The Late Blight Pathogen, *Phytophthora infestans*

Interaction with the Potato Plant and Inoculum Sources

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Abstract

The oomycete, *Phytophthora infestans,* is one of the most important plant pathogens worldwide. This thesis focuses on the late blight pathogen and its host, potato (*Solanum tuberosum*), and the interaction between these two organisms.

Resistance to *P. infestans* was evaluated histologically, using leaves of potato cultivars with varying levels of general resistance. Preinfectional events could be ruled out as discriminating factors determining the level of resistance in the cultivars studied. After penetration of the pathogen into the leaf tissue there were significant differences between cultivars in hyphal growth and branching.

The biochemical response of the tubers to pathogens, more specifically the accumulation of sesquiterpenoid metabolites and their biological effect on *P. infestans* was examined. Two unique sesquiterpenoid metabolites were isolated from tubers. The influence on mycelial growth of *P. infestans* of these compounds and of five naturally occurring plant sesquiterpenoids was tested. The two metabolites, isolated from tubers, induced a slight growth stimulation. All other compounds tested, suppressed the growth of the pathogen.

The population structure of *P. infestans* in one single field in southwest Sweden was analysed with the aim to study the origin of the primary infections. Mitochondrial DNA, mating type and SSR-genotype were used as markers. Some foci were monomorphic for all markers, while other foci displayed a large proportion of unique genotypes. This was taken as evidence that inoculum had come both from tubers and oospores within this field.

The dynamics of an epidemic and sexual reproduction of *P. infestans* was investigated in an experimental field inoculated with six different isolates of the pathogen. Three weeks after inoculation sampling was done. The following year, *P. infestans* isolates were baited from soil samples taken from the field. Parentage analysis, based on SSR markers, showed that recombinant genotypes from the inoculum isolates were present in the soil samples. These findings demonstrate that oospores produced during a summer epidemic in Sweden can overwinter and cause infection the next year.

Keywords: infection process, oospores, *Phytophthora infestans*, phytoalexins, late blight, resistance, sexual reproduction

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Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I   Berggren, B., Widmark, A.-K., Umaerus, V. (1988). The expression of general resistance to late blight (*Phytophthora infestans*) in potato leaves. *Potato Research* 31, 611-616.

II Engström, K., Widmark, A.-K., Brishammar, S., Helmersson, S . (1999). Antifungal activity to *Phytophthora infestans* of sesquiterpenoids from infected tubers. *Potato Research* 42, 43-50.

III Widmark, A.-K., Andersson, B., Cassel–Lundhagen A., Sandström, M., Yuen, J. (2007). *Phytophthora infestans* in a single field in southwest Sweden early in spring: symptoms, spatial distribution and genotypic variation. *Plant Pathology* 56, 573-579.

IV Widmark, A.-K., Andersson, B., Sandström, M., Yuen, J. Tracking *Phytophthora infestans* with SSR markers within and between seasons (manuscript).

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Prologue

This is the beginning of the late blight history:

> "*A fatal malady has broken out amongst the potato crop. On all sides we hear of destructions. In Belgium the fields are said to have been completely desolated. There is hardly a sound sample in Covent Garden Market*".

E.C. Large begins his book "The advance of the fungi" (1946) with this citation from The Gardeners' Chronicle and Agricultural Gazette, August 23rd, 1845.

1 Introduction

Potato late blight is one of the most devastating plant diseases world–wide and is feared globally by farmers and industry. During favourable conditions all above ground parts of the potato crop can be destroyed within a week. The control of the disease has traditionally relied on foliar applications of fungicides, resulting in a very high input of pesticides in the potato production. The annual cost caused by late blight is estimated to over 4 billons € worldwide (Haverkort *et al.*, 2008). Besides the high costs for fungicides there is a societal resistance today against using potentially harmful chemicals. Research on late blight is not only of scientific interest, but is important both from an economical as well as an environmental perspective.

The late blight pathogen *P. infestans* is a challenge to study. It is a microorganism that behaves as a biotroph in nature but it can also be maintained on artificial media. It is able to actively seek infection sites on its host plant both above and below ground. It can form thick–walled resting spores that can overwinter in the soil, flagellated spores that can swim, and spores that can be dispersed with the wind. Then we have the host plant, the potato. Both underground parts and the canopy can be infected. The tubers are often stored before consumption and infected tubers can be totally destroyed during storage. Diseased tubers can bring inoculum that spreads to other parts of the plant, to other plants in the field and to other fields. By global trade the inoculum can cross continents and oceans.

Since the dawn of plant pathology, potato late blight has tended to take center stage. More than 150 years ago Miles J Berkeley published the paper in the Journal of Horticulture Society of London in which he attributed a fungus as the cause of the new potato disease in Europe (Large, 1946). Since then intensive research on potato late blight has been carried out

continuously often resulting in more questions than answers. Late blight research has focused on varying topics under different periods and new techniques often decide the research area.

The work included in this thesis reflects some of the trends during the most recent decades of late blight research. The focus is on the late blight pathogen *Phytophthora infestans* (Mont.) de Bary and on the interaction with its host plant, the potato (*Solanum tuberosum* L.). The pathogen's behaviour on leaves from different potato cultivars with varying levels of general resistance was examined. The main object was to find out where in the infection process the pathogen is hampered by host resistance (paper I). The biochemical response of the potato tubers to pathogens, more specifically the accumulation of sesquiterpenoid metabolites and their effect on the late blight pathogen, was investigated (paper II). The population structure of *P. infestans* in one single field in southwest Sweden was analysed with the aim to study the origin of the primary infections in the field (paper III). An experimental field was inoculated with six isolates of *P. infestans*. The following year, isolates were captured from soil collected in the field. Parentage analysis was performed on these isolates to trace the origin of the progeny (paper IV).

2 The host

Potato (*Solanum tuberosum* L.) is the fourth most important food crop in the world after wheat, maize and rice. It is grown in more than 100 countries. It is cultivated under temperate, subtropical and tropical conditions. Even if potato production has declined in Europe and the US it has increased in the developing countries, notably China and India, resulting in an increase in global production in the last twenty years. The temperature is the limiting factor for production: tuber growth is inhibited below 10°C and above 30°C. Because of this, potato is planted early in spring in temperate regions and grown under the coolest period of the year in the hot, tropical regions (www.potato2008.org). Potatoes are more nutritious, faster growing and need less water than any other major crop (Anonymous, 2008). Since potato is vegetatively propagated it is vulnerable to diseases. The late blight disease caused by *P. infestans* is considered to be a major constraint for potato production wherever potato is grown. Due to this, commercial potato production would hardly exist without routine use of fungicides.

2.1 The introduction of the potato plant in Europe and Sweden

The cultivated potato originated in the Andes in Peru around Lake Titicaca, where a wide variety of primitive cultivars and wild species-relatives still exists (Spooner *et al.*, 2005). The conquistadors brought not only gold but also potatoes on their ships back to Europe. However, no one knows exactly when the potato was introduced in Europe. From the beginning it was mostly grown in gardens as a botanical curiosity. In Sweden, Olof Rudbeck planted potato in the Uppsala Botanical garden in 1658. Jonas Alströmer began to cultivate potato on his farm outside Alingsås in 1724, propagated for potato cultivation, and is seen as the father of Swedish potato production. However, in the 18th century potato mostly was grown in

home gardens. It was not until Swedish soldiers returning from the Pomerian War (1757-1762), during which they had learnt to appreciate potato, that the production increased. The use of potato for production of alcohol also helped to establish the crop. In Sweden, potato cultivation reached a peak around the year 1900 with around 150 000 ha (Osvald, 1965).

3 The pathogen

3.1 The arrival of the late blight pathogen to Europe

A new potato disease struck the American east coast in 1843. In Europe, it was observed in Belgium and England in 1844. The next year it suddenly appeared in Belgium and Netherlands in June. The speed with which the disease spread all over Europe was frightening. When the potato crops were destroyed the rural people suffered from starvation and infectious diseases. The situation became worst in Ireland. The Irish people were poor and had almost nothing but potato to eat (Large, 1946; Bourke, 1991; Turner, 2005). The "Irish Famine", which caused mass mortality and emigration, is well known even outside the field of plant pathology.

If the pathogen that started the historical late blight epidemics came from Mexico or South America has been a scientific debate almost since the first appearance of the disease. (Bourke, 1964; Andrivon, 1996; May & Ristaino, 2004). There is also an ongoing scientific debate whether the origin of *P. infestans* is Mexico or South America. Central Mexico has been proposed as the center of origin since until recently it was only in this region both mating types were found to be present in a 1:1 ratio. In addition, the late blight population has very high genotypic and phenotypic variation in this area. There is also a gene–for–gene relationship between some resistant wild Mexican potato species and *P. infestans* (Niederhauser, 1991; Grünwald *et al.*, 2001). However, more recently, a South American origin has been suggested, based on studies of mitochondrial and nuclear loci in *P. infestans* and the closely related *Phytophthora andina* (Adler *et al.*, 2004; Gómez-Alpizar *et al.*, 2007).

