	0	1	1	0	1	1	1	1	1	0	0	0	1	1	1	1	A	1
14	1	0	1	1	1	0	1	1	0	1	1	1	0	1	0	0	1	1	1	1	1	B	1
15	1	0	1	1	1	0	1	1	1	1	1	0	1	1	0	0	0	1	1	1	1	A	1
16	1	1	0	1	1	0	0	1	1	0	1	1	1	0	1	0	1	1	1	1	1	A	1
17	1	0	1	1	1	0	1	1	0	1	1	1	1	0	0	0	1	1	1	1	1	A	11
18	1	1	1	1	1	0	1	1	0	1	1	1	0	0	1	0	1	1	1	1	1	A	2
19	1	0	1	1	1	0	1	0	1	1	1	1	1	0	1	0	1	1	1	1	1	A	1
20	1	0	1	0	1	1	1	0	0	1	1	1	1	1	1	0	1	1	1	1	1	A	1
21	1	1	1	1	1	0	1	0	0	1	1	1	1	1	0	0	1	1	1	1	1	B	2
22	1	1	1	1	1	0	1	1	0	1	1	1	1	1	0	0	1	1	1	1	1	A	2
23	1	0	1	1	1	0	1	1	1	1	1	1	1	0	0	1	1	1	1	1	1	A	1
24	1	0	1	0	1	1	1	1	1	1	1	1	1	0	1	0	1	1	1	1	1	A	1
25	1	0	1	0	1	1	1	1	0	1	1	1	1	1	1	0	1	1	1	1	1	A	1
26	1	1	1	0	1	1	1	0	1	1	1	1	1	1	0	0	1	1	1	1	1	A	1
27	1	0	1	0	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	A	1
28	1	0	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	A	77
29	1	1	1	1	1	1	1	0	1	1	1	1	1	1	0	0	1	1	1	1	1	A	2
30	1	0	1	0	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	A	2
31	1	1	1	1	1	0	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	A	11
32	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	0	1	1	1	1	1	A	5
33	1	1	1	1	1	1	1	0	1	1	1	1	1	1	1	0	1	1	1	1	1	A	1
34	1	0	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	A	1
35	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0	1	1	1	1	1	A	1

[a] Fragment numbers correspond to those used by Goodwin *et al.* (1992b,c).

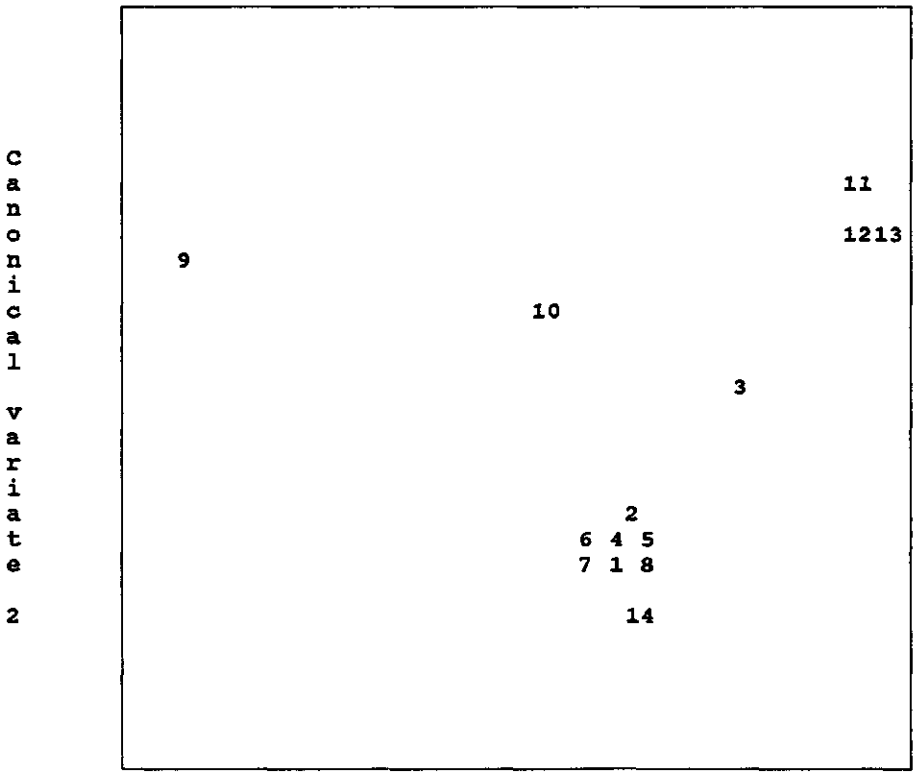

Figure 3. Plot of the canonical variate means in the first two dimensions that represent the 14 fields (1-14) in which isolates of *Phytophthora infestans* were collected. Mitochondrial DNA type (A or B) and the 16 polymorphic RG-57 fragments were used as input in the canonical variate analysis. The *x*-axis shows the first canonical vector and represents 40.5 % of the total diversity. The *y*-axis shows the second canonical vector and represents 31.3 % of the diversity. The first and second canonical variate means showed ratios of among-field to within-field variation in each dimension of 8.2 and 6.3, respectively. Clustering can be observed among isolates collected in fields 11-13 (community gardens) from the west region and among isolates collected in fields 1, 2, 4-8 and 14 (commercial potato fields) in the northeast, north, northwest and southwest regions (see Table 1). Genotypic differentiation is clearly present among isolates collected in fields 9 and 10, fields 11-13, and fields 1, 2, 4-8 and 14.

Seventy-seven out of the 153 isolates had genotype 28 (Table 3). This genotype was found in 10 fields and in every region except the west. Eleven isolates had genotype 17, which occured only in fields 12 and 13 in the west (Table 1). Genotype 31 was identified in 11 isolates, all from field 11 in the west. Two isolates from field 2 (northeast) and three from field 6 (north) had genotype 32. In field 9 (central), five isolates had genotype 6. Genotypes 22 and 29 were each found twice, genotype 22 in fields 3 (northeast) and 11 (west) and genotype 29 in fields 2 (northeast) and 14 (southwest). Four genotypes (10, 18, 21 and 30) were each found twice in the same field. Twenty-one genotypes were found in only one isolate each (Table 3).

Occurrence of mitochondrial DNA types

Two mitochondrial DNA types were observed among the 153 isolates analyzed. They are identical to the A and B types described by Goodwin *et al.* (1991) that correspond to types I and II described by Carter *et al.* (1990, 1991). Isolates that belonged to the same RG-57 genotype always had the same type of mitochondrial DNA. Type A, found in 30 genotypes among 143 isolates, was most common and was found in isolates from all 14 fields. Type B was found in 10 isolates with five genotypes. These isolates were collected in gardens in the central and west regions, where the frequency of type B was 0.27 and 0.08, respectively.

Hierarchical components of genotypic diversity

When total genotypic diversity, estimated by the Shannon diversity index based on data obtained with probe RG-57, was partitioned into hierarchical components by field within regions, the within-fields component of diversity was 52%, the among-fields within-regions component was 8%, and the among-regions component was 40%.

Genotypic differentiation among fields

The 14 fields were used as groups in a CVA to search for genotypic differentiation among fields (Fig. 3). The first canonical variate accounted for 40.5% of the diversity and the second for 31.3% of the diversity. It is evident from Figure 3 that fields 1, 2, 4-8, and 14 are clustered. This cluster comprises commercial potato fields located in four regions. Fields 11-13, all in the west, form another cluster. Isolates from field 9, collected from tomato plants in a community garden complex (Table 1), were the most distinct from other isolates. Isolates from field 13 were also collected from tomato plants but were very similar to the isolates collected from potato in the neighboring field 12. Field 3, which contained many different genotypes (Table 1), is situated in the CVA plot somewhat away from the cluster of other commercial potato fields. The CVA plot shows that the isolates from community gardens (fields 9-13 from the central and west regions) form different clusters than the isolates from commercial potato fields. All the commercial potato fields except field 3 form one cluster regardless of their geographic locations.

DISCUSSION

DNA fingerprinting of isolates with probe RG-57 revealed significantly more diversity in the *P. infestans* population in The Netherlands than had been detected previously. Population analysis based on allozymes, mating type, and metalaxyl resistance identified 17 genotypes among 205 isolates (Fry *et al.*, 1991), compared to 35 genotypes revealed among 153 isolates by RG-57 DNA fingerprinting. Combining all markers distinguishes 45 genotypes among the 153 isolates.

Several populations that showed limited or no diversity in previous studies (Fry *et al.*, 1991) were demonstrated to consist of different RG-57 genotypes. For example, the combination of allozymes, mating type, and metalaxyl resistance differentiated three genotypes among 17 isolates from field 14 in the southwest, whereas probe RG-57 revealed 12 genotypes among 14 isolates analyzed. Thus, allozyme uniformity does not necessarily imply lack of genetic diversity in *P. infestans* populations. This is especially true in The Netherlands, where only two alleles for *Gpi* and two for *Pep* have been found (Fry *et al.*, 1991). Similar findings were reported recently by Goodwin *et al.* (1992c), who used RG-57 DNA fingerprinting to analyze the Mexican *P. infestans* population structure.

In the hierarchical sampling scheme, most of the overall genotypic diversity occurred at the lowest level, within the fields. This high within-field component of diversity was largely due to the many unique genotypes in fields 2, 9, 10 and 14. The low level of genotypic diversity among fields within regions is indicative of high levels of gene flow within regions, either from migration of airborne sporangia or from common sources of seed potatoes. Migration probably occurs freely over large parts of The Netherlands, which would explain why genotype 28 was found in five of the six regions. In the west, the only region where genotype 28 was absent, the two genotypes identified (17 and 21) were found nowhere else. This region has very little commercial potato production, which may limit migration from the rest of the country.

The genotypic differentiation among regions is primarily due to the west and central regions, which differed from the other regions, as can be seen in the CVA plot (Fig. 3). The clustering of commercial potato field isolates in the CVA suggests a common source of inoculum for these fields. Multivariate analysis, in this case CVA, is more sensitive in revealing genotypic differentiation associated with geography than single-locus methods (Yeh *et al.*, 1985). Further investigations are needed to determine why isolates collected in community gardens in the west and central regions are different from each other as well as different from isolates collected in commercial potato fields in other regions.

Partial host substructuring was observed within the *P. infestans* population in The Netherlands (Fig. 3). The isolates in fields 9 (central) and 13 (west) were collected from tomato. Six of the seven genotypes found in field 9 were unique to this field, but the remaining genotype was also present in the potatoes in the neighboring field 10 (Table 1). And the single genotype identified in isolates collected from tomatoes in field 13 was also found in potatoes in the neighboring field 12. Evidently, some genotypes can occur on both hosts in The Netherlands.

Hence, population substructuring by host, as reported in the literature (Fry *et al.*, 1991; Giddings & Berg, 1919; Turkensteen, 1973), was not complete.

The occurrence of two types of mitochondrial DNA in *P. infestans* isolates from The Netherlands is comparable to the situation in the United Kingdom (Carter *et al.*, 1990). The low frequency of isolates with type B can be explained by limited introduction of this type or by selection against this type. Why isolates with type B were restricted to the community gardens is unclear. Type B was found on both potato and tomato in the central region but only on potato in the west. Type B was found in A1 as well as A2 mating type isolates. Type A is most common throughout the world outside central Mexico (Goodwin, 1991). Type B has been found in northern Mexico and in the countries where A2 mating type isolates have been introduced, such as Poland, The Netherlands, Japan, Brazil, Egypt, the United Kingdom and the United States (Carter *et al.*, 1990; Goodwin, 1991). Most likely, type B was introduced with the "new" *P. infestans* population.

The introduction of A2 mating type isolates into Europe has opened the possibility of sexual reproduction of *P. infestans*. Pairing of A1 and A2 mating type isolates from The Netherlands *in vitro* resulted in viable progeny (Goodwin *et al.*, 1992b). The pattern of diversity we found in The Netherlands is consistent with the hypothesis that sexual reproduction generates new genotypes and that occasional fit recombinants (e.g., genotype 28) become widespread as a result of rapid asexual reproduction. However, testing this hypothesis will be difficult because of the many genotypes that can survive asexually between seasons.

Because RG-57 DNA fingerprinting has also been used to asses genotypic diversity in *P. infestans* populations in central and northern Mexico (Goodwin *et al.*, 1992c), we can compare those populations with our isolates from The Netherlands. The only difference in the fingerprint analyses was the scoring of fragment 14a, which was not scored in the fingerprints of the Mexican isolates because it often comigrates with fragment 14. However, fragment 14a is found in Mexican isolates. All the fragments identified in the isolates collected in The Netherlands are also present in Mexican isolates, and isolates collected in central Mexico appear to have a few additional polymorphic RG-57 fragments (11, 12, 15, 22, 23, 24a and 25a) at low frequencies (Goodwin *et al.*, 1992c). This indicates that the present Dutch isolates belong to a subpopulation that originated from the *P. infestans* population in Mexico.

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REFERENCES

Bowman, K.O., Hutcheson, K., Odum, E.P. & Shenton, L.R., 1971. Comments on the distribution of indices of diversity. p 315-359 in: Patil, G.P., Pielou, E.C. & Waters W.E. (eds.). Statistical Ecology Volume 3. Many species populations, ecosystems, and system analysis. The Pennsylvanian State University Press, University Park and London. pp 462.

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

Carter, D.A., Archer, S.A., Buck, K.W., Shaw, D.S. & Shattock, R.C., 1991. DNA polymorphisms in *Phytophthora infestans*: the UK experience. p 272-294 in: Lucas, J.A., Shattock, R.C., Shaw, D.S. & Cooke, L.R. (eds.). *Phytophthora*. British Mycological Society, Cambridge University Press, Cambridge. pp 447.