3.2 The potato murrain

When late blight first appeared in Europe it was not clear what caused this new disease, "the potato murrain" as it was called. At that time the idea that plant diseases were due to fungi was rather unorthodox and even heretical. The first to identify a fungus as the cause of the disease and give a detailed description of the pathogen was the Belgian mycologist, Marie-Anne Libert in 1845, who named it *Botrytis vastatrix*. A bit later the same year, Jean Francis Camille Montagne described the potato pathogen and gave it the name *Botrytis infestans*. The following year the reverend Miles J Berkeley published a paper, in which he attributed a fungus as the cause of the new disease. However, even if fungal mycelium was observed on the plant the general view was that it was a consequence of the disease and not the cause (Large, 1946; Bourke, 1991; Zadoks, 2008). The controversy continued until 1876, when Anton de Bary showed that the disease only developed on potato plants dusted with fungal spores and not on the untreated ones. He also demonstrated that tubers could be infected by watering sporangia into the soil. He was the first to observe the motile zoospores and described the life-cycle of the late blight pathogen. Anton de Bary renamed the pathogen to *Phytophthora infestans*, which means 'infectious plant destroyer' (Large, 1946; Turner, 2005). The same year, Robert Koch demonstrated that anthrax was caused by a bacterium that he called *Bacillus anthracis* (Kronvall, 2000). These discoveries helped pay the way for a general acceptance of the fact that diseases can be caused by microorganisms.

In Sweden, the 1845 epidemic of this new potato disease spread to Uppsala. As in other European countries, there was a debate about the cause of the disease. The famous mycologist Elias Fries participated in the debate from the beginning, and interestingly, was against the fungal theory. He suggested that the main causes were a combination of an excess of water during the growing season and the rich supply of nitrogen amended in the soil with new agricultural practises. He also believed that the potato plant became exhausted and degenerated without a re-generation through seeds (Eriksson, 1884).

3.3 Taxonomy and biology of *P. infestans*

The genus *Phytophthora* belongs to the oomycetes of the kingdom Stramenophila, a group of microorganisms that morphologically resemble fungi, but are more closely related to aquatic organisms such as brown algae, golden-brown algae and diatoms (Förster, 1990; Dick, 2001). The oomycetes include a diverse range of water moulds as well as pathogens of

most classes of organisms ranging from vertebrates to plants. In contrast to higher fungi, they have a non-septated (coenocytic) mycelium. During their life-cycle they form motile spores (zoospores) with two flagella making them able to swim. This can be seen as an indication of an aquatic ancestry. The genus *Phytophthora* includes more than 60 species, mainly parasitic on various plant hosts (Erwin & Ribeiro, 1996).

The oomycetes are diploid for the major part of their life-cycle. The cell wall consists mainly of cellulose and other glucans (Bartnicki-Garcia, 1968), in contrast to the true fungi where chitin is the major cell wall component. Within the oomycetes, the genus *Phytophthora* lacks the ability to synthesize sterol and thiamine (Erwin & Ribeiro, 1996), and consequently needs to acquire these essential compounds from the host plant.

P. infestans is a hemibiotrophic pathogen with a narrow host range. Most host plants belong to the *Solanum* and *Lycopersicum* genera (Erwin & Ribeiro, 1996). The two main crop hosts are potato and tomato (*Solanum lycopersicum* L. also known as *Lycopersicum esculentum* Mill.).

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4 The disease – life cycle

4.1 Effects on the plant

When leaf tissue becomes infected and invaded the photosynthetic tissue will be destroyed resulting in less assimilates. Both the quality and the quantity of the harvest will be affected. Tubers infected during the growing season can rot during storage due to blight and secondary infections caused by other organisms.

4.2 Asexual cycle

Phytophthora infestans can infect all parts of the potato plant except roots (Fehrman & Dimond, 1967). Sporangia are formed on infected leaf surface in humid weather and are spread by the wind or splashed by water to other plants. If there is free water on the leaves and the temperature is below 16°C, motile zoospores can be released from sporangia. Both sporangia and zoospores can infect the plant but zoospores are believed to be more important. The zoospores encyst and form germ tubes that swell to appressoria. An infection peg is formed and the pathogen infects the plant by direct penetration through epidermal cells or through stomata. After penetration, an infection vesicle is formed and mycelium grows both inter- and intracellularly (Fig 1:1). Haustoria occur occasionally (Fig 1:2). A few days after infection the mycelium emerges through the stomatal openings and new sporangia are formed. (Grenville-Briggs & van West, 2005). The sporangia will be released when there is a drop in air humidity. Tubers are easily infected in rainy weather when the sporangia are washed down in the soil and zoospores are released. The zoospores might penetrate the tubers

through wounds, lenticels and eyes (Robertson, 1991). Infected tubers can act as inoculum sources and start an epidemic the following year.
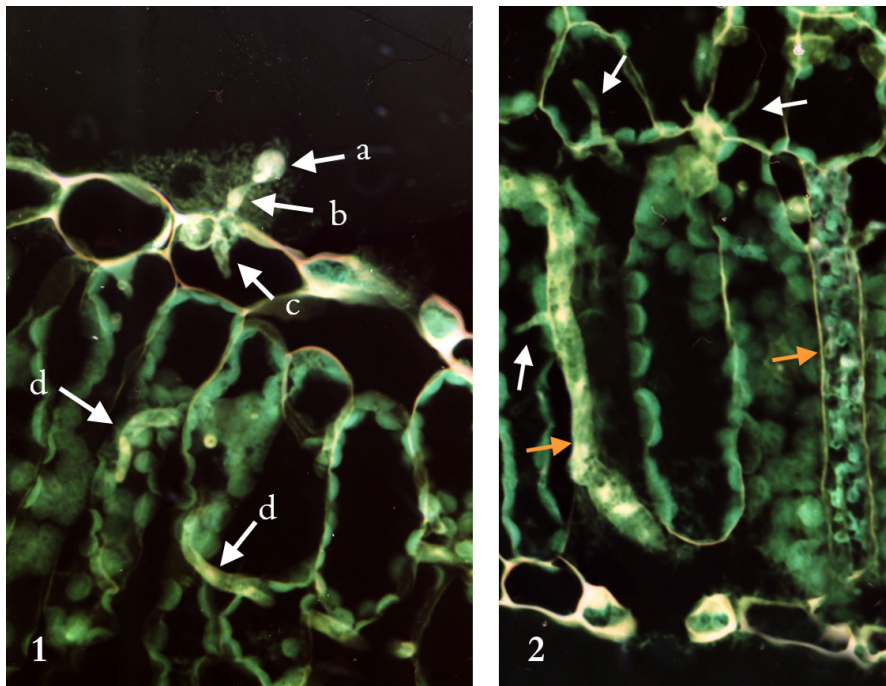
Figure 1. 1: Infection process of *P. infestans* in potato leaf tissue. a: encysted zoopore, b: appressorium, c: infection vesicle, d: hyphae. 2: Haustoria. White arrows indicate haustoria. Red arrows indicate hyphae.

4.3 Sexual cycle

When plants are infected with isolates of both mating types, sexual reproduction with oospore formation may occur. Oospores are more abundantly formed in stems than in foliage, probably because the stems survive blight attack longer than leaves (Frinking *et al.*, 1987; Mosa *et al.*, 1991). For the same reason, more oospores are produced on cultivars with medium high resistance than on susceptible ones (Drenth *et al.*, 1995; Hanson & Shattock, 1998). When infected plant debris fall to the ground and decomposes, the oospores are released into the soil. Very little is known about oospore germination in the soil and the mechanism by which potato plants are infected by the oospores (Andrivon, 1995; Drenth *et al.*, 1995).

4.4 Overwintering

Zoospores, sporangia and free-living mycelia in the soil are considered to be short-lived (Lacey, 1965). This means that without sexual reproduction the pathogen is forced to survive between seasons as living mycelium in its host plant, i.e seed tubers, tubers left in the field (volunteers) or piles of waste potato (Shattock, 1976). In the Netherlands, overwintering infected tubers in fields and piles are considered to be the most important primary inoculum sources (Zwankhuisen *et al.,* 1998; Zwankhuisen *et al.*, 2000). However, in the Nordic countries infections from these sources are of less importance compared to areas with milder winters. Consequently, seed borne inoculum is considered to be the quantitatively most important source of *P. infestans* inoculum in this region (Lehtinen & Hannukkala, 2004).

In contrast to the asexually derived spores, the sexually produced oospores are more robust and can overwinter in soil. Accordingly, in a population with both mating types present, the late blight pathogen has an additional survival strategy, independent of its host.

4.5 Control of the disease

When the late blight appeared in Europe for the first time, it was not known that the disease was caused by a microorganism. Adequate chemical control measures were not available until the end of the 1880s when Millard discovered that copper salts mixed with lime were effective to control mildew on grape vine. This mixture also showed to be efficient against potato late blight but was not generally used to control this disease until after the First World War. However, agronomic measures such as hilling and vine-killing prior to lifting became common practises in the end of the 19th century (Turner, 2005), and are still common. Today late blight is controlled by a combination of sanitary measures, crop rotation, resistant varieties and chemical treatment. Commercial potato production would hardly exist without routine use of fungicides. However, decreasing the chemical impact on the environment is now on the agenda. Moreover, applying fungicides is expensive with costs for chemicals and fuel. In Sweden today, the consumer's demand for organic potato is higher than what the growers can supply. Farmers in the developing countries can usually not afford the most modern fungicides. Of the various means of control available, host resistance is the most attractive, from both an environmental and economical perspective.

5 Plant resistance

5.1 Plant defence

Of all microorganisms on earth very few are able to colonize living plants. Also, the host range of a plant pathogen is usually limited and restricted to a few or a single plant species. This means that plants are not passive when they are exposed to microbial attack but are, in fact, resistant to most microorganisms and have an array of chemical and physical barriers that a potential pathogen must overcome. There are two types of defence, *constitutive* and *active* (induced). Examples of constitutive defence are a thickened cuticle and constitutively produced secondary metabolites. Also, crop architecture may have an effect on spore deposition and microclimate, affecting the plant's susceptibility to plant pathogens (Niks & Rubiales, 2002). Such mechanisms provide a generalized protection throughout the lifetime of the plant. In contrast, active defence will not be triggered until a pathogen starts to attack the plant. Surprisingly, constitutive defence in potato against *P. infestans* is rarely described in the literature. This type of resistance today often appears to be forgotten or neglected, especially by molecular biologist working in the late blight research field. On the other hand, active defence is an expanding research area in the current biocomputional and genomic era.

5.2 Types of resistance

Genetically controlled disease resistance in plants against pathogens are usually classified as *race-specific* or *general* resistance. By classical breeding both types of resistance to *P. infestans* have been introduced into potato. Much general knowledge about plant defence has been accumulated but which

factors that are most important in each pathogen-host plant encounter have been more difficult to elucidate.

Breeding for late blight resistance is a slow process. Resistance to late blight is only one of several traits that must be included in a new cultivar and potato varieties are more commonly chosen on the basis of consumers preferences, agronomic familiarity, yield and storability, than for disease resistance. For example, in Sweden 2009, cv. King Edward, despite its high susceptibility to late blight, was the most grown of all table potato varieties (Ingvar Nilsson, personal communication).

In the first half of the last century, race-specific (vertical) resistance was introduced in *Solanum tuberosum* by crosses with *Solanum demissum* Lindl. Black *et al.* (1953) described a detailed pattern of interaction between physiological races of *P. infestans* and host genotype. (For example, race 4 of *P. infestans* can infect potato that possesses the resistance gene R4). The specific resistance is assumed to be based on a gene-for-gene relationship (Flor, 1971), where an avirulence gene product is recognized by a dominant R-gene in the host. In such an incompatible reaction a hypersensitive response (HR) is triggered. The initially infected cells and the surrounding cells die and the disease is completely inhibited. Races of the pathogen lacking avirulence genes corresponding to the host's R-genes do not trigger HR and readily infect the host tissue. Race-specific resistance in potato against *P. infestans* was initially very successful, but later proved not to be durable because the pathogen evolved new virulent races in response to the selection pressure applied by widespread cultivation of resistant varieties with race-specific resistance (Malcomson, 1969; Umaerus *et al.*, 1983). In a recent study of the Nordic late blight population only "complex" races of *P. infestans* were found, i.e lacking several avirulence genes (Lehtinen *et al.*, 2008). Race-specific resistance has been the most investigated host-pathogen relationship because it is the easiest to work with. In potato, however, this monogenic resistance against *P. infestans* has little practical importance.

Single R-gene resistance against late blight has lost attraction mainly because of the lack of durability. Whether these single genes are realized by classical breeding or introduced via genetic modification will probably not affect the durability. One argument in favour of resistance brought by genetic engineering is that it may be a faster way of acquiring a resistant variety compared to classical breeding. However, to get a transgenic cultivar out on the market in Europe, with all legislations and restrictions concerning GMO (genetically-modified-organisms) will at present be a time-consuming and expensive process. Also, the consumers attitude in

Europe towards GMO food today is sceptical. A current research program in the Netherlands is based on genetic engineering, but only with genes from wild potato species. The hope is that the authorities and the public will accept these "cisgenic" potatoes more easily than "transgenic" ones (Haverkort *et al.*, 2008*)*.

General resistance, (also called horizontal resistance or field resistance), is assumed to be polygenic and is therefore considered to be more stable than race-specific resistance since the pathogen population must change several loci to adapt to the host (Umaerus *et al.*, 1983). In addition, less selection pressure is thought to be placed on the pathogen with this type of resistance than with race-specific resistance since the plant is not totally immune. In this context it must be stressed that durability of the resistance is almost as important as the level of resistance. Umaerus predicted, that future field resistant cultivars might have such levels of resistance that fungicidal treatment would not be necessary (Umaerus, 1970). Today we can see that he underestimated the adaptive capacity of the late blight pathogen and with a sexually reproducing population of *P. infestans* present in Europe, we can expect even faster population changes than earlier. According to Vanderplank (1963), horizontal resistance is not considered to give total immunity but to slow down the growth of the pathogen and to confer equal protection against all races of the pathogen. General resistance is also influenced by environmental factors such as day length and the nutritional status of the plant. Plant age also affects the host's susceptibility to late blight (Umaerus, 1970).

Niederhauser (1991) noted that much information has been accumulated on the use of race-specific R-genes but very little is known about the more durable general resistance such as what basic chemical and physical factors contribute to this type of resistance and how such factors are genetically controlled. According to Bradshaw (2009) four types of general resistance is now recognized: Late maturity is one trait often associated with general resistance. Some quantitative trait loci (QTL) have been defined in the potato genome, which have a large effect on general resistance. However, QTL-isolate interactions occur which questions the durability of this type of general resistance. There is also one type of general resistance associated with defeated R-genes (se below). Finally, there is resistance that is not associated with any of these types, and which does not show resistance-isolate interaction.

Most isolates of *P. infestans* found in the fields today are complex races able to overcome several of the known R-genes. Therefore, selection of resistance against late blight is usually made in an R-gene free population of

potato clones. However, residual effects of defeated R–genes have been reported for several crop plants (Pedersen & Leath, 1988). In an experiment, Stewart *et al.* (2003) crossed R–gene and R–gene-free cultivars. When the progeny was tested in a field trial the group that had inherited the R–genes were significantly more resistant than the R–gene free group. They concluded that the beneficial effect of the presence of the R–gene may be a true residual effect by the defeated gene or by other genes closely linked to the R–gene.

According to Vanderplank (1975), vertical and horizontal resistance genes differ from each other. Vertical resistance genes do not participate in horizontal resistance and vice versa. However, the general view today is that the defence reactions in the plant to *P. infestans* is of quantitative rather than qualitative nature. (e.g. Fritzmeyer *et al.*, 1987; Kamoun *et al.*, 1999; Desender *et al.*, 2007). For example, in several cytological investigations it has not been possible to see any qualitative differences in the phenotypic expression of race-specific and general resistance. The HR, traditionally associated with R–gene mediated resistance has been observed in all types of interactions between *P. infestans* and its host (Wilson & Coffey, 1980; Coffey & Wilson, 1983; Geés & Hohl, 1988; Vleeshouwers *et al.*, 2000). However, the timing and the number of HR responding cells suggest a correlation between resistance level and HR effectiveness (Vleeshouwers *et al.*, 2000), implying a central role for HR in all resistance interactions.

5.3 The expression of general resistance to late blight (*Phytophthora infestans*) in potato leaves (paper I)

Van der Zaag (1959) divided general resistance into several components: the chance of infection, (i.e. the chance of a spore penetrating the leaf), the extent of spread of the mycelium and the rate and the number of sporangia formed. Umaerus & Lihnell (1976) showed that resistant potato cultivars had a lower infection frequency, smaller lesions and lower sporulation capacity than more susceptible cultivars. The objectives in our study (paper I) were to try to determine when and where in the infection process the resistance against *P. infestans* is first expressed and whether there are some varietal differences. Different potato cultivars with varying levels of general resistance of the same maturity class and with no known R–genes were infected with a complex race of *P. infestans*. The hypothesis was that the resistance was expressed before penetration. This was based on the observations made by Lapwood (1968) and Bignell (1975) who found

longer germ tubes on the leaf surface of resistant hosts than on susceptible ones.

5.3.1 Preinfectional events

In our study (paper I) six potato cultivars with different levels of general resistance were used and preinfectional parameters such as frequency of spore germination and appressoria formation, site of appressoria formation, and germ tube length were measured. We did not find any significant statistical differences in the parameters measured. Our conclusion was that the host genotype did not influence any preinfectional events in the pathogen's morphogenesis in the material studied. These results are contradictory to those earlier reported by Bignell (1975) and Lapwood (1968). Both Bignell and Lapwood used the cultivar Pimpernel in their investigations. The longer germ tubes observed on this *S. tuberosum* cultivar may be a response to a rather unusual form of constitutive defence present in cv. Pimpernel. Interestingly, Oyarzún *et al.* (2004) who studied the preinfectional stages of *P. infestans* on accessions of the potato species *S. phureja*, observed that the germ tubes were longer on resistant clones.

With our techniques it was not possible to detect the actual penetration moment but Wilson & Coffey (1980) who made a thorough cytological investigation, noted fewer numbers of penetrations of *P. infestans* on cv. Pimpernel which expressed a higher level of general resistance compared to cvs. Majestic (susceptible) and Shamrock (resistant). However, Geés & Hohl (1988), could not find any difference in penetration frequency of epidermal cells of potato foliage between cultivars with either general or race-specific resistance. They concluded that prepenetration and penetration events are non-discriminating factors in specific and general resistance to late blight. Vleeshouwers *et al.* (2000) cytologically examined a diverse set of potato cultivars and wild Solanum species. They observed differences in penetration frequency of *P. infestans* on different Solanum clones but this was not correlated to resistance level. In contrast, Rubio-Covarrubias *et al.* (2006) found that resistance to penetration of *P. infestans* into the epidermal cells of the host plant was correlated with the resistance level in the five potato cultivars studied. In summary, it is not possible to answer the question whether the penetration of epidermal cells is influenced by the host genotype or not. The disparate results obtained may be explained by the fact that different potato cultivars and *P. infestans* isolates were used in the studies.