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

Deahl, K.L., Goth, R.W., Young, R., Sinden, S.L. & Gallegly, M.E., 1991. Occurrence of the A2 mating type of *Phytophthora infestans* in potato fields in the United States and Canada. American Potato Journal 68: 717-726.

Feinberg, A.P. & Vogelstein, B., 1983. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Analytical Biochemistry 132: 6-13.

Fry, W.E., Drenth, A., Spielman, L.J., Mantel, B.C., Davidse, L.C. & Goodwin, S.B., 1991. Population genetic structure of *Phytophthora infestans* in the Netherlands. Phytopathology 81: 1330-1336.

Giddings, N.J. & Berg, A., 1919. A comparison of the late blights of tomato and potato: A Preliminary Report. Phytopathology 9: 209-211.

Gittins, R., 1985. Canonical Analysis: A review with applications in ecology. Berlin Springer-Verlag, Biomathematics 12. pp 351.

Goodwin, S.B., 1991. DNA polymorphisms in *Phytophthora infestans*: the Cornell Experience. p 256-271 in: Lucas, J.A., Shattock, R.C., Shaw, D.S. & Cooke, L.R. (eds.), *Phytophthora*. British Mycological Society, Cambridge University Press, Cambridge. pp 447.

Goodwin, S.B., Allard R.W., Hardy, S.A. & Webster, R.K., 1992a. Hierarchical structure of pathogenic variation among *Rhynchosporium secalis* populations in Idaho and Oregon. Canadian Journal of Botany 70: 810-817.

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

Goodwin, S.B., Spielman, L.J., Matuszak, J.M., Bergeron, S.N. & Fry, W.E., 1992c. Clonal diversity and genetic differentiation of *Phytophthora infestans* populations in northern and central Mexico. Phytopathology 82: 955-961.

Hohl, H.R. & Iselin, K., 1984. Strains of *Phytophthora infestans* with A2 mating type behavior. Transactions of the British Mycological Society 83: 529-530.

Hooker, W.J., 1981. (ed.) Compendium of potato diseases. American Phytopathological Society. St. Paul. MN. pp 123.

Klimzack, L.J. & Prell, H.H., 1984. Isolation and characterization of mitochondrial DNA of the oomycetous fungus *Phytophthora infestans*. Current Genetics 8: 323-326.

Lewontin, R.C., 1972. The apportionment of human diversity. Evolution Biology 6: 381-398.

Malcolmson, J.F., 1985. *Phytophthora infestans* A2 compatibility type recorded in Great Britain. Transactions of the British Mycological Society 85: 531.

Mosa, A.A., Kato, M., Sato, N., Kobayashi, K. & Ogoshi, A., 1989. Occurrence of the A2 mating type of *Phytophthora infestans* on potato in Japan. Annals Phytopathology Society Japan 5: 615-626.

Shaw, D.S., Fyfe, A.M., Hibberd, P.G. & Abdel-Sattar, M.A., 1985. Occurrence of the rare A2 mating type of *Phytophthora infestans* on imported Egyptian potatoes and the production of sexual progeny with A1 mating types. Plant Pathology 34: 552-556.

Spielman, L.J., Drenth, A., Davidse, L.C., Sujkowski, L.J., Gu, W.K., Tooley, P.W. & Fry, W.E., 1991. A second world-wide migration and population displacement of *Phytophthora infestans*? Plant Pathology 40: 422-430.

Tantius, P.H., Fyfe, A.M., Shaw, D.S. & Shattock, R.C., 1986. Occurrence of the A2 mating type and self fertile isolates of *Phytophthora infestans* in England and Wales. Plant Pathology 35: 578-581.

Turkensteen, L.J., 1973. Partial resistance of tomatoes against *Phytophthora infestans*, the late blight fungus. Agricultural Research Report No. 810. Instituut voor Plantenziektenkundig Onderzoek, Wageningen, Nederland. Mededeling. No. 633. pp. 88.

Yeh, F.C., Cheliak, W.M., Dancik, B.P., Illingworth, K., Trust, D.C. & Pryhitka, B.A., 1985. Population differentiation in logdepole pine, *Pinus contorta* spp. *latifolia*: A discriminant analysis of allozyme variation. Canadian Journal of Genetics and Cytology 27: 210-218.

Zhang, Q., Webster, R.K. & Allard, R.W., 1987. Geographical distribution and associations between resistance to four races of *Rhynchosporium secalis*. Phytopathology 77: 352-357.

Chapter 7

Acquisition of sexual reproduction and rapid evolution of *Phytophthora infestans*

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European Journal of Plant Pathology (1994) in press, in adapted form

ABSTRACT

Sexual reproduction is generally thought to result in faster rates of evolution (Maynard-Smith, 1971; Stearns, 1987; Michod & Levin, 1988). However, few have been the opportunities to assess how acquisition of sexual reproduction affects the immediate evolution of a species. Here we demonstrate that acquisition of sexual reproduction in the eukaryotic plant pathogen *Phytophthora infestans* has resulted in (i) an increase in virulence diversity, (ii) the appearance of new virulence factors, (iii) a dramatic increase in overall genetic diversity, (iv) an alternative mode of overwintering in soil by means of oospores, and (v) possible faster rates of adaptation to environmental changes.

INTRODUCTION

The oomycetous fungus *Phytophthora infestans* (Mont.) de Bary causes late blight, one of the most devastating diseases of potato worldwide. In Europe the first late blight epidemic occurred in 1845 and this resulted in the notorious Irish famine. *P. infestans* is heterothallic with two known mating types, A1 and A2. When A1 and A2 mating type thalli grow in close vicinity sexual spores (i.e. oospores) can be formed. Formation of male (antheridia) and female (oogonia) sexual organs on each thallus is induced by hormones (Ko, 1980). Physical contact of an antheridium and an oogonium of opposite mating types leads to the production of oospores. Oospores have been found in central Mexico, which is thought to be the center of origin of *P. infestans*. There two interacting species, *P. infestans* and its solanaceous hosts, *Solanum* spp., co-evolved (Gallegly & Galindo, 1958; Smoot *et al.*, 1958). The isolate(s), which initially originated from this area and spread worldwide, had the A1 mating type only. From 1845 until 1980 indeed only A1 mating type isolates were found in Europe, restricting *P. infestans* for at least 135 years to asexual reproduction. Around 1980 A2 mating type isolates appeared in Europe (Hohl & Iselin, 1984), Africa, Asia and North America (Spielman *et al.*, 1991; Fry *et al.*, 1992, 1993), most likely due to a new escape from central Mexico. Around the same time outbreaks of the disease in The Netherlands and other European countries began to occur more often and became more difficult to control. It was hypothesized that these phenomena were caused by the appearance of A2 mating type isolates which in the presence of the already existing A1 mating type isolates, enabled *P. infestans* to reproduce sexually. In the case of a plant pathogen the change from asexual to sexual reproduction, and the resulting increased adaptability and ability to survive outside the host, may interfere drastically with the regular disease control methods. In order to determine whether acquisition of sexual reproduction was truly the cause of the observed phenomena we first analyzed the changes in genetic diversity in *P. infestans* populations by comparing virulence properties and DNA fingerprints of A1 mating type isolates collected before 1980 (called "old" isolates) and A1 and A2 mating type isolates collected after 1980 (called "new" isolates).

RESULTS AND DISCUSSION

Among the "old" isolates, diversity for virulence was limited to only five of the eleven known virulence factors and these five (i.e. 1, 2, 3, 4, 10) corresponded to the resistance genes used in the common potato cultivars grown in the Netherlands (Fig. 1). Only eight different races (defined by their ability to overcome specific resistance genes, or combinations of specific resistance genes, in the host) were identified among 148 isolates tested (Table 1). After 1980, the same five virulence factors occurred but now they rapidly appeared in many new combinations and as a consequence new races developed. When only the "old" virulence factors 1, 2, 3, 4 and 10 were taken into consideration 26 races with different virulence patterns were found among 253 isolates tested. New virulence factors (i.e. 5, 6, 7, 8, and 11), not strictly required to colonize the prevailing potato cultivars grown in The Netherlands, showed up (Fig. 1) and increased the total number of races with different virulence patterns from 26 to 73 among the 253 isolates tested (Table 1). Obviously, the diversity for virulence patterns increased significantly, a conclusion supported by statistical analysis using the normalized Shannon diversity index (Sheldon, 1969; Goodwin *et al.*, 1992b) (Fig. 2c).

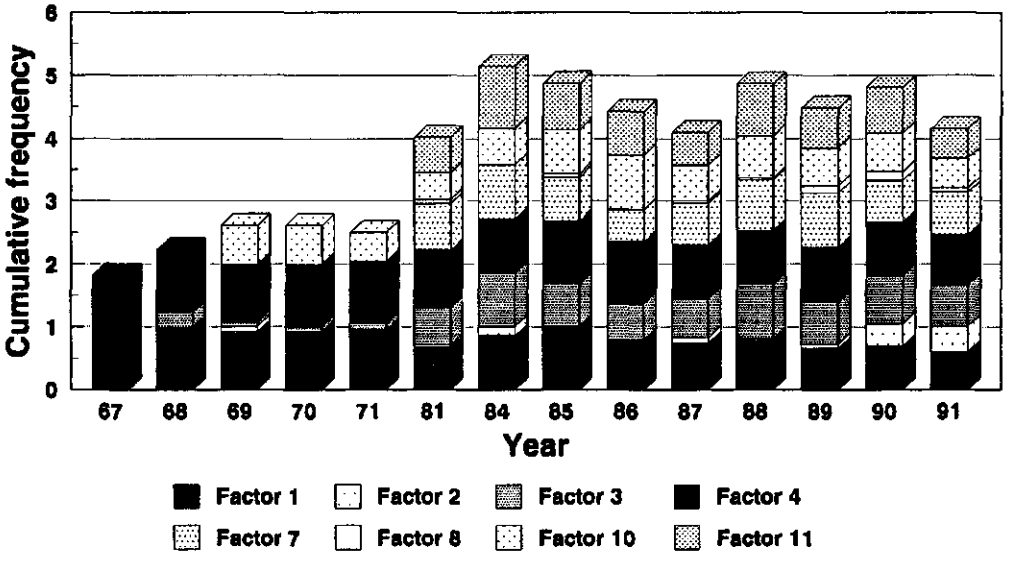

Figure 1. Yearly cumulative frequency of virulence factors in 148 "old" (1967-1971) and 253 "new" (1981-1991) *P. infestans* isolates. For the number of isolates and races per year see Table 1. Virulence factors 5 and 6 are not included in the bars. They were not found in the "old" isolates and only at very low frequency in the "new" isolates (0.011 and 0.023, respectively).

Table 1. The number of *P. infestans* isolates analyzed for virulence (1), DNA fingerprinting (2) and the number of different races and RG-57 genotypes identified in "old" and "new" isolates.

	Virulence		DNA fingerprinting	
Year[1]	#Isolates	Races[2]	#Isolates	RG-57 genotypes
66			1	1
67	39[3]	5		
68	63[3]	3	1	1
69	13[3]	4		
70	13[3]	4	1	1
71	20[3]	4		
74			1	1
78			1	1
Totals "old"	148	8[4]	5	1
81	46[5]	19 (12)	5	5
84	7	5 (5)	4	4
85	16	6 (4)	13	13
86	14	8 (4)	9	7
87	23	15 (12)	17	17
88	32	15 (8)	30	25
89	56	25 (14)	57	40
90	44	29 (18)	30	24
91	15	12 (9)	14	12
Totals "new"	253	73[6] (26)	179	134

[1] The year when the isolates were collected.
[2] In brackets: the number of races when only virulence factors 1,2,3,4, and 10 are considered.
[3] Data collected and published by Mooi (1967, 1968, 1971).
[4] These races contain different combinations of virulence factors 1,2,3,4,10.
[5] The virulence data from 38 of these isolates were collected by L.C. Davidse, unpublished.
[6] These races contain different combinations of virulence factors 1,2,3,4,5,6,7,8,10,11.

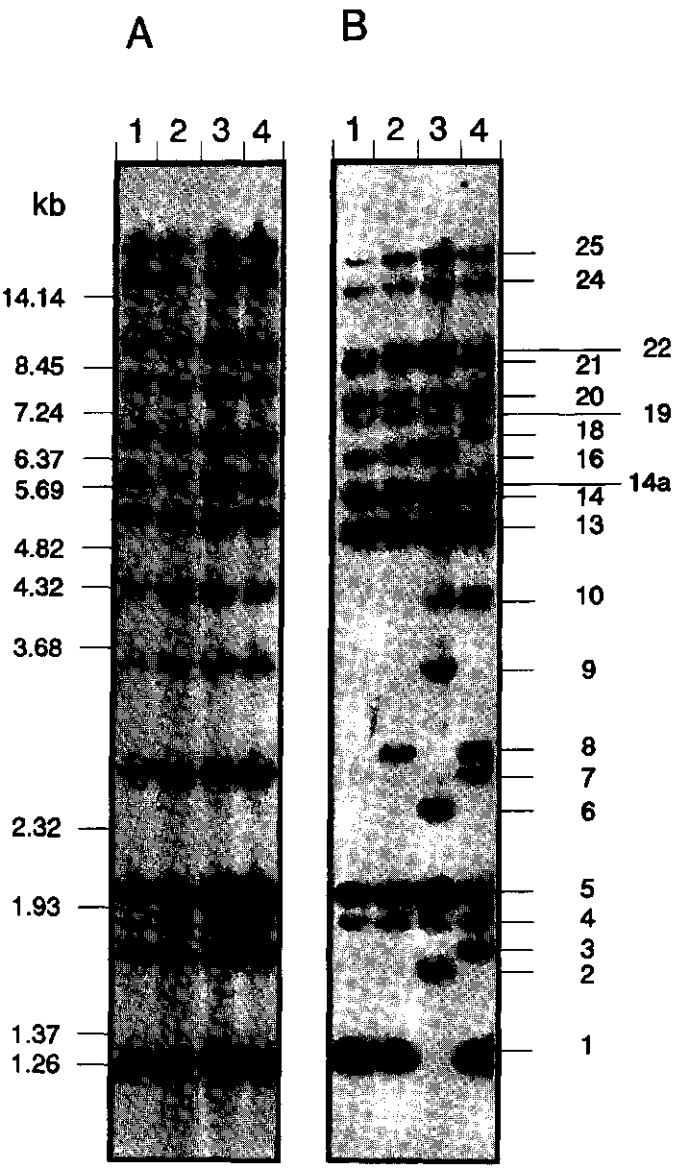

Figure 2a. RG-57 DNA fingerprinting patterns from "old" and "new" *P. infestans* isolates. The "old" isolates shown in (a) were collected in 1966 (1), 1968 (2), 1974 (3) and 1978 (4). The "new" isolates shown in (b) were collected in 1986 (1) and in 1988 (2, 3 and 4). Size markers in kilobases (kb) are indicated on the left. RG-57 fragment numbers, corresponding to those used by Goodwin *et al.* (1992a,b) and Drenth *et al.* (1993), are indicated on the right. Fragments 9a, 12, 17, 23 and 24a are missing in the four RG-57 genotypes shown here but are present in other "new" RG-57 genotypes not shown in this figure.