5.3.2 Postinfectional events, varietal differences

Leaf discs from three potato cultivars with different levels of general resistance were inoculated (paper I). The cvs. Bintje, SV76127 and Matilda with low, medium and high levels of resistance, respectively were included in the analysis. Small leaf tissues were embedded, sectioned and stained. In this histological examination there appeared to be varietal differences to the pathogen attack. Already 12 hrs postinfection fewer hyphae were observed in cv. Matilda than in the two other more susceptible cultivars. The few hyphae noticed in cv. Matilda initially grew faster than in cvs. Bintje and SV76127, but after 24 hrs the hyphal growth rate was significantly lower. After 48 hrs we observed differences between all three cultivars in hyphal growth rate and number of observed hyphae per section. In cv. Matilda there were also fewer hyphal branches observed compared to the other two cultivars.
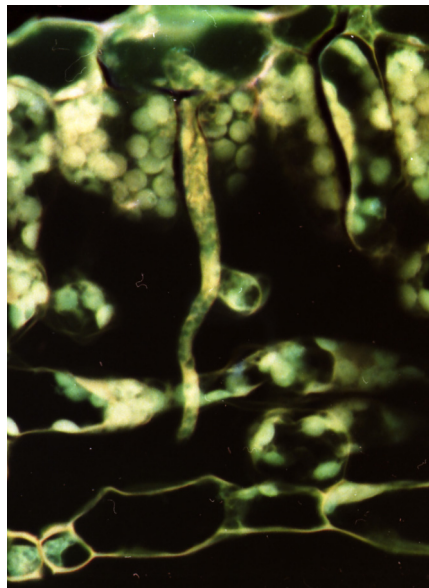
Figure 2. A nonbranching intercellular hypha of *P. infestans* in cv. Matilda, 24 h postinfection.

5.3.3 Conclusions

The conclusions from this study (paper I) are that the resistance is expressed early in the infection process but usually not before penetration and that prepenetration events are unlikely as differential factors for estimating the degree of general resistance in the plant cultivars studied. Since the level of general resistance against late blight in different potato cultivars assumingly

comprises both constitutive barriers and inducible defence reactions in varying proportions and combinations, screening for general resistance, even in the future, probably must be carried out in the traditional way in greenhouses and field trials.

6 The biochemical era - in search for resistance factors

6.1 Induced resistance

No matter what the mechanisms of stimulation of the plant defence are, they require recognition of the pathogen as "non-self" and consequently signal transductions to activate different cellular responses. However, the observations of induced resistance reviewed by Chester (1933) and later verified by Kuć *et al.* (1959) were largely ignored at the time when they were published. According to Kuć (2001), the work with induced resistance in his laboratory that started in the late 1950s and up to 1980s, was greeted with curiosity and was often thought to be "somehow mistaken". But now this concept is considered to be "self-evident and obvious" (Kuć, 2001). Agents that elicit the plant defence (elicitors) can be pathogen derived substances or compounds released from the plant-cell wall caused by pathogen activity. Based on the similarity to the self and non-self recognition models of the animal innate immune system the plant pathogen-derived elicitors often today are called PAMPs or MAMPs (pathogen- or microbe-associated molecular patterns). PAMPs are considered to be conserved molecules that are displayed or secreted on the surface of the pathogen (Bent & Mackey, 2007). Examples on elicitors from *Phytophthora infestans* are cell-wall glucans (Friend, 1991), arachidonic acid (Bostoc *et al.*, 1983) and elicitins. Elicitins are extracellular proteins that are produced by all tested *Phytophthora* and *Pythium* species (Pernollet *et al.*, 1993; Kamoun *et al.* 1994). In the *Phytophthora parasitica-Nicotiana* pathosystem elicitins have been shown to act as avirulence factors (Kamoun *et al.*, 1994). However, though elicitins are produced by *P. infestans* mycelium when cultured *in vitro*, they are down-regulated during the infection process and they do not

appear to be involved in the defence response against the pathogen in the potato plant (Kamoun *et al.*, 1997). This means that *P. infestans* recognizes the potato plant as a host and is also able to avoid recognition by the host plant. In tobacco, elicitins produced by *P. infestans* have been shown to be involved in non-host interaction (Kamoun *et al.*, 1998).

The recognition of conserved features of the pathogen on the plant cell surface receptors will induce altered cytoplasmic Ca^{2+} levels, activation of MAPK (mitogen-acitvated kinase) cascade and production of reactive oxygen species and nitric oxide (Nürnberger & Scheel, 2001). These early events can transduce additional intracellular signals leading to expression of defence responses. Examples of such responses are the synthesis of phenolics and proteins in the cell wall, rapid cell collapse and death, accumulation of antimicrobial compounds and the synthesis of hydrolytic enzymes, e.g. chitinases and glucanases, (Kombrink & Somssich, 1995; Keen, 1999). Two stress-induced main pathways are known: the salicylate- and jasmonate-dependent pathways. The salicylate pathway is known to be triggered by pathogen attack and the jasmonate pathway mainly by wounding but also by microorganisms. The salicylate-pathway is closely correlated to HR. There are also cross-talks between the salicylate- and jasmonate-pathways that often is counteractive but synergistic interactions have also been reported (Koornneef & Pieterse, 2008).

To be able to colonize, a plant a pathogen must be overcome the plant defences. Evasion of recognition or suppression of the plant defence response are two ways for a pathogen to invade plant host tissue. Detoxification of antimicrobial compounds is another approach used by some plant pathogens (Morrisey & Osbourn, 1999). Examples on suppressors identified from *P. infestans* are glucans (Doke *et al.*, 1979; Andreu *et al.*, 1998), and extracellular protease inhibitors (Tian *et al.*, 2004) Instead of using terms like avirulence factor, elicitor, toxin etc. a new term "effector*", has been introduced since the term "effector" is neutral. The underlying assumption of much current research on plant pathogen interactions today is that effectors known only by their avirulence or elicitor functions may have an unknown virulence activity (Kamoun, 2007). Effector research is at present mainly focused on protein effectors assumed to be secreted, based on computational analyses of genomic data.

6.2 Phytoalexins

The idea to vaccinate plants and in that way make the plant immune arose much earlier than the concept "induced resistance". In 1933, Chester

reviewed 200 publications describing a phenomenon he termed physiological acquired immunity (Chester, 1933). He looked for proteins, which like antibodies, are capable of binding other proteins. This idea was taken up again by Müller & Börger (1940) who stated that: "protective immunization today is an indispensable tool in medicine and veterinary medicine which probably could be applied also to plants". Müller and Börger demonstrated that by inoculating with an avirulent race of *P. infestans*, the tuber became "immunized" and virulent races could subsequently not attack the tuber (Müller & Börger, 1940). They proposed the term phytoalexins (PAs) to describe compounds inhibitory to fungal growth that assumingly were formed by potato tuber slices when inoculated with avirulent races of the late blight pathogens. However, they did not look for the PA compounds in potato tubers.

The phytoalexin concept became popular because of the analogies with the immune response in mammals. Several definitions of PAs have been suggested and in 1981 scientists assembled and tried to agree upon a definition for phytoalexins. The working definition arrived at by consensus at the meeting was that "Phytoalexins are low-molecular weight, antimicrobial compounds that are both synthesized by and accumulated in plants after exposure to micro-organisms" (Paxton, 1981). This definition does not require evidence that the compounds are involved in resistance. Many of the compounds defined as PAs have been shown to have antimicrobial effect *in vitro*. However, in general, PAs are comparatively weak as antibiotics (Kuć, 1995; Smith, 1996). For example, as seen in our work (Engström *et al.*, 1999), the fungicide metalaxyl was inhibitory at a much lower concentration than the PA rishitin.

Substances defined as phytoalexins are diverse compounds, predominantly phenolics, isoprenoids and acetylenes. Each plant family tends to produce the same class of closely related compounds. Usually accumulation of PAs occur faster and to a higher level in an incompatible reaction than in a compatible one but PAs have also been found in healthy plant tissue (Smith, 1996). The synthesis of PAs can be induced by pathogens but also by stress-compounds of abiotic origin such as heavy metal salts, cold and UV light (Ebel, 1986; Smith, 1996). When potato tubers are stressed by pathogens they produce, among other substances, sesquiterpenoid metabolites. Sesquiterpenes are a class of terpenes that consist of three isoprene units and have the molecular formula $C_{15}H_{24}$. The first postinfectional sesquiterpenoid isolated from tubers was rishitin (Katsui *et al.*, 1968) which accumulated in high amount, (i.e. 100 µg/g of fresh tissue), in tuber slices from the cv. Rishiri infected with an incompatible

race, but only in small amounts when inoculated with a compatible race. Based on this result it was assumed that rishitin was one of the main components in tuber resistance against the late blight pathogen.

6.3 Antifungal activity to *Phytophthora infestans* of sesquiterpenoids from infected tubers (paper II)

The research on phytoalexins in tubers has been focused on the major sesquiterpenoid metabolites rishitin, lubimin and solavetivone found in tubers (Desjardins *et al.*, 1995). The objectives of our study (paper II) were to investigate whether there were other sesquiterpenoid PAs in the potato tuber and if they possibly had a stronger antimicrobial effect.

Two sesquiterpenoid metabolites had earlier been isolated and characterized from *Phoma*-infected tubers by Malmberg and Theander (1980). They are 2-(1', 2'-dihydroxy-1'-methylethyl)-6, 10-dimethyl-spiro-[4,5]dec-6-en-8-on (**1**) and its glucoside 2'-O-ß-D-glucopyranoside (**2**) (Fig 3). Our aim was to purify these compounds from *P. infestans*-infected tubers and compare their antimicrobial effect with other more well-known sesquiterpenoids such as rishitin and solavetivone, (Fig 3). We were also interested to see if there were differences between the aglycone (**1**) and its glucoside (**2**). Surprisingly, we could not find **1** and **2** in *P. infestans*-infected tubers. This was an unexpected observation of selective occurrence of postinfectional metabolites. Therefore we extracted them from naturally infected *Phoma foveata* and *Fusarium* spp.–infected tubers instead and the components were tested in a bioassay. The other substances tested were rishitin and solavetivone from potato tubers and also three commercially available sesquiterpenoid compounds, abscisic acid, cedrol and farnesol (Fig 3), which are known to occur in healthy plant tissue but not associated with post–infectional response.

Bioassays of sesquiterpenoid PAs in potato have been done on germination of zoospores (Ishiaka *et al.*, 1969; Harris & Dennis, 1976), germ tube growth (Ishiaka *et al.* 1969; Sato *et al.*, 1985), and mycelial growth (Beczner & Ersek, 1976; Hohl *et al.*, 1980; Stössel & Hohl, 1981). Since we assumed that the synthesis of PAs does not start before penetration we did not use any preinfectional event such as zoospore germination or germ tube growth. If resistance is expressed after penetration of the plant, it is obvious that only assays using hyphae would be appropriate to use in the bioassays.
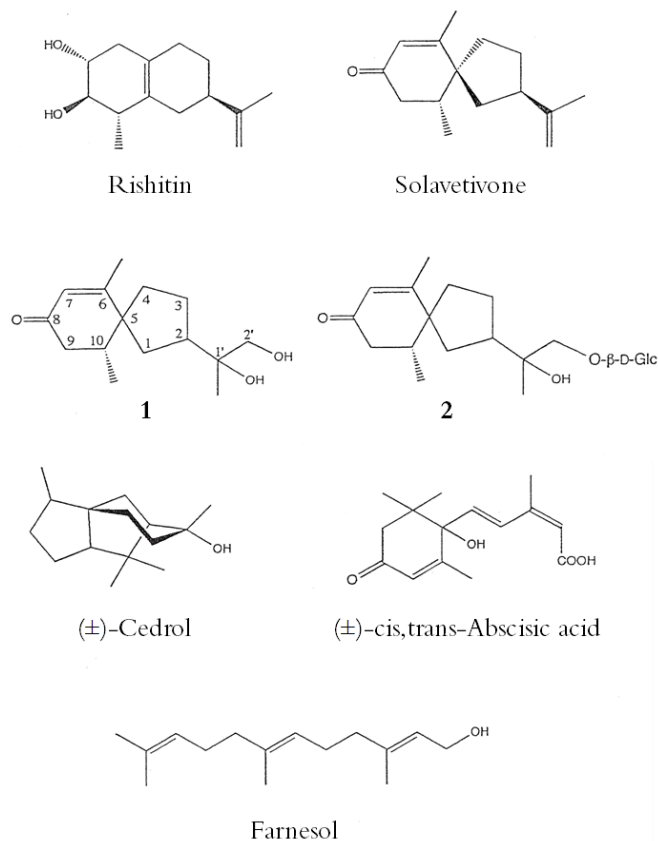
Figure 3. Structures of the sesquiterpenoids in the bioassay.

In our experiments (paper II) all sesquiterpenoids tested suppressed the growth of the pathogen except the glucoside (**2**) and its aglycone (**1**) which induced a weak growth stimulation. At a concentration of 50 or 100 μg/ml agar, rishitin and cedrol showed the strongest inhibitory effect. Solavetivone, abscisic acid and farnesol were somewhat less inhibitory. The inhibition of mycelium growth rate of *P. infestans* by these compounds has been measured earlier (Beczner & Ersek, 1976; Hohl *et al.*, 1980; Stössel & Hohl, 1981). In comparison with these reports we observed lower inhibition. However, it is difficult to compare the results from different bioassays. Various factors can influence the toxicity of the compounds such as substrate composition, pH, temperature, isolates of the pathogen, the timing of the growth measurements (Harris & Dennis, 1976; Smith, 1982; Stössel & Hohl, 1981), and the purity of the compounds tested. It cannot be excluded that if traces of contaminants do occur they may have an effect on the obtained results.

Lipophilicity has been suggested to determine the level of the antimicrobial effect. Kodama *et al.* (1985) found that some sesquiterpenoid PAs from tobacco had antimicrobial properties while their much more hydrophilic glycosides were inactive. Laks & Pruner (1989) found a close correlation between the lipophilicity of flavonoid PA analogues and their inhibitory effect on the pathogens *Aphanomyces euteiches* and *Fusarium solani*. Compound **1** is more hydrophilic than solavetivone and much more so as a glucoside. However, this does not explain the stimulatory effect of **1** and **2**. One suggestion is that *P. infestans* can use these compounds as nutrients. If so, this might explain why **1** and **2** are not found in *P. infestans* infected tubers.

Skipp & Bailey (1977) tested the antimicrobial effect of isoflavonoid PAs purified from leguminous plants on several pathogens. They did not find any evidence that differential sensitivity to PAs was related to pathogenicity. However, direct toxicity may not be the mechanisms by which sesquiterpenoid PAs work. Hohl *et al.* (1980) found that sesquiterpenoid PAs from tuber tissue inhibited glucanase activity, important for the late blight pathogen's growth in the tuber tissue. Clearly, in vitro tests can never reflect what is happening in living cells but only be used for comparative studies of antimicrobial activity between different compounds.

Usually, sesquiterpenoid PAs accumulate more rapidly and to a higher level in incompatible interactions than in compatible ones (Rohwer *et al.*, 1987). However, sesquiterpenoid metabolites have also been found in healthy tubers (Kuć *et al.*, 1976; Schöber, 1978). Schöber (1980) did not find any correlation between the level of general resistance and the amount of accumulating sesquiterpenoid metabolites. Bostock *et al.* (1983) found that tubers infected directly after harvest did not accumulate sesquiterpenoid metabolites although they showed resistance reactions similar to those observed in stored tubers. These reports clearly show that sesquiterpenoid metabolites cannot be the only determinants of resistance to the late blight pathogen in potato tubers.

For other pathogens e.g. the necrotrophic fungi *Gibberella pulicaris* (anamorph *Fusarium sambucinum*), genetic analysis has shown that virulence is correlated with the ability to detoxify the sesquiterpenes rishitin and lubimin, although by itself this is not sufficient for virulence (Desjardin *et al.*, 1992). However, the oomycete, *P. infestans* has not been shown to detoxify sesquiterpenoid metabolites (Kuć, 1982; Desjardin *et al.*, 1992).

In tobacco and tomato sesquiterpenoid metabolites have been found in infected leaves. However, Rohwer *et al.* (1987) and also Ertz & Friend (1993) reported lack of these metabolites in potato foliage. In a recent study,

the authors detected rishitin in potato leaves from cv. Kennebec after infection (Wang *et al.*, 2008). One explanation for these conflicting results could be that cv. Kennebec has some unusual terpenoid metabolism. However, the authors used thin-layer-chromatography (TLC) for estimating the amount of sesquiterpenoid metabolites. Based on our own experience this rather crude technique cannot clearly discriminate between fatty acids and sesquiterpenoids.

The conclusions from our study (paper II) are that the types of sesquiterpenoid PAs that accumulate in tubers are affected by the pathogen involved. Whether this is due to different capabilities to metabolize the compounds or to different influences on the host metabolism is not known. Many sesquiterpenoids inhibit *P. infestans* growth and those synthesized in potato do not have a greater effect than sesquiterpenoid substances from other sources tested. It does not seem reasonable that sesquiterpenoid metabolites in tubers are the main resistance factors in the *P. infestans*-potato tuber system. It is more probable that the resistance is a concerted action of a multitude of resistance factors with varying contributions in different cultivar-isolate combinations.

The interest in phytoalexins and especially in sesquiterpenoid PAs in the potato plant has declined. To cite Kuć (1995): "this does not mean that they no longer are important. It merely means that new defence-associated compounds such as chitinases, PR proteins, active oxygen species, jasmonates etc have been discovered and are in style".

6.4   The role of other secondary metabolites in resistance

Other biochemical factors than sesquiterpenes that have been implicated in resistance to *P. infestans* include phenols, glycoalkaloids, and the polymers suberin and lignin, which are involved in wound healing. For example, Schöber (1971) found that the amount of the phenolic compound chlorogenic acid accumulating after cutting potato tuber tissue was directly related to field resistance of potato cvs. to *P. infestans*. Ampomah & Friend (1988) found a correlation between the level of general resistance to late blight and the amount of lignin deposited in tuber discs. Evers *et al.* (2003) reported that the lignin content increased upon infection in the leaves of a resistant potato cultivar but not in a susceptible hybrid. However, it must be emphasized that if lignin and chlorogenic acid accumulate more rapidly and to a higher level in resistant than in susceptible potato cultivars in response to stress, it does not necessarily mean that these compounds are involved in disease resistance. It just shows that the host response is stronger. Genetic

analysis with mutants or treatments with inhibitors are two different ways to confirm whether a stress response mechanism is involved in resistance or not. Under short day conditions the potato plant is more susceptible to late blight infection (Umaerus, 1960; Ullrich & Krug, 1965). One very light-dependent enzyme is phenylalanine ammonia-lyase, a key-enzyme in the phenylpropanoid pathway. Treatment of potato leaves with PAL-inhibitor prior to infection resulted in a complete collapse of late blight resistance (Parker *et al.*, 1991). Yao *et al.* (1995), working with transgenic tubers with reduced phenylpropanoid synthesis, found that those were more susceptible to late blight than non-transformed ones. They also observed a direct link between the availability of phenolics and the strength of the cell walls. All these results indicate that phenolic compounds may play a critical role in the defence response to *P. infestans*.