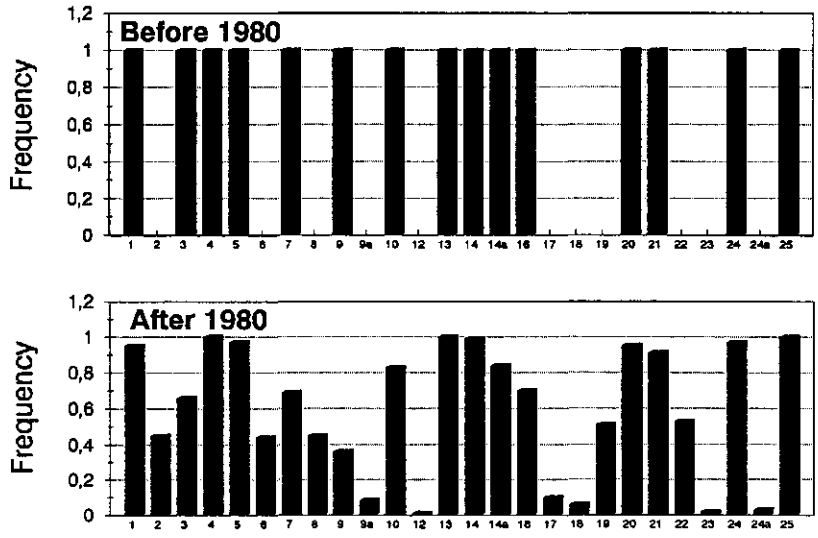

Figure 2b. Relative frequency of RG-57 hybridizing fragments in 5 "old" and 179 "new" *P. infestans* isolates. The fragment numbers indicated on the x axis correspond to those indicated on the autoradiograph in (A). The total number of different genotypes that were identified are summarized in table 1. When "old" UK and German isolates are included the relative frequency does not change.

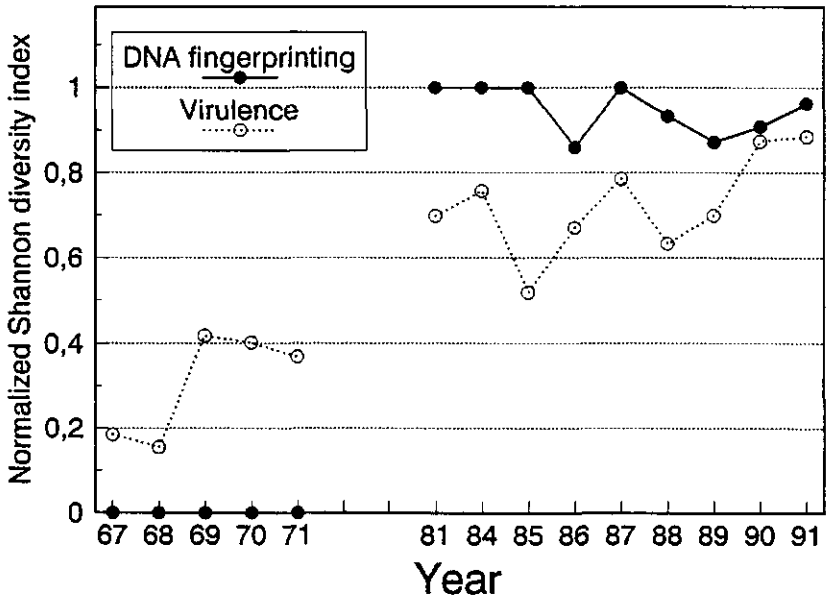

Figure 2c. Genetic diversity as calculated by the normalized Shannon diversity index (3) for virulence and DNA fingerprinting by probe RG-57 for *P. infestans* populations collected between 1967 and 1991.

For DNA fingerprinting the moderately repetitive DNA probe RG-57 was used. This probe, obtained from a genomic *P. infestans* library, hybridizes to several unlinked loci (Goodwin *et al.*, 1992a) and provides isolate-specific DNA fingerprints (Goodwin *et al.*, 1992a,b; Drenth *et al.*, 1993). On genomic blots containing DNA isolated from "old" *P. infestans* isolates, collected in The Netherlands between 1966 and 1978, probe RG-57 hybridizes to fifteen different DNA fragments, none of which is polymorphic (Fig. 2a,b). Sixteen other "old" isolates collected in Europe before 1980 and available for DNA fingerprinting analyses (ten from Germany and six from the United Kingdom) exhibit the same RG-57 genotype (data not shown). One of the six UK isolates (collected in 1978) is slightly different. Although this isolate displays the same RG-57 pattern as the others, it has one additional RG-57 hybridizing fragment. The DNA fingerprinting data show that over at least 12 years the *P. infestans* population in western Europe consisted of one RG-57 genotype which is consistent with the notion that *P. infestans* reproduced asexually. From the "new" isolates collected between 1981 and 1991 in The Netherlands, 179 were analyzed by DNA fingerprinting and among those 134 different RG-57 genotypes were found (Table 1). The number of hybridizing RG-57 fragments per isolate varied between 10 and 19, and in total, 26 different RG-57 hybridizing fragments could be detected of which 23 were polymorphic (Fig. 2a,b). Apparently, genetic diversity of the *P. infestans* population increased dramatically and this is again supported by statistical analyses (Fig. 2c). Every isolate collected in 1981 (5 in total), 1984 (4), 1985 (13) and 1987 (17) represented a unique genotype (Table 1). Only thirteen of the 134 different RG-57 genotypes, about 10 %, were identified in at least two subsequent years, suggesting that these isolates survived asexually as mycelium in stored seed potatoes, potato refuse piles or volunteer potatoes. Hence, every year the genetic structure of the fungal population was different and this is typical for a sexually reproducing population with non-overlapping generations (Maynard-Smith, 1971).

The observation that the predominant RG-57 genotype found in "old" isolates was not found in "new" isolates proves the hypothesis by Spielman *et al.* (1991) that the current, "new", population consisting of A1 and A2 mating type isolates completely replaced the "old" A1 mating type population. There are ample indications that the "new" population again originated from Mexico (Spielman *et al.*, 1991; Niederhauser, 1991). Many different virulence factors and complex races are known to occur there (Rivera-Peña, 1990). All RG-57 hybridizing fragments, which are found in the "new" *P. infestans* population in The Netherlands, were also found in Mexican *P. infestans* isolates (Goodwin *et al.*, 1992b; Drenth *et al.*, 1993).

Both virulence and DNA fingerprinting analyses demonstrated a marked increase of genetic diversity, in *P. infestans* populations collected after the appearance of "new" A1 and A2 mating type isolates in 1980 (Fig. 2c). Our results strongly suggest that around 1980 the *P. infestans* population has acquired the ability of sexual reproduction which, in turn, is the driving force behind the high level of genetic diversity. To support this suggestion we searched for the occurrence of oospores in the field and investigated whether the Dutch climate allows survival of oospores in soil between growing seasons. In 1992 we found oospores in blighted potato and tomato plants in the field. Previously we showed that the isolates 80029 (A1 mating type) and 88133 (A2) can produce viable sexual progeny *in vitro* (an *in vitro* produced oospore is shown in Fig. 3a) and that their sexual and asexual progeny can be distinguished by DNA fingerprinting (Goodwin *et al.*, 1992a). We used the same isolates for oospore production *in*

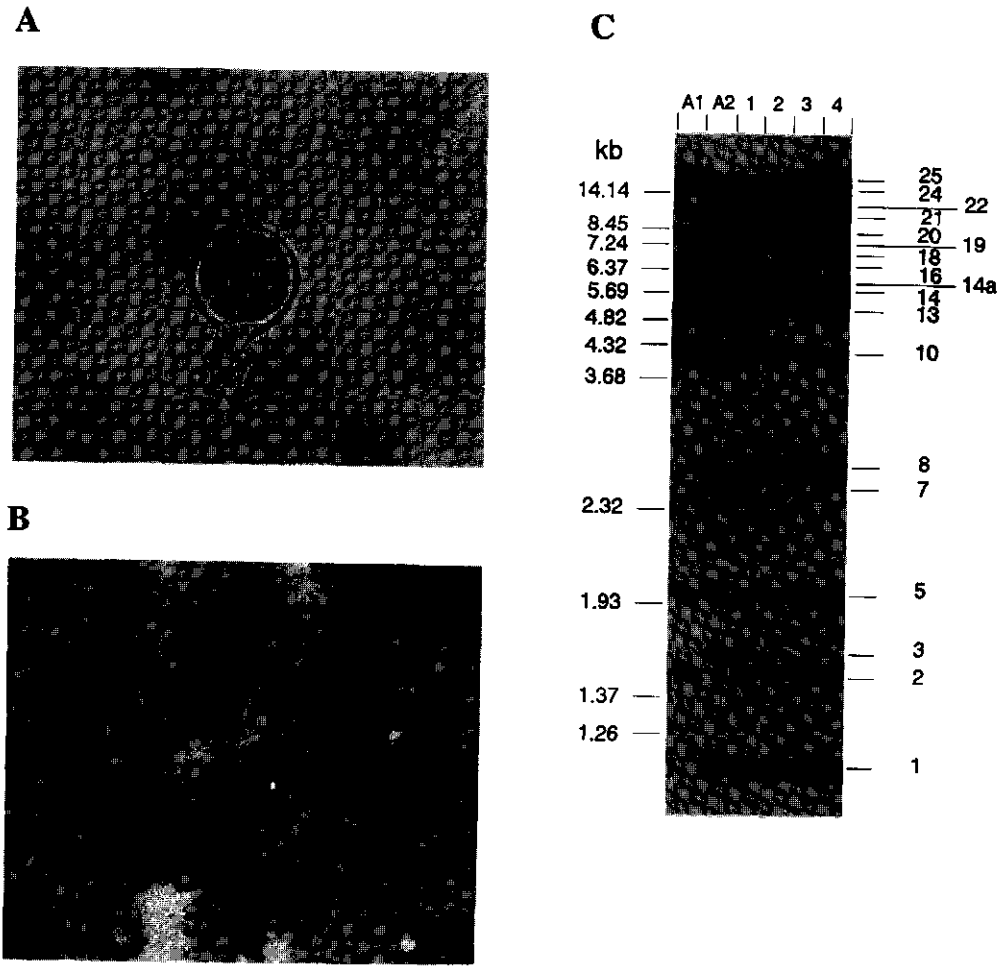

Figure 3. (A) *In vitro* produced oospore of *P. infestans* (4). (B) *In planta* produced oospores of *P. infestans* (5). (C) RG-57 DNA fingerprinting patterns of the *P. infestans* A1 mating type isolate 80029 (A1) and A2 mating type isolate 88133 (A2), and four sexual progeny (1, 2, 3 and 4) obtained from eight months old, overwintered oospores produced in leaves infested with the isolates 80029 and 88133. Besides the three recovered isolates of which the DNA fingerprints are shown here, seventeen others were analyzed and, based on their RG-57 DNA fingerprints, they all appeared to be sexual progeny from 80029 and 88133 (6).

planta (Fig. 3b). Infested leaves, containing oospores, were mixed with soil and exposed to natural weather conditions during the winter of 1992-1993. After eight months the soil still contained infectious material. DNA fingerprinting confirmed that infections on leaves, which contacted the soil containing the oospores, indeed resulted from oospores and were not caused by mycelium or vegetative spores from any of the two parental isolates (Fig. 3c). In addition, we found that soil samples taken in June 1993 from potato fields, which had been inoculated with a mixture of the isolates 80029 and 88133 the year before, were still infectious. DNA fingerprinting showed that these infections were caused by progeny from the two parents used to inoculate the field plots in 1992.

In summary, we have shown that there has been a sudden change in the level of genetic diversity in the Dutch *P. infestans* population around 1980. The change from little, if any, diversity in the "old" population to a very high level of diversity in the "new" population coincided with the appearance of new A1 and A2 mating type isolates in Europe. We demonstrated that oospores can overwinter in Europe and we conclude that sexual reproduction of *P. infestans* in the field is the cause of the high level of genetic diversity.