Except for the sesquiterpenoids at least two other isoprenoids, the steroid glycoalkaloids (SGA) α-solanine and α-chaconine, known to be toxic to microorganisms (Sinden *et al.*, 1973), may be associated with resistance. However, Shih *et al.* (1973) reported that the accumulation of α-solanine and α-chaconine at the surface of cut tuber slices is markedly suppressed by inoculation with *P. infestans* and that sesquiterpenoid metabolites increased instead. At wounding SGA synthesis increases, but a metabolic switch from SGA to sesquiterpene biosynthesis has been shown to occur at infection with an incompatible race of *P. infestans* (Zook & Kuć, 1991). Both sesquiterpenoid PAs and SGAs are controlled by the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase. It has been shown that closely related genes coding for different isozymes of this reductase are expressed by wound induction or with inoculation with *P. infestans*, respectively (Choi *et al.*, 1992). This beautifully demonstrates how the plant is programmed to segregate processes as wound repair from those elicited from pathogens and often directed to HR.

As Friend (1991) states: "it must be emphasized that many investigations of the biochemistry of the late blight-potato interaction have used cut tuber slices or discs and that these are wounded tissue". Also, most studies have been made on potato cultivars with R-genes inoculated with compatible and incompatible races of the pathogen. Moreover, it must be stressed that although *P. infestans* is considered to be mainly a leaf pathogen, and that the leaves are the relevant organs for successful late blight resistance (Thurston & Schulz, 1981), most investigations have been made on tubers.

7 The new population of *P. infestans*

7.1 World-wide migrations of *P. infestans*

The late blight disease was not as problematic during the 1970s and 1980s as it is today and there was a hope to control the pathogen with resistant varieties and new types of fungicides (Struik *et al.*, 1997). Then something happened. In 1984, Hohl and Iselin reported the appearance of A2 mating types in Switzerland. This set off mating type surveys all over the world and soon it was reported that the A2 mating type had spread not only to Europe but worldwide (e.g. Malcomson, 1985; Tantius *et al.*, 1986; Kadir & Umaerus, 1987; Mosa *et al.*, 1989). This worldwide emergence of the A2 mating type has been explained by migration. In the mid–1970s a shipment of tubers was imported into continental Europe and a decade later tomato fruits and transplants were imported to USA. Each of these shipments, containing blight–affected plant material, was imported from Mexico (Niederhauser, 1991; Fry *et al.*, 1993). Convincing support for the migration hypothesis has been supplied by genetic studies demonstrating that the first detection of A2 outside Mexico coincides with the appearance of new alleles at different loci in the *P. infestans* genome (Spielman *et al.*, 1991, Drenth *et al.*, 1994).

Besides the presence of the A2 mating type, there were also other indications that the European late blight population had changed. Day and Shattock (1987) noted increased aggressiveness in isolates collected after 1982 in England and Wales, compared to isolates collected earlier. Several studies revealed that the *P. infestans* populations in Europe have become more phenotypically and genotypically diverse (e.g. Drenth *et al.*, 1994; Flier & Turkensteen, 1999; Sujkowski *et al.*, 1994). The replacement of the

old population has been explained by an increased fitness in the new population compared to the old one.

Before 1970s the European populations of *P. infestans* appear to have been dominated by a single A1 clonal lineage, US-1. This lineage has the mitochondrial (mt) haplotype Ib, a specific allozyme genotype, (Gpi 86/100, pep 92/100) and a characteristic fingerprint pattern based on RFLP with probe RG57. US-1 isolates have been found in at least 19 countries on all continents, except Australia and Antarctica prior to 1970s (Goodwin *et al.*, 1994), supporting the clonal theory. In a survey in the 1950s, Smooth *et al.* (1958) included isolates from the United States, Canada, Western Europe, South Africa, and the West Indies, and discovered that all isolates were of the A1 mating type, except for some isolates from Mexico that were of the A2 mating type. The presence of only one mating type in the sampled material indicates that *P. infestans* only could reproduce clonally at that time. However, we do not know much about the presence of the A2 mating type outside Mexico before 1950s and how many migrations there have been. Shaw (1991) speculated that A2 could have been present all the time but undetected, because it was present only at a very low frequency. Also Ko (1994) argues, based on his own experiments that A1 self-fertile strains can produce A2 progeny, that the A2 mating type have been present all the time. Ristaino *et al.* (2001) investigated old herbarium specimens from the 19th century from Europe and United States and could not detect haplotype Ib in the specimens examined, indicating that at least one more migration has occurred after the historical one in the 1840s. If the historical late blight was of both A1 and A2 mating type is not known at present. Probably the scientific debate will continue about the origin and migrations of *P. infestans*.

7.2   The fate of Matilda

In our investigation (Berggren *et al.*, 1988), cv. Matilda was shown to be very resistant to a complex race of *P. infestans*. There were very few successful infections and no sporulation. Today, in Sweden, cv. Matilda can be heavily infected even though it is still more resistant than for example cv. Bintje. Erjefält (1999) made a study 1996-98 to investigate whether the resistance level in some cultivars (Bintje, Ofelia, Apell, Matilda) still were at the same level as in trials 1992-95. He showed that the resistance level in cv. Apell was the same as in the earlier ratings but had decreased in the others. This indicates that the population of *P. infestans* has changed during these years in Sweden.

7.3   Consequences of both mating types present

There is strong circumstantial evidence that *P. infestans* is frequently reproducing sexually in the Netherlands (Drenth *et al.*, 1994), Poland (Sujkowskij *et al.*, 1994; Śliwka *et al.*, 2006), Estonia (Runno-Paurson *et al.*, 2009) and in the Nordic countries (Andersson *et al.* 1998; Brurberg *et al.*, 1999; Lehtinen *et al.*, 2004; Flier *et al.*, 2007; Widmark *et al.*, 2007). It is also evident from a survey carried out in some European countries that the *P. infestans* populations are mainly clonal in France, UK and Switzerland (Flier *et al.*, 2007). Probably *P. infestans* reproduces sexually in other parts of Europe and more thorough surveys of genotypic variation and presence of oospores are necessary to get a clear picture of the European *P. infestans* populations.

The sexually formed oospores are hardy thick walled resting spores that can survive harsh condition. These spores give the pathogen the ability to survive outside its host plant e.g. in the soil between cropping seasons. Also, sexual recombination increases the adaptive capacity (Barton & Charlesworth, 1998; Wills, 2003). In addition, primary infections caused by overwintering oospores in the soil are hard to detect and control by fungicide application since the infection starts in the lower part of the canopy. A very small proportion of infected tubers will give rise to infected shoots (Hirst & Stedman, 1960; van der Zaag, 1959. This means that with an additional inoculum source present, such as oospores in the soil, the inoculum dosage will increase considerably. However, presence of oospores *per se* does not necessarily mean that both mating types of *P. infestans* are present. Self-fertility and oospore-production induced by other *Phytophthora* species, other fungi (Shaw, 1991) and fungicide treatments (Groves & Ristaino, 2000) have been reported.

8 The Nordic Blight

8.1 Nordic population studies of *P. infestans* indicate sexual reproduction

In the Nordic countries, during the last decade, there are observations of earlier infections of late blight and the impression that more fungicides are needed to control the disease than earlier. A sexually reproducing population of *P. infestans* with overwintering oospores has been suggested to be one of the underlying causes for these phenomena. In 1996, a potato field trial was established in a field in mid-Sweden, partly overlapping a field trial from 1994 where different fungicide dosages were applied. Two weeks after emergence heavy infections of late blight were observed and the infection foci corresponded almost perfectly with the infections in the 1994 trial (Andersson *et al.*, 1998). This is a solid indication of soil borne inoculum. This was a contributing factor behind intense late blight research activities in the Nordic countries aimed at analysing the population structure and the occurrence of sexual reproduction. In summary, these reports demonstrate that the Nordic population of late blight can be characterized by the coexistence of both mating type in the same field, oospores often found in blighted leaves and high genotypic and phenotypic variation (Brurberg *et al.*, 1999; Hermansen *et al.*, 2000; Dahlberg *et al.*, 2002; Lehtinen & Hannukkala, 2004). The results indicate that sexual reproduction of *P. infestans* does occur frequently in the Nordic countries and that oospores serve as primary inoculum although direct evidence is not provided.

8.2 Monitoring *P. infestans* population structure

With the emergence of the new population of late blight, monitoring the population structure of *P. infestans* has been on the agenda. Marker systems have changed when new techniques have become available. In chronological order, the most used techniques for late blight population studies have been: Isozymes, RFLP (restriction fragment length polymorphism), mitochondrial haplotype, AFLP (amplified fragment length polymorphism), SSRs (simple sequence repeats, microsatellites). Since there have been different marker system used over time and in different laboratories there are difficulties to compare data obtained concerning *P. infestans* populations. Isozymes and mitochondrial haplotype have very low resolution. RFLP has higher resolution than isozymes and mitochondrial haplotype but it is time consuming and requires large amounts of DNA. AFLP has a comparatively high resolution but is not always suitable for diploid organisms since it cannot distinguish between homozygotes and heterozygotes. However, SSRs are co-dominant markers (both alleles at a locus revealed), and are therefore very useful to trace the origin of isolates or the sources of infection and identify clonal lineages. Moreover, SSRs are highly polymorphic, well-defined, and easy to score (Cooke & Lees, 2004). However, SSRs will not reveal the race structure in a late blight population. In the future, when presumably new techniques with high throughput techniques combined with lowered costs are available, sequencing the total genome of different isolates of *P. infestans* may become routine procedure in population studies of this organism.