Whether or not sexual reproduction has advantages over asexual reproduction has been a point of much debate (Stearns, 1987; Michod & Levin, 1988; Charlesworth, 1989). In the case of *P. infestans* the advantages are obvious. Firstly, *P. infestans* is a hemibiotrophic pathogen of which the asexual spores, in contrast to oospores, cannot survive for long periods outside the host plant. With the production of oospores *P. infestans* has acquired the ability to survive in soil between growing seasons, thereby increasing its possibilities to colonize new host plants and to propagate. Secondly, sexual reproduction results in a wider variety of genotypes and this, in turn, leads to an increased ability to respond to changes in the environment and to selection pressure (Maynard-Smith, 1978). This is illustrated by the speed at which the "new" *P. infestans* population developed resistance to the fungicide metalaxyl. Soon after introduction of the "new" population in Europe, around 1980, metalaxyl resistant isolates appeared in the field (Davidse *et al.*, 1981). In some countries, where the "new" sexually propagating population did not yet appear, metalaxyl is still used successfully to control late blight. So far, metalaxyl resistant isolates have not been detected in "old" asexually propagating *P. infestans* populations throughout the world (Fry *et al.*, 1993). Thirdly, the increased diversity of virulence patterns and the appearance of new virulence factors, through sexual recombination, enables the fungus to avoid recognition by any specific resistance gene, or combinations of resistance genes, in the potato population.

Established disease management strategies are based on the biological characteristics of the "old" asexual *P. infestans* population. With the asexual propagating population infections originated from mycelium retained in seed potatoes or in potato refuse piles which initiated typical focal epidemics. Hence, control measures involved disease free seed potatoes and removal of potato refuse piles. With the acquisition of sexual reproduction, resulting in the presence of oospores in soil, late blight epidemics can start anywhere in the field as soon as potatoes have emerged. Thus, a general epidemic, originating from oospores, may appear despite sufficient removal of infected tubers containing mycelium. Moreover, in a sexually propagating population more aggressive strains can easily emerge and this will seriously interfere with the current efforts of breeders to create potato cultivars with durable resistance. At present it is difficult to envisage all practical implications for crop protection of a sexually propagating and

more diverse *P. infestans* population and, therefore, it is a challenge for the future to cope with sexual reproduction in the potato late blight fungus.

NOTES

1. Virulence

Virulence was determined by assaying compatibility and incompatibility in a differential set of potato lines. Detached leaflets from different potato lines carrying single resistance genes 1 to 11, with the exception of 9, and combinations 1-2, 1-3, 1-4, 2-3, 2-4, 1-2-3, 1-3-10, 1-4-10 and 1-2-3-4, were sprayed with a spore suspension of 10^4 spores per ml. After six days of incubation at 15 °C, 16 hours light, the leaflets were examined for the occurrence of late blight lesions. If sporulation was observed, the interaction was rated compatible; if no sporulation was observed, the interaction was rated incompatible. For most isolates the virulence was determined at least twice. The same differential set and method was used by J.C. Mooi (1967, 1968, 1971) (148 "old" isolates, 1967-1971) and L.C. Davidse (1981) (38 "new" isolates, 1981).

2. DNA fingerprinting

DNA isolation, *Eco*RI digestion, Southern blotting and hybridization with RG-57 were performed as described (Drenth *et al.*, 1993; Drenth & Govers, 1993).

3. Data analysis

Genetic diversity was measured using the normalized Shannon diversity index: $H_s = H/H_{MAX}$, in which H is the usual Shannon diversity index over genotypes, and H_{MAX} is ln(N), the maximum diversity for a sample of size N. This statistic is relatively stable when sample sizes vary (Sheldon, 1969).

4. Oospore production *in vitro*

For oospore production *in vitro* the *P. infestans* isolates 80029 (A1 mating type) and 88133 (A2) were co-cultivated on rye A agar (Caten & Jinks, 1968) and incubated at 18 °C. After 2 weeks oospores were formed.

5. Oospore production *in planta*

For oospore production *in planta* a mixture of spores of the isolates 80029 and 88133 was inoculated on leaves of the susceptible potato cultivar Bintje. After 10 to 15 days incubation at 10 °C oospores were formed. The oospores were visualized by squashing the leaves on a microscope slide.

6. Oospore survival

The putative sexual progeny was recovered as follows: 16 days after inoculation of potato leaves with a mixture of spores of 80029 and 88133 and incubation at 10 °C the necrotized leaves, which contained $\pm$ 5 x 10^3 oospores per cm^2, were mixed with soil. The soil was exposed to natural weather conditions during the winter of 1992-1993. After eight months the soil was transferred to a flat tray and mixed with water (2 volumes). Potato leaves of the susceptible potato cultivar Bintje were allowed to float on the water surface. After 5 to 7 days sporulating lesions appeared on the leaves and from the spores pure *P. infestans* cultures were obtained. RG-57 DNA fingerprinting was performed to identify sexual progeny from the two parental isolates used to produce the oospores.

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REFERENCES

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

Charlesworth, B., 1989. The evolution of sex and recombination. Trends in Ecology and Evolution 9: 264-267.

Davidse, L.C., Looijen, D., Turkensteen, L.J. & Wal, D. van der, 1981. Occurrence of metalaxyl-resistant strains of *Phytophthora infestans* in the Netherlands. European Plant Protection Organization Bulletin 15: 403-409.

Drenth, A., Goodwin, S.B., Fry, W.E. & Davidse, L.C., 1993. Genotypic diversity of *Phytophthora infestans* in The Netherlands revealed by DNA polymorphisms. Phytopathology 83: 1087-1092.

Drenth, A. & Govers, F., 1993. DNA fingerprinting of the potato late blight fungus, *Phytophthora infestans*. p 223-230 in: Schots, A., Dewey, F.M. & Oliver, R. (eds.). Modern assays for plant pathogenic fungi: Indentification, detection and quantification. CAB international. pp 267.

Fry, W.E., Goodwin, S.B., Matuszak, J.M., Spielman, L.J., Milgroom, M.G. & Drenth, A., 1992. Population genetics and intercontinental migrations of *Phytophthora infestans*. Annual Review of Phytopathology 30: 107-129.

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

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

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

Goodwin, S.B., Spielman, L.J., Matuszak, J.M., Bergeron, S.N. & Fry, W.E., 1992b. Clonal diversity and genetic differentiation among *Phytophthora infestans* populations in northern Mexico. Phytopathology 82: 955-961.

Hohl, H.R. & Iselin, K., 1984. Strains of *Phytophthora infestans* with A2 mating type behaviour. Transactions of the British Mycological Society 83: 529-530.

Ko, W.H., 1980. Hormonal regulation of sexual reproduction in *Phytophthora*. Journal of General Microbiology 116: 459-463.

Maynard-Smith, J., 1971. What use is sex ? Journal of Theoretical Biology 30: 319-335.

Maynard-Smith, J., 1978. The evolution of sex. Cambridge University Press, Cambridge.

Michod, R.E. & Levin, B.R., 1988. The evolution of sex: An examination of current ideas. Sinauer Associates, Sunderland, Massachusetts, USA. pp 342.

Mooi, J.C., 1967. Onderzoek naar het voorkomen van "hogere" fysio's van *Phytophthora infestans* in de Groninger Veenkoloniën en aangrenzend gebied in 1968. Resultaten proefvelden fabrieksaardappelen 1967: 43-46.

Mooi, J.C., 1968. Onderzoek naar het voorkomen van "hogere" fysio's van *Phytophthora infestans* in de Groninger Veenkoloniën en aangrenzend gebied in 1968. Resultaten proefvelden fabrieksaardappelen 1968: 54-57.

Mooi, J.C., 1971. Het optreden van fysio's van *Phytophthora infestans* in de Groninger Veenkoloniën en omgeven gebied in 1970 en 1971. Resultaten proefvelden fabrieksaardappelen 1971: 62-65.

Niederhauser, J.S., 1991. *Phytophthora infestans*: the Mexican connection. p 25-45 in: Lucas, J.A., Shattock, R.C., Shaw, D.S. & Cooke, L.R. (eds.). *Phytophthora*. British Mycological Society, Cambridge University Press, Cambridge. pp 447.

Rivera-Peña, A., 1990. 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.

Sheldon, A.L., 1969. Equitability indices: dependence on the species count. Ecology 50: 466-467.

Smoot, A.J., Gough, F.J., Lamey, H.A., Eichenmuller, J.J. & Gallegly, M.E., 1958. Production and germination of oospores of *Phytophthora infestans*. Phytopathology 48: 165-171.

Spielman, L.J., Drenth, A., Davidse, L.C., Sujkowski, L.J., Gu, W.K., Tooley, P.W. & Fry, W.E., 1991. A second world-wide migration and population displacement of *Phytophthora infestans* ? Plant Pathology 40: 422-430.

Stearns, S.C., 1987. The evolution of sex and its consequences. Experientia. Supplementum: 55. Birkhäuser Verlag, Basel. pp 403.

Chapter 8

Formation and survival of oospores of *Phytophthora infestans* under natural conditions

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ABSTRACT

The oomycete *Phytophthora infestans* is able to produce oospores in leaves of potato and tomato plants after inoculation with a mixture of A1 and A2 mating type isolates. Here various conditions for oospore formation in leaves were analyzed. In experiments under controlled conditions, oospores were produced in potato leaves at temperatures ranging from 5 to 25 °C. In leaves of potato cultivar Bintje incubated at 15 °C oogonia and antheridia were observed six days after inoculation and thick walled oospores appeared nine to ten days after inoculation. In field experiments potato and tomato plants grown under natural conditions in The Netherlands were inoculated with a mixture of A1 and A2 mating type isolates. Oospores were found in leaves and stems of potato cultivars Bintje, Irene and Pimpernel and in leaves, stems and fruits of tomato cultivar Moneymaker within two weeks after inoculation. A bioassay was developed to test the infectiosity of soil infected with oospores. To determine whether the late blight infections derived from infectious soil were caused by oospores, DNA fingerprinting with probe RG-57 was used to distinguish asexual and sexual progeny of *P. infestans*. We demonstrated survival of oospores of *P. infestans* in soil during the winter of 1992-1993.

INTRODUCTION

Potato late blight caused by the oomycetous fungus *Phytophthora infestans* (Mont.) de Bary is one of the most devastating pathogens of potatoes worldwide. *P. infestans* is heterothallic with two known mating types, A1 and A2. Interaction between hyphae of opposite mating type can result in the formation of oospores.

Central Mexico was the only place where, before 1980, A1 and A2 mating type isolates were present and, since the 1950s, oospores were discovered in infected potato leaves (Gallegly & Galindo, 1958; Smoot *et al.*, 1958). In most potato growing areas of the world only A1 mating type isolates were found. Therefore, in those areas *P. infestans* could exclusively reproduce asexually and overwinter as mycelium in potato seed tubers in storage or in potato tubers in cull piles and in volunteer potatoes.

In the early 1980s, however, A2 mating type isolates were discovered in Switzerland (Hohl & Iselin, 1984) and subsequently in many other countries in Europe, Africa, Asia and north and south America (Spielman *et al.*, 1991; Fry *et al.*, 1993; Drenth *et al.*, 1993ab). Population genetic studies, based on two polymorphic allozymes, revealed that since 1980 "new" A1 and A2 mating type populations were established in Europe and replaced the "old" A1 mating type population present before 1980 (Spielman *et al.*, 1991; Fry *et al.*, 1991, 1992, 1993). Additional studies, based on DNA fingerprint analyses and virulence factors, demonstrated that after 1980 the level of genetic diversity in the Dutch *P. infestans* populations increased dramatically (Drenth *et al.*, 1994). Over 90 % of the isolates identified each year had a unique genotype, suggesting that every year late blight was caused by different genotypes.

Moreover, we showed that oospores can survive in soil between growing seasons and that since 1980 the *P. infestans* population in The Netherlands propagates sexually apart from asexually (Drenth *et al.*, 1994b).

The production of oospores by *P. infestans* in the field and their survival under natural conditions has become an object of study since the introduction of A2 mating type isolates. Oospores of *P. infestans* were observed in stems but rarely in leaves of potato and tomato plants grown in the greenhouse and inoculated with mixtures of A1 and A2 mating type isolates of *P. infestans* (Frinking *et al.*, 1987; Mosa *et al.*, 1991). Small numbers of oospores were also found in tubers (Grinberger *et al.*, 1989). In Europe, oospore formation in potato leaves in the field was reported in Germany and in The Netherlands (Götz, 1991; Drenth *et al.*, 1994b).

Oospores of oomycetous fungi are, in general, endogenously dormant, tolerant to adverse conditions, and capable of long term survival (Ribeiro, 1983). Oospores from *Phytophthora fragariae*, the causal agent of red stele root in strawberry, can survive for at least three years in soil (Duncan, 1980; Duncan & Cowan, 1980). Oospores of *Peronospora destructor*, the causal agent of downy mildew in onion, remained infectious for up to 25 years in soil despite continuous exposure to natural weather conditions, including 1549 occasions of frost (McKay, 1957).

Survival and infectivity of *P. infestans* oospores is not yet studied in detail. Perches and Galindo (1969) showed that soil, collected from a Mexican field two years after that field was occupied by a severely blighted potato crop, was still infectious. In greenhouse experiments they showed that potatoes planted in this soil were infected on the lower part of the stems and on the leaves, close to or in contact with the soil. *P. infestans* could be isolated from this soil using selective media and the authors suggested that oospores were responsible for the infectivity of the soil.

Oospores of *Phytophthora* spp. are rather insensitive to fungicides (Duncan, 1985b) but quite sensitive to heat treatments of 45 °C (Duncan, 1985a). Thus, some *Phytophthora* spp. (i.e. *P. cambivora*, *P. cryptogea*, *P. cinnamomi*) can be controlled by soil solarization (Juarez-Palacios *et al.*, 1991). Asynchronous germination is typical of oospores and most likely will enhance survival by ensuring a continuous supply of infective propagules in soil (Hord & Ristaino, 1991). Despite many studies (reviewed in Ribeiro, 1983), factors affecting germination of oospores are not well understood.

With the introduction of "new" *P. infestans* A1 and A2 mating type populations in Europe together with evidence for the occurrence of sexual reproduction (Drenth *et al.*, 1994b) there is the need to determine to what extent oospores contribute to late blight epidemics and which factors influence production, survival, germination and infectivity of oospores of *P. infestans*. Here we describe the effect of temperature and the resistance level of potato cultivars on the development and production of oospores in potato leaf discs. A bioassay has been developed to obtain late blight infections from soil containing oospores. This bioassay was used to determine whether oospores, exposed to natural weather conditions in soil during the winter of 1992-1993, remained viable and retained infectivity. In order to prove that oospores

caused the infections sexual hybrid progeny was distinguished from asexual progeny using DNA fingerprint probe RG-57.

MATERIALS AND METHODS

P. infestans isolates

Two *P. infestans* isolates, 80029 (A1 mating type) and 88133 (A2 mating type) collected from an infested potato field in The Netherlands in 1980 and 1988, respectively, were used throughout this study. Sexual progeny of these isolates can be distinguished from the parental isolates and naturally occurring infections by RG-57 DNA fingerprint analyses (Goodwin *et al.*, 1992; Drenth & Govers, 1993).

Oospore formation

For studies on oospore formation in leaves under controlled conditions potato cultivars Bintje (susceptible), Irene (moderately susceptible) and Pimpernel (moderately resistant) were used. In all these experiments plants were inoculated with a mixture of sporangiospores (10^4 sp/ml) containing equal amounts of both isolates 80029 and 88133. In Petri dishes (∅ 9 cm) containing 20 ml of water, ten potato leaf discs (∅ 18 mm), cut with a cork borer, were placed upside down and inoculated in the middle with 10 μl of the sporangiospore mixture. To study the effect of temperature on the production of oospores the leaf discs were incubated at temperatures varying from 5 to 25 °C at a light intensity of 10 klx, 16 h per day. Every day the leaf discs from a single Petri dish were collected and frozen at -20 °C. The frozen leaf discs were homogenized for 30 sec (polytron homogenizer) in two ml of water and the number of oospores counted using a hemocytometer.

To study oospore formation in the field, 40 small plots (1.2 x 1.2 m) containing either potato cultivar Bintje, Irene or Pimpernel, or tomato cultivar Moneymaker were inoculated in August 1992. Inoculation was conducted in the evening by spraying the sporangiospore mixture (25 ml per plot) with a rotary hand sprayer (De Villbis atomizer). Leaf and stem samples of potato plants and leaf, stem and fruit samples of tomato plants were collected 13 days after inoculation. In July 1993, two plots (6 x 6 m) of potato cultivar Bintje were inoculated in a similar way. Leaves with late blight lesions were collected at day 7, 10 and 17 after inoculation.

Oospores in leaf tissue were detected after squashing the leaves on a microscope slide or clearing the leaf tissue. Leaves were cleared by autoclaving in 80 % ethanol at 115 °C for 5 min, incubation for 30 min in 1 M NaOH at 80 °C and incubation for a few minutes in NaClO (4% available chlorine) at 60 °C, until the leaves were clear, and than mounted on microscope slides.

Oospore survival

Survival and infectivity of oospores of *P. infestans* was studied using either soil containing

oospores in pots, or field plots artificially inoculated with *P. infestans* isolates 80029 and 88133. The soil infested with oospores was exposed to natural weather conditions during the winter 1992-1993.

To produce sufficient amounts of oospores for longevity trials, leaves of the potato cultivar Bintje floating upside down in large trays (37 x 57 cm) containing 2 liters of water were inoculated with the sporangiospore mixture. The leaves were incubated at a light intensity of 10 klx, 16 h per day at 10 °C. Sixteen days after inoculation the leaves, containing approximately 5.000 oospores/cm^2, were briefly homogenized in a blender. The homogenized oospore suspension was added to pots (∅ 13 cm, volume 800 cm^3) with field soil which were buried at ground level. Per pot 660.000 oospores were added resulting in 825 oospores/cm^3 soil. The pots were exposed to natural weather conditions. Soil from the first pot was analyzed for infectivity after seven days exposure to natural weather conditions. After 5.5, 7 and 8 months of exposure to natural weather conditions infectivity of soil in four additional pots was assessed using the bioassay described below.

To study survival of oospores at extreme temperatures, soil from one pot was homogenized, and divided into nine portions of 100 gram. The portions were incubated for 48 hours at different temperatures ranging from -80 °C to 50 °C (temperature intervals shown in table 2). The infectivity of the heated soil with oospores was assessed using the bioassay described below.

To study survival of oospores under field conditions eight plots (5 x 5 m, heavy clay soil) planted with potato cultivars Bintje, Irene and Pimpernel (two rows of each cultivar, 75 cm distance between rows) and two plots (5 x 5 m) with tomato plants (cultivar Moneymaker) were prepared. Plants were inoculated in September 1992, a time when late blight still occurs in The Netherlands, with 1 liter per plot of a sporangiospore mixture with isolates 80029 and 88133 (5400 spores/ml). Late blight symptoms were visible seven days after inoculation. All potato tubers were harvested in October 1992. The remaining blighted potato leaves and stems and the blighted tomato plants were mixed through the soil. In May 1993, all ten plots were planted with potato cultivar Bintje. In July 1993, soil was randomly collected from each plot to asses infectivity of the soil using the bioassay described below. On August 30, 1993 when late blight was present on most plants of cultivar Bintje in all ten plots, ten lesions were randomly collected from diseased plants of each plot. DNA fingerprint analyses were performed on these isolates to determine whether they originated from oospores formed the previous year.

Bioassay

The bioassay is based on the observations that the rate of oospore germination increases after incubation of oospores in large volumes of water and that zoospores of *Phytophthora* spp. have a negative geotaxis (Cameron & Carlile, 1977). Soil (1 kg) containing oospores is transferred to a 30 x 45 x 28 cm plastic container with a transparent lid and mixed with 2 liters of water. After two days of incubation at 15 °C, 20 leaves of the susceptible potato cultivar Bintje were placed upside down on the water surface and incubated at 15 °C at a light intensity of 10 klx,

16 h per day. After 5 to 7 days sporulating lesions appeared on the leaves. Spores from the lesions are transferred to potato tuber slices. The mycelium is allowed to grow through the tuber slices after which the sporangiospores produced were transferred to selective media to obtain pure cultures.

DNA fingerprinting

For DNA fingerprinting, the moderately repetitive DNA probe RG-57 was used. This probe, obtained from a genomic *P. infestans* library, hybridizes to several unlinked loci and provides an isolate-specific DNA fingerprint (Goodwin *et al.*, 1992). DNA isolation, *Eco*RI digestion, Southern blotting and hybridization with probe RG-57 were performed as described before (Drenth & Govers, 1993; Drenth *et al.*, 1993).

RESULTS AND DISCUSSION

Oospore formation

In leaf discs of potato cultivar Bintje, incubated at 15 °C, oogonia and antheridia were observed five to six days after inoculation. After eight days, a few oospores were observed and nine to ten days after inoculation, many thick walled oospores were present in the leaf discs (Fig. 1).

Oospores of *P. infestans* were produced in leaf discs of potato cultivar Bintje between 5 and 25 °C. At 10 °C more than 6000 oospores per cm^2 leaf tissue were observed. At 15 °C more than 2000 but at 20 °C less than 1000 oospores per cm^2 leaf tissue were observed (Fig. 2a). At 5 and 25 °C only limited numbers of oospores were observed (< 10 oospores/cm^2, data not shown). At 5 °C mycelium of *P. infestans* colonized the leaves slowly and at 25 °C leaf tissues of potato deteriorates quickly leaving limited time for the production of oospores. Apparently, at 10 °C the *P. infestans* mycelium in leaf discs of potato cultivar Bintje grows at such a rate that there is ample time for oospore formation.

In leaf discs of the moderately resistant potato cultivar Pimpernel incubated at 15 °C more oospores were found than in the leaf discs of the susceptible cultivar Bintje and moderately susceptible cultivar Irene (Fig. 2b). A similar finding was reported by Estrada (1967) using potato cultivar Bintje (susceptible) and Atzimba (moderately resistant). Abundant oospore production in moderately resistant potato cultivars can be explained by the fact that leaf discs of these potato cultivars show delayed deterioration resulting in extra time for production of oospores.

In 1992, plants of potato cultivars Bintje, Irene and Pimpernel and plants of tomato cultivar Moneymaker, all grown under natural field conditions, were inoculated with a suspension of sporangiospores containing a mixture of A1 and A2 mating types. Thirteen days after inoculation, oospores were found in leaves and stems of the potato plants and in leaves, stems and fruits of tomato plants. In the field experiment conducted in 1993, oogonia and antheridia were observed in the leaves of potato cultivar Bintje ten days after inoculation, and

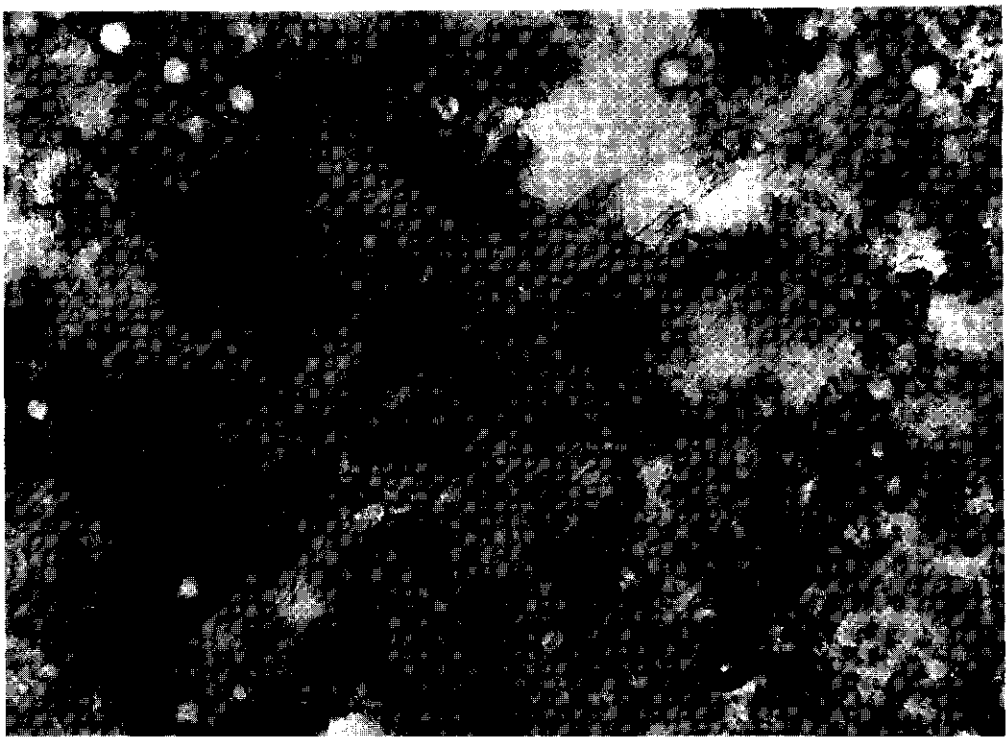


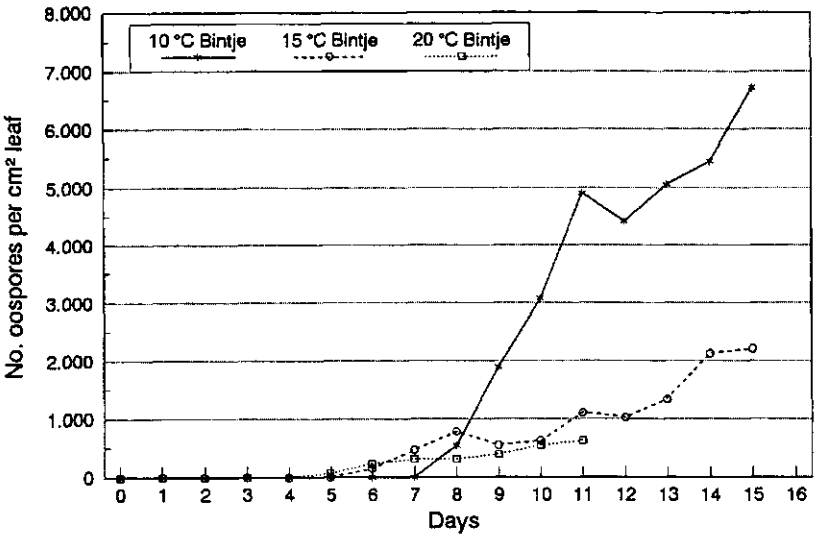
Figure 1. Oospores of *P. infestans* formed in leaves of potato cultivar Bintje ten days after inoculation with a mixture of equal amounts of sporangiospores (10^4 spores/ml) of *P. infestans* isolates 80029 (A1 mating type) and 88133 (A2 mating type). Leaves were incubated at 15 °C at a light intensity of 10 klx, 16 h per day.

seventeen days after inoculation numerous oospores were detected. From these results it can be concluded that climatic conditions in The Netherlands allow formation of oospores in potato plants as well as in tomato plants.