8.3 *Phytophthora infestans* in a single field in southwest Sweden early in spring: symptoms, spatial distribution and genotypic variation (paper III)

In southwest Sweden, some fields have been used for early potato production every year for 50 years or more. Previously, late blight was not considered a problem since the potato was harvested before the blight appeared. However, from the early 1990s fields with late blight were found in this region. At that time, these were the first late blight infections observed during the Swedish growing season. For example, late blight could be observed as early as in the beginning of May in those fields. After planting, the early potato crop usually is covered with fleece and the symptoms were observed as soon as the fleece was removed. In some fields disease foci could be found in the same spot from year to year, indicating soil borne inoculum as source of infection.

A new approach of determining the source of primary inoculum was taken by a genotype study of the population of *P. infestans* in a single potato field southwest Sweden (paper III). The assumptions were: a) that tubers as the source of primary infections result in infection foci individually caused by a single or very few genotypes, since it is unlikely that a tuber carries several genotypes or that a number of infected tubers are planted together; b) infection foci caused by oospores consist of many genotypes, each coming from infections from different oospores. Mating type, mitochondrial haplotype and microsatellites were used as markers, with the aim of determining the infection source. Sampling was performed very early in the growing season before any symptoms were observed in other fields in the vicinity. In the investigated field, infected plants were found in six discrete foci. Symptoms were almost exclusively observed in the lower part of the canopy with numerous infections on stems and on leaves touching the ground. Both mating types were present and haplotypes Ia and IIa were detected. Among 61 isolates analysed with microsatellite markers, 14 multilocus genotypes were distinguished based on six polymorphic loci. Out of six foci, three foci included 3–6 unique genotypes each (Fig 4).
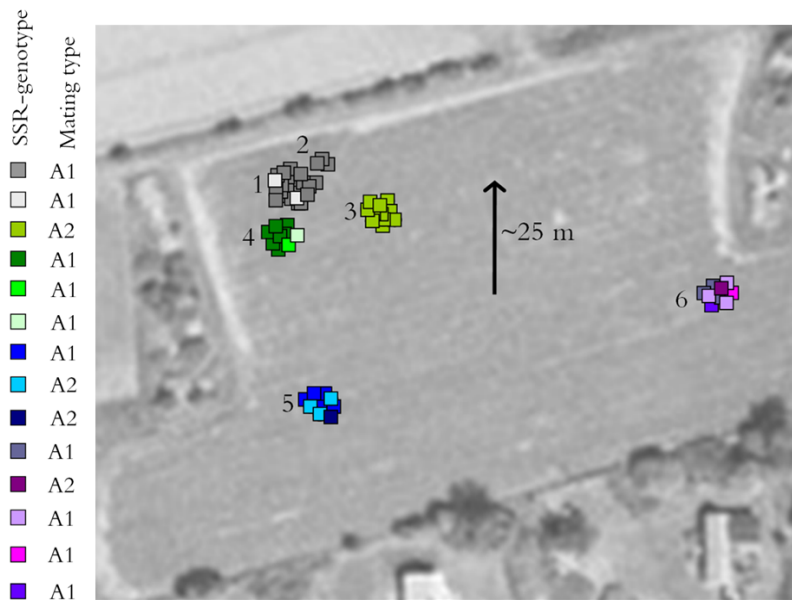

Figure 4. Aerial photograph of the sampled field in southwest Sweden in 2001 with SSR–genotypes of *P. infestans* in the different foci by colours.

The presence of several genotypes and within one single focus is difficult to explain as something else than oospore derived infections. These findings suggest that soil borne inoculum contributed significantly to initiate late blight epidemics in the investigated field.

Today, there are few reports from the potato district in Southwest Sweden about early infections in the potato crop. The potato growers in this area have become aware of the high risk of late blight early in the season and have taken precautions. The potato crop is grown under fleece cover for a shorter period and as soon as the cover is removed it is sprayed with fungicides.

8.3.1 Earlier infections with oospores?

A long term study in Finland showed that the onset of the late blight epidemic was 9 days earlier in fields were potato was grown after potato as compared to fields with alternate crops between the potato crops in data from 1998-2002, while no difference could be observed in data from 1992-1997 (Hannukkala *et al.*, 2007). In a Nordic study, Danish data showed that 1-2 years interval between the potato crops gave earlier attacks of late blight compared to 3 or more years (Bødker *et al.*, 2006). This indicates that oospores give rise to earlier infections than infected seed tubers. We do not know why. One explanation might be that the infection of the potato plant from oospores is a faster process than infection from blighted tubers. However, since very few infected tubers will give rise to an infected plant (van der Zaag, 1956; Hirst & Stedman, 1960), the total amount of inoculum in a field will increase considerably with an additional inoculum source such as oospores present in the soil. Early infections especially in the lower part of the canopy, are difficult to observe. An increased number of infections result in more observations of late blight that may be interpreted as "earlier".

8.3.2 Can we cope with the oospores in the soil?

Oospores are robust propagules but they will not survive forever in the soil. In a study performed in the Netherlands viable oospores were found in sandy soil up to 34 months in pot experiments during field conditions (Turkensteen *et al.*, 2000). From Central Mexico survival of oospores in the soil for two years has been documented, but vitality and infectivity decreased significantly (Fernandez-Pavia *et al.*, 2004). As mentioned above, Nordic studies showed earlier infection of late blight without crop rotation. This indicates that longer crop rotation periods can reduce the risk of soil borne infections of late blight.

Although oospores are long-lived in the soil, they can be parasitized by various microorganisms. Sneh and co-workers (2007) found that more than a dozen soil microorganisms are capable of infecting oospores of *Phytophthora*. Bio-control measures may be a possible approach to decrease the amount of oospore inoculum in the soil.

8.4 Tracking *Phytophthora infestans* with SSR markers within and between season – a field study in Sweden (paper IV)

Our results from a single field in southwest Sweden (paper III) indicate that a substantial portion of the late blight attacks in the investigated field was derived from oospores. However, to provide experimental evidence that clearly confirms that oospores in the soil act as a primary inoculum source during field conditions is difficult. Drenth *et al.* (1995) developed a soil bioassay to test survival of oospores in the soil. They inoculated small field plots with two isolates of different mating type, sampled soil the next season from the plots and the single-lesion isolates obtained using the bioassay were subjected to DNA fingerprint analyses. By using RFLP with probe RG-57 as marker they identified recombinants of the parental generation and concluded that oospores can survive winter in the Netherlands.

Figure 5. Soil bioassay for detecting soil borne inoculum (oospores) of *Phytophthora infestans*

Our intention was to look at the oospore survival during Swedish winter conditions, using the same soil bioassay technique but instead of RFLP

using SSRs as markers since the resolution is much higher and parentage analysis therefore can be more accurate. We were also interested in investigating if there were some correlation between the frequency of the genotype detected in the field at the end of the season and the reproductive fitness of the inoculum isolates.

The dynamics of an epidemic generated by inoculation with six different A1 and A2 isolates of *P. infestans* in an experimental potato field in mid Sweden was investigated (paper IV). Three weeks after inoculation single-lesion leaflets were sampled and isolates characterized using microsatellites (SSRs) and mating type as markers. Among the 151 isolates analysed, the inoculum genotypes constituted more than 80% of the population. Three other genotypes were also detected forming the remaining 20%, probably from infected seed or nearby fields. The following year, *P. infestans* isolates were baited from soil samples taken from this field and six novel genotypes were identified. Genotypes from the previous summer population were not detected (Fig 6).

Parentage analysis of the genotypes recovered from the soil was consistent with them being recombinants from the previous summer's population. These findings demonstrate that oospores produced during a summer epidemic in Sweden can overwinter and cause infection the next year.

Despite an abundant oospore formation observed the previous summer in the experimental field, only 5 of twelve soil samples yielded infected leaflets in the bioassay. Lehtinen and Hannukkala (2004) obtained infection from only three of sixteen soil samples from fields with early infections presumably caused by oospores. They only obtained infectious soils from two experimental fields where potatoes had been grown in monoculture without fungicide application for the last decade. Based on these results, the sensitivity of the bioassay for quantifying the inoculum in the soil may be questioned. However, the amount of oospores in the soil can not be used as a single measure of the inoculum potential of the soil, because only successful matings will result in the formation of oospores capable of germination and infection (Pittis & Shattock, 1994; Fay & Fry, 1997; Knapova *et al.*, 2002; van Bekkum *et al.*, 2007).

8.4.1 Can oospores germinate and infect during the whole growing season?

Van Bekkum *et al.* (2007) assumed that oospores in the soil germinate continuously and release sporangia during large parts of the growing season. They speculated that tubers in the end of the growing season might be infected from oospore infested soil. Germinated oospores in leaf tissue have