Oospore survival

Soil containing oospores from one pot which was exposed for seven days to natural weather conditions was tested for infectivity in the bioassay. DNA fingerprint analyses of the isolates that caused the late blight lesions in the bioassay revealed that 25% of the infections was caused by hybrid progeny from the parental isolates whereas 75% of the lesions was caused by one of the parental isolates, 80029 and 88133, used to produce the oospores (Table 1). Hence, vegetative spores or mycelium in plant debris can survive for at least one week in soil. Moreover, oospores are able to germinate and cause infections as soon as they are formed. Götz (1991) even observed

A



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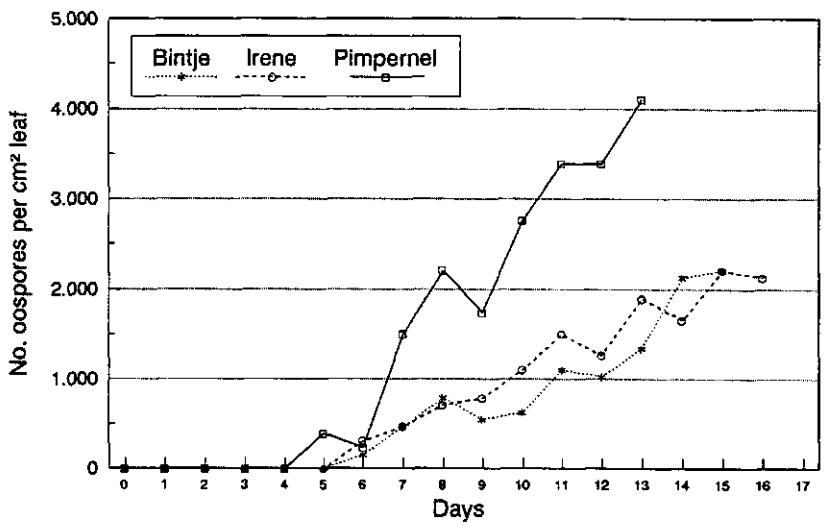

Figure 2. A The number of oospores in leaf discs of potato cultivar Bintje (susceptible) plotted against time after inoculation. The leaf discs were incubated at different temperatures. B The number of oospores in leaf discs of potato cultivar Bintje (susceptible), Irene (moderately susceptible) and Pimpernel (moderately resistant) plotted against time after inoculation. The incubation temperature was 15 °C.

Table 1. Survival and infectivity of oospores of *P. infestans* in pots with soil after exposure to natural weather conditions in The Netherlands for varying times during the winter of 1992-1993.

Date[a]	Exposure in soil	Number of isolates tested	Percentage of hybrids[b]
16 November 92	7 days	12	25
21 April 93	5.5 months	25	100
2 June 93	7 months	23	100
11 June 93	7 months	15	100
12 July 93	8 months	21	100

[a] Oospores were added to the soil on 9 November, 1992.

[b] "Hybrid" means sexual progeny from isolates 80029 and 88133.

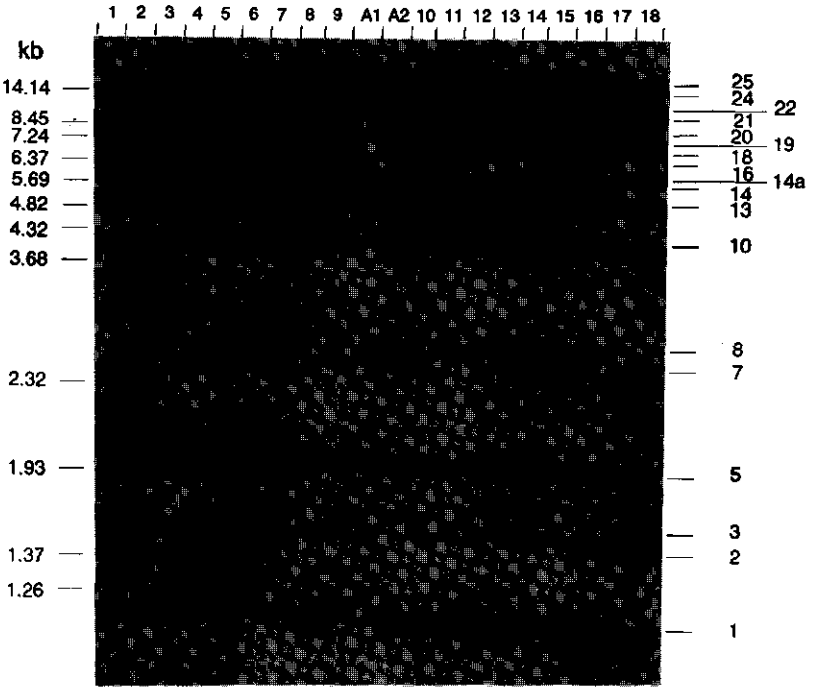

Figure 3. Autoradiograph of a Southern blot after hybridization with probe RG-57 showing DNA fingerprint patterns of the parental *P. infestans* isolates 80029 (A1) and 88133 (A2) and 18 hybrid progeny (1-18). The hybrids were isolated from lesions on leaves which in the bioassay were brought in contact with soil containing oospores. The soil was exposed to natural weather conditions for 8 months. Size markers in kilobases (kb) are indicated on the left. Fragment numbers, corresponding to those used by Goodwin *et al.* (1992) and Drenth *et al.* (1993a) are indicated on the right.

Table 2. Survival and infectivity of oospores of *P. infestans* maintained in soil which was first exposed to natural weather conditions for 8 months and subsequently treated at various temperatures for 48 hours.

Temperature	Lesions[a]	Number of isolates tested	Percentage of hybrids[b]
-80 °C	+	3	100
-20 °C	+	3	100
15 °C	+	8	100
30 °C	+	4	100
35 °C	+	3	100
40 °C	-	.	.
45 °C	-	.	.
50 °C	-	.	.

[a] Presence (+) and absence (-) of lesions in the bioassay.
[b] "Hybrid" means sexual progeny from isolates 80029 and 88133.

germinated oospores in potato leaf tissue. Hence, oospores do not only act as a source of inoculum for the following season but they can also play a role to initiate new epidemics after a spell of unfavorable conditions within a season.

After exposure of pots with infested soil to natural weather conditions for 5.5, 7 and 8 months the infectivity of the soil was tested in the bioassay. The soil remained infectious. DNA fingerprint analyses revealed that all late blight lesions in the bioassay were caused by hybrid progeny. As none of the lesions were caused by parental isolates, mycelium and vegetative spores did not survive up to 5.5 months in soil (Table 1).

To test the sensitivity of the oospores for extreme temperatures, infested soil of the eight months treatment, was exposed for two days to temperatures ranging from -80 to 50 °C (Table 2). Soil exposed to temperatures of 40 °C or higher did not give rise to late blight lesions in the bioassay indicating that the oospores did not survive temperature treatments higher than 35 °C (Table 2). However, oospores could survive temperatures as low as -80 °C and up to 35 °C. DNA fingerprint analyses confirmed that the lesions originated from hybrid offspring, hence oospores (Table 2).

Survival and infectivity of oospores in soil collected from fields on which blighted potato plants were present ten months before were also tested in the bioassay. Plant debris containing oospores was left in the field plots in 1992 to act as inoculum source. Soil samples gave rise to late blight lesions on potato leaves in the bioassay. DNA fingerprint analyses of isolates obtained from three of these lesions revealed that one of the lesions was caused by a hybrid offspring

originating from parental isolates 80029 and 88133.

On the same field plots potatoes were planted in 1993. Late blight lesions appeared when the plants were four months old. Isolates were collected from lesions on stems and leaves and 76 of these were subjected to DNA fingerprint analyses. It appeared that one lesion was caused by a sexual progeny from the parental isolates. The other 75 analyzed lesions were caused by other *P. infestans* isolates which were not related to the isolates that caused the infections at those fields the previous year. In August 1993 the cool wet weather conditions favoured the development of potato late blight epidemics throughout The Netherlands and it is obvious that sporangia or zoospores were introduced into the field plots and caused infections.

We conclude that oospores of the "new" *P. infestans* population can survive in soil during the winter in The Netherlands and are able to infect potato plants during the growing season. This conclusion is supported by the results of similar experiments conducted in the United Kingdom which showed that oospores of *P. infestans* were viable and able to germinate after exposure to natural weather conditions in soil for eight months (J.E. Pittis & R.C. Shattock, personal communication).

There is limited information concerning the mechanism by which potato plants are infected by oospores under field conditions. We hypothesize that during periods of high rainfall, oospores present on and in soil germinate and that the motile zoospores move to the surface, where they infect leaves and stems of potato and tomato plants in contact with the soil surface. Splash dispersal during rainfall and overhead sprinkling irrigation may even lead to infections higher up in the leaf canopy. Resulting lesions will sporulate to start late blight epidemics.

It is expected that oospores of *P. infestans* can survive for many years in soil analogous to oospores of other oomycetous plant pathogens (McKay, 1957; Duncan, 1980; Duncan & Cowan, 1980). In our experiment on survival and infectivity of oospores many pots containing oospores remain exposed to natural weather conditions for unlimited times. In the coming years we will get a more detailed picture of the longevity of oospores of *P. infestans* in soil.

Sexual reproduction of *P. infestans* and the presence of oospores in soil acting as an inoculum source, in addition to overwintering mycelium in potato tubers, surely has an influence on the epidemiology of the late blight disease and this, in turn, has many consequences for the control of late blight on potatoes. First, oospores in soil can start an epidemic whenever weather conditions are favorable for the fungus and potato plants (planted or volunteer) are present. This will lead to earlier and more massive starts of late blight epidemics. Second, oospores will most likely infect leaves in the lower part of the leaf canopy. Protectant fungicides, mainly applied to the top of the canopy, protect the lower leaf canopy in general less effectively. Third, sexual reproduction will result in genetically more diverse *P. infestans* populations with a high adaptability to environmental conditions and control strategies (Drenth *et al.*, 1994b). Fourth, rapid germination of oospores present in potato leaves and stems will enable the fungus to survive unfavorable weather conditions within the season, and maybe even survive treatments with systemic fungicides, while they easily can start colonizing the remaining healthy foliage as soon as conditions become conducive for the disease again. Thus, the change from exclusively

asexual reproduction, before 1980, to mixed sexual/asexual reproduction after 1980 (Drenth *et al.*, 1994) will have severe consequences for the strategies to control *P. infestans* in The Netherlands. Control of *P. infestans* will certainly become difficult in the years to come.

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REFERENCES

Cameron, J.N. & Carlile, M.J., 1977. Negative geotaxis of zoospores of the fungus *Phytophthora*. Journal of General Microbiology 98: 599-602.

Drenth, A., Goodwin, S.B., Fry, W.E. & Davidse, L.C., 1993a. Genotypic diversity of *Phytophthora infestans* in The Netherlands revealed by DNA polymorphisms. Phytopathology 83: 1087-1092.

Drenth, A. & Govers, F., 1993. DNA fingerprinting of the potato late blight fungus, *Phytophthora infestans*. p 223-230 in: Schots, A., Dewey, F.M. & Oliver, R. (eds.). Modern assays for plant pathogenic fungi: Identification, detection and quantification. CAB international. pp 267.

Drenth, A., Turkensteen, L.J. & 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, I.C.Q. & Govers, F., 1994. DNA fingerprinting uncovers a new sexually reproducing population of *Phytophthora infestans* in the Netherlands. European Journal of Plant Pathology (in press).

Duncan, J.M., 1980. Persistence of mycelium of *Phytophthora fragariae* in soil. Transactions of the British Mycological Society 75: 383-387.

Duncan, J.M. & Cowan, J.B., 1980. Effect of temperature and soil moisture content of persistence of infectivity of *Phytophthora fragariae* in naturally infested field soil. Transactions of the British Mycological Society 75: 133-139.

Duncan, J.M., 1985a. Effect of temperature and other factors on in vitro germination of *Phytophthora fragariae* oospores. Transactions of the British Mycological Society 85: 455-462.

Duncan, J.M., 1985b. Effect of fungicides on survival, infectivity and germination of *Phytophthora fragariae* oospores. Transactions of the British Mycological Society 85: 585-593.

Estrada, S.P., 1967. Importancia de las oosporas de *Phytophthora infestans* (Mont.) de Bary como inoculo primario del "tizon tardio" de la papa. MsC thesis, Chapingo, Mexico. pp 43.

Frinking, H.D., Davidse, L.C. & Limburg, H., 1987. Oospore formation by *Phytophthora infestans* in host tissue after inoculation with isolates of opposite mating type found in the Netherlands. Netherlands Journal of Plant Pathology 93: 147-149.

Fry, W.E., Drenth, A., Spielman, L.J., Mantel, B.C., Davidse, L.C. & Goodwin, S.B., 1991. Population genetic structure of *Phytophthora infestans* in the Netherlands. Phytopathology 81: 1330-1336.

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

Fry, W.E., Goodwin, S.B., Matuszak, J.M., Spielman, L.J., Milgroom, M.G. & Drenth, A., 1992. Population genetics and intercontinental migrations of *Phytophthora infestans*. Annual Review of Phytopathology 30: 107-129.

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

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

Götz, E., 1991. Untersuchungen zum auftreten des A2-paarungstyps bei *Phytophthora infestans* (Mont.) de Bary in Ostdeutschland. Potato Research 34: 233-237.

Grinberger, M., Kadish, D. & Cohen, Y., 1989. Occurrence of the A2 mating type and oospores of *Phytophthora infestans* in potato crops in Israel. Phytoparasitica 17: 197-204.

Juarez-Palacios, C., Felix-Gastelum, R., Wakeman, R.J., Paplomatas, E.J. & Devay, J.E., 1991. Thermal sensitivity of three species of *Phytophthora* and the effect of soil solarization on their survival. Plant disease 75: 1160-1164.