been observed by Götz (1991). However, to my knowledge, it is not known if oospores in the leaves on the potato plants can germinate and introduce new genotypes within a growing season.
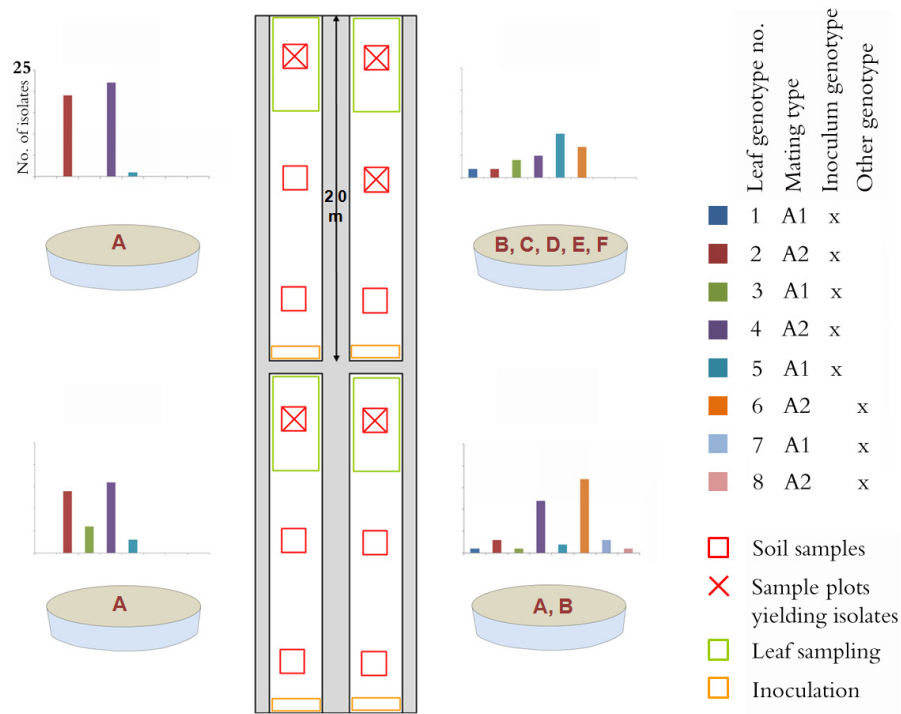
Figure 6. Schematic presentation of a field trial in Sweden 2001 inoculated with five genotypes of *P. infestans*. The genotypes isolated from leaves collected three weeks after inoculation are numbered 1–8. The number of these genotypes found in each experimental block are illustrated as bars in the four bar charts. The 6 genotypes captured from soil the next season are marked A–F. Parentage analysis indicates that soil genotype A is a recombinant of leaf genotype 4 X 5 and soil genotype B is a recombinant of leaf genotypes 1 X 4. Soil genotype C is a recombinant of leaf genotypes 4 X 7 or 5 X 8. Soil genotype D has a unique allele not present in either leaf or soil samples.

8.5 Why sex?

The late blight pathogen has been very successful all over the world without sex for more than hundred years. However, it is also clear that it has been more or less replaced by a new population that is more diverse and has the ability to reproduce sexually. Sexual recombination may have been hindered between the new and old populations since the old population usually was polyploid (Tooley & Therrien, 1991). So the question is, what is the advantage with sexual reproduction? It enables separation of harmful mutants from beneficial ones and the combination of beneficial mutations from separate ancestries. On the other hand, sexual reproduction breaks up well-adapted genotypes (Barton & Charlesworth, 1998). This results in a progeny that is on average less fit than a successful clonal lineage. In crossing experiments with *P. infestans* isolates, less fit progeny than the parental generation has earlier been reported (Mayton *et al.*, 2000). In our investigation (paper IV), the isolates captured from soil generally grew poorly, showed low sporulation and were difficult to mate, compared to isolates collected during a summer epidemic. Maybe an optimal situation for the late blight pathogen is when the climatic conditions are favourable for survival and spread of a particularly fit clonal lineage all over the years and only occasionally sexual reproduction does occur. When conditions change, (e.g. weather, cultivar, fungicides), it can be replaced by a rare genotype more adapted to the new environment (McDonald & Linde, 2002). For instance in UK, with rather mild winters compared to the Nordic countries, one clonal lineage "blue 13", a complex race with high aggressiveness, is predominant and is emerging in other parts of Europe (Lees *et al.*, 2009). It will be extremely interesting to follow the emergence and decline of "Blue 13" in Europe.

9 The genomic era - new tools

The genomes of *P. infestans*, *P. capsici* and *P. sojae* have recently been sequenced and are now available (Haas *et al.*, 2009). New insights about *Phytophthora* have been achieved very fast (Lamour *et al.*, 2007), and probably there will be a rapid increase of research articles concerning the gene structure and function of *P. infestans* within the near future. The availability of the genome sequence has also resulted in more scientists becoming interested in late blight research and an expansion of the late blight research community. For example, in Sweden, the number of researchers in this area has increased considerable the last five years.

The genome of *P. infestans* is characterized by an abundance of transposon elements and hypervariable regions indicating a fast evolving organism (Kamoun, 2003). This explains on a genetic level the high phenotypic variation observed in this pathogen even within clonal lineages both in the old and new population of *P. infestans* (Caten & Jinks, 1968; Goodwin *et al.*, 1995; Abu-El Samen *et al.*, 2003; Guo *et al.*, 2009).

9.1 Effector genes

Effector proteins, by definition, are able to modulate the plant's defence and can be secreted either in the extracellular space of the plant or translocated directly into the cell cytoplasm (Kamoun *et al.*, 2006). In oomycetes, most attention has been on the so-called RXLR effectors. All oomycete Avr genes identified to date have an RXLR motif (van Poppel *et al.*, 2008). (RXLR=arginine, any amino-acid, leucine, arginine). However, the effector secretome, (proteins exported from the cell), of oomycetes has been shown to be unexpectedly complex. There are hundreds of potential RXLR effector genes in the genomes of the oomycetes so far studied (Jiang *et al.*, 2008). Recent genomic research has shown that the RXLR motif in

P. infestans resembles a motif in the malaria parasite *Plasmodium falciparum*. In *P. falciparum*, this motif functions in delivering proteins into the red blood cells and the RXLR motif in *P. infestans* has been shown to have a similar function, i. e. mediating protein transport into the plant cytoplasm (Whisson *et al.*, 2007). The *Phytophthora* secretome is at present a subject of intensive research and the near future will be a very exiting time for all working in the late blight research field when the secrets of the secretome are revealed. Blocking the effector delivery system of the late blight pathogen suggested by Panstruga & Dodds (2009) may be an efficient strategy to control the disease.

Today, in all parts of the world we have mostly complex races of the pathogen that have lost their avirulence function against most of the known R-genes. In the Nordic countries all pathotypes of *P. infestans* are complex and it is difficult to find isolates unable to grow on potatoes possessing R3 and R4 resistance genes (Lehtinen *et al.*, 2008). The selection pressure to retain the avirulence functions is obviously low so the question still remains: Why have they ever been there? The Avr3 effector can suppress the HR induced by another *P. infestans* protein (INF1), indicating a virulence function (Bos *et al.*, 2006). A fitness reduction would be expected if important virulence functions are lacking. However, the putative virulence function of Avr 3 does not seem to be important for the present Nordic late blight population.

9.2 The host range is limited despite the adaptibility of the pathogen

Most research has been focused on the resistant interaction and not on the susceptible one. For a plant pathogen with a very high adaptive capacity such as *P. infestans*, single gene resistance in the host plant is easy to overcome. However, despite *P. infestans* variability it still prefers a few members of the Solanacea as hosts. Usually we culture *P. infestans* on a medium based on boiled peas for propagation and on a mixture of pea and rye for sporulation. We have tried a substrate based on boiled potato leaves and from our observations it is quite clear that pea and rye broth are better nutrient sources than extract of potato leaves. These observations indicate that *P. infestans* grows on the potato plant because it can infect it, not because it is an optimal nutrient source. Why can *P. infestans* usually not infect living plants from other plant families despite its adaptibility? With the sequence of the pathogen now available we have an opportunity to unravel the molecular determinants for host specificity.

10 Concluding remarks

As long as man cultivates potato he also cultivates its companion *P. infestans*. Much hope has been put on resistance breeding in order to cope with this pathogen. In the 1980s a new more aggressive population of the late blight pathogen emerged. Resistance in some old potato cultivars has now been rendered unusable. The fungicides applied do not affect the new population of *P. infestans* as well as the old one. The soil is infested with oospores waiting for an opportunity to infect new potato plants. With the presence of both mating types and subsequent sexual reproduction we can expect enhanced capability of the late blight pathogen to adapt to different factors such as weather, fungicides and cultivar resistance.

Late blight research has focused on varying aspects under different periods. New techniques usually decide the research area and with every new research trend new hope arises that "this time the late blight problem will be solved". Nevertheless, the pathogen continues to defeat us. Even though there is an immense amount of articles published during 150 years of late blight research, many questions concerning the biology of *P. infestans* are still unanswered. The secret life of *P. infestans* in soil must be studied further. "How and under which conditions do the oospores infect the potato plant" and "how does the infection from infected tubers usually spread to above-ground parts of the plants", are urgent questions to be answered. Since the knowledge of the mechanism of disease resistance would allow the breeder to select for certain traits at an early stage in a breeding programme, the search for resistance factors must continue. Scientific knowledge is now emerging very fast in the late blight research field and one hopes that exploiting this knowledge will bring new potato varieties with durable resistance against late blight within reach.

The population of *P. infestans* has changed and will continue to change. We have experienced that this pathogen has a very high adaptive capacity.

The expected warmer winters in the future in the Nordic countries will probably have a great influence on the late blight population structure, since survival of clones between growing seasons will be easier. The climatic change will also affect the entire agriculture system, what we cultivate and where. Increased temperature and more rain during the growing season will be favourable for the late blight disease. With more late blight in the fields, the conflict between the need for more fungicides to save the crop and the public's demand for less use of chemicals will probably increase. *P. infestans* is obviously a very adaptive pathogen and therefore it is important to monitor the genotypic and phenotypic changes in the late blight populations and above all, to learn about the evolutionary forces that drive these changes.

The adaptibility of *P. infestans* is impressive. If we wish to grow potato in the future we must use the scientific knowledge available in order to control the disease and continue our research to developing effective, environmental friendly late blight specific fungicides, cultivars with durable resistance and reliable forecasting systems. What to some extent is lacking in the late blight research field is synthesis and overview. Despite that intensive research on late blight has been carried out for more than 150 years by thousands of scientists this organism still defeats us. If all research on late blight were aimed at combating the disease and to obtain healthy potato crops maybe we would be able to subdue *P. infestans*.

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