Hohl, H.R. & Iselin, K., 1984. Strains of *Phytophthora infestans* with A2 mating type behaviour. Transactions of the British Mycological Society 83: 529-530.

Hord, M.J. & Ristaino, J.B., 1991. Effects of physical and chemical factors on the germination of oospores of *Phytophthora capsici* in vitro. Phytopathology 81: 1541-1546.

Mckay, R., 1957. The longevity of the oospores of onion downy mildew *Peronospora destructor* (Berk.) Casp. Scientific Proceedings of the Royal Dublin Society. New Series 27: 295-307.

Mosa, A.A., 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.

Perches, E.S. & Galindo, A.J., 1969. Supervivencia del *Phytophthora infestans* (Mont) de Bary del Tizón tardio de la papa y jitomate. Agrociencia 5: 92-98.

Ribeiro, O.K., 1983. Physiology of asexual sporulation and spore germination in *Phytophthora*. p 55-70 in: Erwin, D.C., Bartnicki-Garcia, S. & Tsao, P.H. (eds.). *Phytophthora*: its biology, taxonomy, ecology and pathology. APS Press, USA. pp 392.

Smoot, A.J., Gough, F.J., Lamey, H.A., Eichenmuller, J.J. & Gallegly, M.E., 1958. Production and germination of oospores of *Phytophthora infestans*. Phytopathology 48: 165-171.

Spielman, L.J., Drenth, A., Davidse, L.C., Sujkowski, L.J., Gu, W.K., Tooley, P.W. & Fry, W.E., 1991. A second world-wide migration and population displacement of *Phytophthora infestans*? Plant Pathology 40: 422-430.

Chapter 9

Summary and concluding remarks

Summary and concluding remarks

The oomycete *Phytophthora infestans* (Mont.) de Bary, the causal agent of late blight, is worldwide one of the most devastating pathogens of potato (*Solanum tuberosum* L.). Since its appearance in Europe in 1845 only A1 mating type isolates were present. In the absence of the A2 mating type, *P. infestans* could only overwinter as mycelium in potato tubers. In the early 1980s however, A2 mating type isolates showed up in Europe. When A1 and A2 mating type thalli grow in close vicinity sexual spores (i.e. oospores) are formed which may enable *P. infestans* to overwinter outside the host plant in soil. The aim of the research described in this thesis was to determine whether the *P. infestans* population in The Netherlands reproduces sexually, whether oospores survive during the winter in soil and whether oospores can cause infections under field conditions in The Netherlands.

Population genetic analyses using two allozyme markers, glucose phosphate isomerase (*Gpi*) and peptidase (*Pep*), demonstrated that not only A2 mating type isolates were introduced in Europe in the early 1980s but also new A1 mating type isolates. Thus, after 1980, we are confronted with an entire new pathogen population, consisting of A1 and A2 mating type isolates. This new population has displaced the old A1 mating type population present before 1980. The appearance of new A1 and A2 mating type isolates and new allozyme alleles (*Gpi* 90 and *Pep* 83) indicate that the appearance of the new *P. infestans* population in Europe was due to migration. Mexico is the most likely origin of the new population, since historically it has harbored both mating types while the *Gpi* 90 and *Pep* 83 alleles occur there in low to moderate frequencies (chapter 3).

Individual genotypes of any organism can be identified with numerous markers which identify one or a few loci or with one marker which identifies multiple loci. DNA probe RG-57, obtained from a random genomic library of *P. infestans*, hybridizes to many fragments of genomic DNA of which at least 17 show independent Mendelian inheritance. The fragment patterns are stable through asexual reproduction. Because of the low probability of unrelated individuals having the same fragment pattern, probe RG-57 provides a specific DNA fingerprint pattern of *P. infestans* genotypes. Therefore, this probe was used for elucidating the level of genetic diversity in populations and for studying the fate of particular genotypes during a polycyclic epidemic in one year or in consecutive years. In addition, probe RG-57 proved to be powerful in distinguishing parental *P. infestans* isolates from their sexual progeny (chapter 4).

Population genetic studies using as markers the allozymes *Gpi* and *Pep*, mating type, and resistance to the fungicide metalaxyl, revealed differences among subpopulations of *P. infestans* collected in 1989 in The Netherlands. Significant differences in genotype frequencies were observed among *P. infestans* isolates collected in different regions, between A1 and A2 mating type populations, and between populations collected from diseased potato and tomato plants. However, complete substructuring was not found. Some allozyme genotypes were widely distributed, whereas others were unique to a single field. Some fields contained a single allozyme genotype, whereas other fields contained different allozyme genotypes (chapter 5).

The two available polymorphic allozyme markers for *P. infestans*, *Gpi* and *Pep*, were insufficient to accurately determine the level of genetic diversity of the *P. infestans* population in The Netherlands. DNA fingerprint analyses of isolates with probe RG-57 revealed a significantly higher level of diversity in the *P. infestans* population collected in 1989 in The Netherlands than the previous allozyme analyses. Thus, allozyme uniformity does not necessarily imply lack of genetic diversity in *P. infestans* populations. The differentiation power of allozyme markers is clearly too limited for detailed genetic studies. Probe RG-57 identified 35 genotypes among 153 isolates collected in 14 fields in The Netherlands. Half of the isolates had the same RG-57 genotype and isolates with this particular RG-57 genotype were found in 10 of the 14 fields sampled from different regions. This suggests a common source of inoculum for these fields and indicates that migration probably occurs freely over large areas within The Netherlands. Most (89%) of the genotypes were only identified in one field and 60% of the genotypes occurred only once in the sampled population. DNA fingerprint analyses showed that substructuring on potato and tomato plants is incomplete, since some genotypes occurred on both host plants. Most of the diversity (52%) was associated with differences within fields, the lowest hierarchical level. The observed pattern of diversity found among *P. infestans* isolates collected in The Netherlands is consistent with the hypothesis that sexual reproduction generates new genotypes and that occasionally fit recombinants reproduce asexually and become widespread. Two types of mitochondrial DNA, A and B, were observed among the Dutch *P. infestans* isolates collected in 1989. Mitochondrial DNA type A is widely distributed throughout the world, whereas type B is restricted to places where A2 mating type isolates are found. Analysis of DNA fingerprint patterns and types of mitochondrial DNA confirmed that the present Dutch *P. infestans* isolates originate from the *P. infestans* population in Mexico (chapter 6).

Isolates of *P. infestans* collected in successive years before and after 1980 were analyzed using virulence factors and DNA fingerprint probe RG-57 with the aim to compare levels of genetic diversity in the different populations and to learn more about the fate of genotypes throughout time. Before 1980 eight different races were found in The Netherlands in which virulence factors 1, 2, 3, 4 and 10 were most common. After 1980 new virulence factors (i.e. 5, 6, 7, 8 and 11) showed up for which the corresponding resistance genes are absent in the potato cultivars grown in The Netherlands. The diversity for virulence increased tremendously. After 1980, 73 different races were detected among 253 isolates analyzed. DNA fingerprint analysis of isolates collected before 1980 revealed that for at least two decades only one RG-57 fingerprint genotype was present in Europe. This "old" RG-57 genotype exhibited limited diversity for virulence to match the resistance genes present in the potato cultivars grown in The Netherlands. Due to the presence of only one mating type this "old" RG-57 genotype could only overwinter as mycelium in potato tubers. After 1980 the level of genetic diversity increased dramatically and many distinct RG-57 genotypes were identified. Among 179 isolates 134 different RG-57 fingerprint patterns were found. About 90% of these 134 genotypes occurred in only one year, indicating that every year the pathogen population consists almost entirely of new genotypes not found in the previous year. The "old" RG-57 genotype, present before 1980,

was not found again after 1980 indicating a complete population displacement. The huge genetic differences between isolates causing late blight in successive years might be the result of sexual reproduction (chapter 7).

Oospores of *P. infestans* were observed in leaves and stems of potato plants of cultivars Bintje, Irene and Pimpernel and in leaves, stems and fruits of tomato plants of cultivar Moneymaker in conditioned climate rooms and in the field after inoculation with isolates of opposite mating type. Oospore production of *P. infestans* in leaves of potato cultivar Bintje occurred at temperatures ranging from 5 to 25 °C. Oospores produced in leaves and exposed to natural weather conditions in unsterilized soil for eight months during the winter of 1992-1993 were still able to infect potato leaves and to cause disease in conditioned bioassays. DNA fingerprint analyses demonstrated unambiguously that the late blight lesions were caused by hybrid, sexual progeny of the two parental isolates used to produce the oospores (chapter 8).

In conclusion, the results presented in this thesis show *(i)* that sexual reproduction of *P. infestans* occurs in The Netherlands, *(ii)* that oospores of *P. infestans* can survive in soil during the winter and *(iii)* that oospores present in soil can cause infections. To explain infection of potatoes by oospores under field conditions the following hypothesis is put forward. During periods of high rainfall oospores present in soil germinate and produce motile zoospores which move to the soil surface to infect leaves and stems of potato and tomato plants that come in contact with the contaminated soil. Splash dispersal during rainfall or overhead sprinkler irrigation may even lead to infections higher up in the leaf canopy. After a few days the resulting lesions will produce sporangia to form a late blight epidemic.

Sexual reproduction of *P. infestans* and the presence of oospores in soil acting as an inoculum source, in addition to overwintering mycelium in potato tubers, surely has an influence on the epidemiology of the late blight disease and this, in turn, has many consequences for the control of late blight on potatoes. First, oospores in soil can start an epidemic whenever weather conditions are favorable for late blight and potatoes (planted or volunteer) are growing on soil contaminated with oospores. This will lead to earlier and more massive breakouts of late blight epidemics. Second, oospores will most likely infect leaves in the lower part of the leaf canopy. Protectant fungicides, mainly applied to the top of the canopy may fail to protect the lower canopy. These factors may increase the chances for the development of initial foci and occurrence of epidemics. Third, sexual reproduction results in genetically more diverse *P. infestans* populations with greater adaptability to environmental conditions and control strategies. Fourth, rapid germination of oospores present in potato leaves and stems enables the fungus to survive unfavorable weather conditions within the season and to start colonizing the remaining healthy foliage again as soon as conditions are favorable for the disease. Thus the change from an exclusively asexually reproducing population and overwintering as mycelium in potato tubers, before 1980, to a population with mixed sexual/asexual reproduction and survival of oospores in soil, after 1980, asks for adjustment of the current practices to control late blight in areas where both mating types of *P. infestans* occur.

Samenvatting en slotbeschouwing

De oömyceet *Phytophthora infestans* (Mont.) de Bary, de veroorzaker van de aardappelziekte, is wereldwijd één van de meest belangrijke pathogenen van de aardappel (*Solanum tuberosum* L.). Tussen 1845, het jaar waarin de aardappelziekte voor het eerst werd waargenomen in Europa, en 1980 zijn hier alleen maar isolaten met het A1 paringstype gevonden. Zonder de aanwezigheid van het A2 paringstype kan *P. infestans* alleen overwinteren in de vorm van mycelium in aardappelknollen. Na 1980 werd ook het A2 paringstype gevonden in Europa. Als A1 en A2 thalli in elkaars nabijheid groeien kunnen sexuele sporen (oösporen) gevormd worden waarmee *P. infestans* buiten de waardplant in de grond kan overleven. Het doel van het in dit proefschrift beschreven onderzoek was te bepalen of de aanwezigheid van A1 en A2 paringstype isolaten in Nederland geleid heeft tot sexuele voortplanting van *P. infestans*, of oösporen die tijdens de sexuele voortplanting gevormd worden gedurende de winter buiten de waardplant kunnen overleven en of deze oösporen het volgende seizoen als een bron van inoculum kunnen dienen.

Populatie-genetische analyses waarbij gebruik gemaakt werd van twee allozym-merkers, glucosefosfaatisomerase (*Gpi*) and peptidase (*Pep*), hebben aangetoond dat rond 1980 niet alleen *P. infestans* isolaten met het A2 paringstype zijn ingevoerd in Europa maar ook nieuwe isolaten met het A1 paringstype. De van oudsher in Nederland aanwezige *P. infestans* isolaten werden na 1980 niet meer teruggevonden. De aardappelziekte in Nederland werd dus na 1980 veroorzaakt door een nieuwe *P. infestans* populatie die zowel isolaten met het A1 paringstype als isolaten met het A2 paringstype bevat. Deze nieuwe populatie heeft blijkbaar de oude populatie zoals die vóór 1980 voorkwam vervangen. Het gelijktijdig verschijnen van nieuwe isolaten met het A1 of het A2 paringstype en nieuwe allozymallelen (*Gpi* 90 en *Pep* 83) duidt op migratie van een nieuwe *P. infestans* populatie naar Europa. Het is aannemelijk dat de oorsprong van deze nieuwe populatie in Mexico ligt omdat daar van oudsher beide paringstypen en de allozymallelen *Gpi* 90 en *Pep* 83 voorkomen (hoofdstuk 3).

Individuele genotypen van nagenoeg ieder organisme kunnen van elkaar worden onderscheiden door gebruik te maken van een groot aantal genetische merkers die één of enkele loci herkennen, of van één genetische merker die een groot aantal loci tegelijkertijd herkent. DNA probe RG-57, die geselecteerd is uit een genomische bank van *P. infestans*, hybridiseert met een groot aantal genomische DNA fragmenten waarvan er tenminste zeventien onafhankelijk van elkaar overerven. De met DNA probe RG-57 verkregen hybridisatiepatronen bleken voor ieder isolaat afzonderlijk stabiel te zijn en ook hun asexuele nakomelingen vertoonden hetzelfde hybridisatiepatroon. De kans dat twee genotypen hetzelfde RG-57 hybridisatiepatroon bezitten is gering en daarom kan deze probe gebruikt worden voor het verkrijgen van specifieke DNA vingerafdrukken van onverwante *P. infestans* isolaten. Probe RG-57 is daarom goed bruikbaar om, enerzijds, de mate van genetische diversiteit te bepalen in populaties van *P. infestans* en anderzijds, het lot van specifieke genotypen te volgen tijdens een polycyclische epidemie binnen één seizoen of in opeenvolgende jaren. Tevens is DNA probe RG-57 geschikt om vegetatieve

nakomelingen te onderscheiden van geslachtelijke nakomelingen (hoofdstuk 4).

Populatie-genetische studies met behulp van de allozym-merkers *Gpi* en *Pep*, paringstype en resistentie tegen het fungicide metalaxyl, toonden duidelijke verschillen aan tussen *P. infestans* populaties die in 1989 verzameld waren in Nederland. Significante verschillen in genotype frequenties werden gevonden tussen *P. infestans* populaties verzameld in verschillende regio's, tussen A1 en A2 paringstype populaties, en tussen populaties verkregen van aangetaste aardappel- en tomateplanten. Echter een complete scheiding tussen subpopulaties werd niet gevonden. Sommige allozym-genotypen kwamen in meer percelen voor terwijl andere slechts binnen één perceel gevonden werden. In sommige percelen werd maar één allozym-genotype gevonden terwijl in andere percelen verschillende allozym-genotypen voorkwamen (hoofdstuk 5).

De twee beschikbare allozym-merkers, *Gpi* en *Pep*, waren niet toereikend om het niveau van genetische diversiteit in de Nederlandse *P. infestans* populatie nauwkeurig te bepalen. DNA vingerafdruk analyses met DNA probe RG-57 lieten een beduidend hoger niveau van genetische diversiteit in de *P. infestans* populatie zien dan de allozym analyse. Uniformiteit voor allozym-merkers hoeft dus niet te duiden op afwezigheid van genetische diversiteit in *P. infestans* populaties. Het onderscheidend vermogen van de voor *P. infestans* beschikbare allozym-merkers is echter onvoldoende voor gedetailleerde populatie-genetische studies. DNA probe RG-57 onderscheidde 35 genotypen in 153 isolaten die verzameld waren in veertien percelen in Nederland. De helft van deze 153 isolaten had hetzelfde RG-57 genotype. Dit genotype bleek in tien van de veertien bemonsterde percelen voor te komen. Dit wijst op een gemeenschappelijke inoculum bron voor dit genotype en het toont aan dat migratie van *P. infestans* over geheel Nederland plaats kan vinden. Het merendeel (89%) van de RG-57 genotypen kwam maar in één perceel voor en 60% van de RG-57 genotypen kwam maar één keer in de populatie voor. Sommige RG-57 genotypen kwamen zowel op aardappel- als tomateplanten voor. Specialisatie op waardplant, indien aanwezig, is zeker niet compleet. Het grootste deel van de genetische diversiteit was geassocieerd met genetische verschillen tussen percelen, het laagste hiërarchische niveau. Het waargenomen patroon van genetische diversiteit van *P. infestans* duidt op het voorkomen van sexuele voortplanting waarbij nieuwe genotypen ontstaan die, indien ze een hoge fitness bezitten, zich door snelle asexuele vermeerdering in de vorm van vegetatieve sporen over Nederland kunnen verspreiden. In de in 1989 verzamelde *P. infestans* isolaten werden twee typen mitochondriaal DNA, A en B, gevonden. Mitochondriaal DNA type A komt wereldwijd voor maar type B wordt alleen gevonden in streken waar ook het A2 paringstype aanwezig is. Analyses van RG-57 genotypen en typen mitochondriaal DNA bevestigden dat de huidige Nederlandse *P. infestans* isolaten voortgekomen moeten zijn uit een *P. infestans* populatie die recent vanuit Mexico ons land is binnengekomen (hoofdstuk 6).

P. infestans populaties verzameld in opeenvolgende jaren vóór en ná 1980 zijn geanalyseerd op het voorkomen van virulentiefactoren en DNA vingerafdrukpatronen met DNA probe RG-57 om het niveau van genetische diversiteit in de verschillende populaties te vergelijken en het lot van genotypen in de tijd te achterhalen. Vóór 1980 werden in 148 isolaten acht verschillende fysio's gevonden en kwamen de virulentiefactoren 1, 2, 3, 4 en 10 algemeen

voor. Ná 1980 verschenen nieuwe virulentiefactoren (5, 6, 7, 8 en 11), waarvan de corresponderende resistentiegenen ontbraken in de in Nederland gebruikte aardappelcultivars. Ook nam de diversiteit voor virulentie na 1980 enorm toe. In 253 isolaten werden 73 verschillende fysio's gevonden. Vingerafdrukanalyse van isolaten verzameld vóór 1980 heeft aangetoond dat gedurende de twee decennia vóór 1980 maar één RG-57 genotype voorkwam in Nederland. Dit "oude" RG-57 genotype bezat een geringe diversiteit voor virulentiefactoren en deze virulentiefactoren correspondeerden met de in de gebruikte aardappelcultivars aanwezige resistentiegenen. Door de aanwezigheid van maar één paringstype kon dit "oude" RG-57 genotype alleen maar vegetatief overwinteren als mycelium in aardappelknollen. Na 1980 nam de genetische diversiteit enorm toe en werden vele verschillende RG-57 genotypen geïdentificeerd. In 179 isolaten werden 134 verschillende RG-57 genotypen gevonden. Het merendeel (90%) van deze 134 RG-57 genotypen werd niet in meerdere jaren gevonden zodat de *P. infestans* populatie na 1980 ieder jaar uit bijna geheel nieuwe genotypen bestond. Het "oude" RG-57 genotype van vóór 1980 werd niet meer gevonden ná 1980 hetgeen wijst op een complete vervanging van de populatie. De grote genetische verschillen tussen isolaten die de aardappelziekte in opeenvolgende jaren veroorzaken, duiden op een actieve sexuele voortplantingscyclus van *P. infestans* in Nederland (hoofdstuk 7).

Oösporen van *P. infestans* zijn aangetroffen in bladeren en stengels van de aardappelrassen Bintje, Irene en Pimpernel en in bladeren, stengels en vruchten van het tomateras Moneymaker onder geconditioneerde omstandigheden en in veldexperimenten, na inoculatie met isolaten van verschillend paringstype. Produktie van oösporen van *P. infestans* werd waargenomen bij temperaturen variërend van 5 tot 25 °C. Oösporen geproduceerd in blad werden gedurende de winter van 1992-1993 blootgesteld aan natuurlijke weersomstandigheden in niet-gesteriliseerde grond. Deze oösporen bleken na acht maanden nog steeds in staat om aardappelbladeren te infecteren en lesies te veroorzaken in een biotoets. Analyses met DNA probe RG-57 toonden aan dat de lesies waren veroorzaakt door sexuele nakomelingen van de beide ouders die in het voorafgaande jaar gebruikt waren om de oösporen te produceren (hoofdstuk 8).

Uit de resultaten van het onderzoek beschreven in dit proefschrift kan geconcludeerd worden dat *(i)* sexuele voortplanting van *P. infestans* plaatsvindt in Nederland, *(ii)* dat oösporen van *P. infestans* gedurende de winter in de grond kunnen overleven en *(iii)* dat oösporen die in de grond aanwezig zijn infecties kunnen veroorzaken. Infectie van aardappelen door oösporen, die in de grond overwinteren kan als volgt worden verklaard. Gedurende periodes met veel neerslag kiemen oösporen en produceren mobiele zoösporen. Deze bewegen naar het grondoppervlak waar stengels en bladeren die in contact komen met de besmette grond, geïnfecteerd worden en er lesies ontstaan. Tijdens neerslag of beregening kunnen opspattende waterdruppels eventueel leiden tot infectie in hoger gelegen bladlagen. Na enkele dagen kunnen deze lesies sporangiën produceren die zorgen voor een verdere opbouw van een epidemie.

Sexuele voortplanting van *P. infestans* en het voorkomen van oösporen in grond die als inoculum bron dienen, naast overwintering als mycelium in aardappelknollen, heeft zeker een

invloed op de epidemiologie van de aardappelziekte en dit heeft een aantal consequenties voor de bestrijding van de aardappelziekte in Nederland. *i)* Oösporen in de grond kunnen in aardappelplanten (geplant of als opslag) een epidemie starten zodra de weersomstandigheden gunstig zijn voor kieming van de oösporen en infectie. Dit kan aanleiding geven tot een eerder optreden en meer veelvuldig voorkomen van epidemieën. *ii)* Door kiemende oösporen zullen waarschijnlijk bladeren in de lager gelegen bladlagen geïnfecteerd raken. Preventieve fungiciden, die voornamelijk toegediend worden aan de bovenste bladlagen, zullen slechts een matige bescherming geven aan de onderste bladlagen. Dit vergroot de kans op het ontstaan van vele afzonderlijke haarden en het uitbreken van epidemieën. *iii)* Sexuele voortplanting leidt tot een grotere genetische diversiteit in *P. infestans* populaties met als gevolg een groter aanpassingsvermogen van de populatie aan veranderende omgevingsfactoren en bestrijdingsmaatregelen. *iv)* Kieming van pas gevormde oösporen in bladeren en stengels van aardappelplanten stelt *P. infestans* in staat ongunstige weersomstandigheden in het groeiseizoen te overbruggen en bij gunstige weersomstandigheden het overgebleven gezonde blad aan te tasten. De overgang van een zich strikt asexueel voortplantende populatie, die overwinterde als mycelium in aardappelknollen, vóór 1980, naar een populatie die zich zowel sexueel als asexueel voortplant, en die nu ook als oösporen in de grond kan overleven, na 1980, vraagt om een aanpassing van de huidige maatregelen ter bestrijding van *P. infestans* in gebieden waar beide paringstypes voorkomen.

List of publications

Drenth, A., Goodwin, S.B., Fry, W.E. & Davidse, L.C., 1993. Genotypic diversity of *Phytophthora infestans* in The Netherlands revealed by DNA polymorphisms. Phytopathology 83: 1087-1092.

Drenth, A. & Govers, F., 1993. DNA fingerprinting of the potato late blight fungus, *Phytophthora infestans*. p 223-230 in: Schots, A., Dewey, F.M. & Oliver, R. (eds.). Modern assays for plant pathogenic fungi: Identification, detection and quantification. CAB international. pp 267.

Drenth, A., Turkensteen, L.J. & Govers, F., 1993. 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, I.C.Q. & Govers, F., 1994. DNA fingerprinting uncovers a new sexually reproducing population of *Phytophthora infestans* in the Netherlands. European Journal of Plant Pathology (in press).

Drenth, A., Janssen, E.M. & Govers, F., 1994. Formation and survival of oospores of *Phytophthora infestans* under natural conditions. Plant Pathology (in press).

Fry, W.E., Drenth, A., Spielman, L.J., Mantel, B.C., Davidse, L.C. & Goodwin, S.B., 1991. Population genetic structure of *Phytophthora infestans* in The Netherlands. Phytopathology 81: 1330-1336.

Fry, W.E., Goodwin, S.B., Matuszak, J.M., Spielman, L.J., Milgroom, M.G. & Drenth, A., 1992. Population genetics and intercontinental migrations of *Phytophthora infestans*. Annual Review of Phytopathology 30: 107-129.

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

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

Spielman, L.J., Drenth, A., Davidse, L.C., Sujkowski, L.J., Gu, W.K., Tooley, P.W. & Fry, W.E., 1991. A second world-wide migration and population displacement of *Phytophthora infestans* ? Plant Pathology 40: 422-430.

Curriculum vitae

André Drenth werd geboren op 16 augustus 1962 te Bellingwolde. Hij groeide op, als vijfde in een gezin van zes kinderen, op het Groninger platteland. Na vier jaar HAVO, één jaar Middelbare Technische School en één jaar werken op het ouderlijk bedrijf begon hij in 1981 aan de middelbare opleiding voor landbouwonderwijs op de Jan Heidema-school te Groningen. De voor deze opleiding vereiste stages werden volbracht in Texas, USA (1982) en in Kenya (1983). In 1984 behaalde hij het MAS diploma en won de Jan Heidema prijs. De studie werd voortgezet op de Hogere Agrarische School te Dordrecht in de richting Agrarische Bedrijfskunde. In 1985 stapte hij over naar de toenmalige Landbouwhogeschool te Wageningen waar in 1986 de propaedeuse met lof werd afgesloten. In maart 1990 studeerde hij af in de richting Plantenveredeling. De doctoraalstudie omvatte als afstudeervakken Plantenveredeling en Moleculaire Biologie. De stage werd in 1988-1989 volbracht bij het Plant Breeding Institute van de universiteit van Sydney, Australië. In januari 1990 begon hij bij de vakgroep Fytopathologie en het instituut voor planteziektenkundig onderzoek (IPO-DLO) met een promotieonderzoek waarvan de resultaten staan beschreven in dit proefschrift. Als onderdeel van het promotieonderzoek werkte hij van december 1990 tot augustus 1991 bij de afdeling Plant Pathology van Cornell universiteit, Ithaca, USA. Vanaf januari 1994 is hij werkzaam bij het Cooperative Research Centre for Tropical Plant Pathology te Brisbane, Queensland, Australië.

Nawoord

Wetenschap is als een estafette, men bouwt voort op het resultaat van anderen. Het is dan ook eenvoudig in te zien dat een gezamenlijke inzet het meest succesvol is. Echter, maar één renner zal, na een eventuele eindsprint, de eindstreep passeren. De schrijver van een proefschrift kan met de laatste renner worden vergeleken.

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André Drenth