How Sexual Reproduction Affects the Population Biology of *Phytophthora infestans*

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Abstract

Phytophthora infestans is a rapidly evolving and highly adaptable pathogen. It is the cause of late blight, one of the most devastating diseases in potato production. Depending on whether both mating types are present or not, *P. infestans* can undergo both asexual and sexual reproduction. In most parts of the world the asexual part of the life cycle is the dominant reproduction system resulting in dominant clonal lineages. However, earlier reports indicate that sexual recombination of the late blight pathogen occurs in the Nordic countries. This thesis includes studies on how this will affect the population biology of *P. infestans*. The results show that the genotypic variation of *P. infestans* in the Nordic countries is high. The highest variation was observed within fields, and no dominating clonal lineages were found. In a field trial planted with artificially inoculated seed, the genotypes originating from the infected tubers had a minor impact on the population biology of *P. infestans* during the season. Immigrating genotypes, which probably originated from potato crops infected by oospores, proved to be more important for the epidemiology of the disease. The presence of the alternative host (hairy nightshade) was shown to result in an increased oospore production and a higher aggressiveness of late blight on potato. From the results it can be concluded that oospores play a major role in the population biology of the late blight pathogen in the Nordic countries. Furthermore, in a study of the variation in effector genes of *P. infestans,* indication of selection pressure towards losing intact Avr4 genes was found. In all studied isolates this frame shift mutation was observed which means that all isolates would be able to infect plants with the R4 resistance gene.

The population biology of the late blight pathogen in the Nordic countries is complex and differs from that in many other parts of the world. The difficulties to control this disease are numerous and the nature of the Nordic population of *P. infestans* threatens to further add to this problem.

Keywords: late blight, hairy nightshade, effectors, SSR, oospores

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Dedication

Till Niclas

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 L. Sjöholm, B. Andersson, N. Högberg, A-K. Widmark & J. Yuen (2012). Genotypic diversity and migration patterns of *Phytophthora infestans*. (submitted)

II L. Sjöholm, J. Yuen & B. Andersson. Immigration and persistence of *Phytophthora infestans* in a single field. (manuscript)

III L. Grönberg, B. Andersson & J. Yuen (2012). Can weed hosts increase the aggressiveness of *Phytophthora infestans* on potato? *Phytopathology* 102(4), 429-433.

IV L. Sjöholm, U. Blandón-Díaz, N. Högberg. Effector variation in a clonal vs. sexual population of *Phytophthora infestans*. (manuscript)

The contribution of Lina Sjöholm to the papers included in this thesis was as follows:

I Planned the study together with co-authors. Collected samples and carried out the laboratory work. Analysed the data and wrote the manuscript with the help from co-authors

II Planned the study together with co-authors. Collected samples and carried out the laboratory work. Analysed the data. Wrote the manuscript assisted by co-authors.

III Planned the study, collected samples, carried out the laboratory work and analysed data in cooperation with co-authors. Wrote a major part of the manuscript assisted by co-authors.

IV Planned the study together with co-authors. Carried out the laboratory work. Analysed the data with the help of co-authors. Wrote a major part of the manuscript assisted by co-authors.

1 Introduction

Agriculture today feeds 6,000 million people. Plant production and yields have increased tremendously during the last 40 years due to irrigation, progress in plant breeding, and inputs such as fertilizers and pesticides. Potato production has increased in many developing countries during the last years but decreased during the same period in the industrialised world. In total, however, it is becoming more important as a staple crop. Globally, potato is the third main food crop after wheat and rice (Nations). In Europe potato is the second most important arable crop. It is cultivated in scales ranging from just a few square meters in back-yard gardens to large scale production on hundreds of hectares. However, even after decades of improvements in potato production in the form of resistance breeding efforts and fungicide development, there are still major constraints to overcome. For example, late blight on potato remains to be one of the most devastating plant diseases. The annual cost of this disease in control efforts and yield loss is estimated to M € 4800 globally (Haverkort *et al.*, 2008).

It is a challenge to study this organism since it is rapidly evolving, has a complex genome and varying population structures in different parts of the world. This thesis contributes to the understanding of the population biology of the late blight pathogen in a sexually reproducing system. It will cover areas such as how the population structure in the Nordic countries differs from other areas, how an alternative host affects the aggressiveness of *P. infestans*, population development during the season and variation in pathogenicity genes (effectors).

2 History and background to the late blight pathogen

"In the wake of the famine agronomists and plant pathologists faced the task of coming to understand the disease and devising ways to combat it. That effort goes on today. It has not, in the main, been a heroic tale of scientific triumph; on the contrary, late blight has allegedly broken the hearts of more agricultural scientists than any other single crop disease" (Turner, 2005).

2.1 History of the pathogen

Late blight caused by *Phytophthora infestans* has caused one of history's most well-known plant disease epidemics, leading to the Irish potato famine in the 1840's and mass emigration and death of millions of people in Ireland. The first occurrence of the disease was reported from the east coast of the U.S around 1843. In 1845, the first outbreaks in Europe were discovered in Belgium. Later the same year it spread to Holland, Germany, England and Ireland (Bourke, 1991). The Irish population was dependent on potato as a main food source and therefore the blight in Ireland caused a famine resulting in a demographic disaster with a population decline of twenty-one percent (Large, 1946, Turner, 2005), one from which Ireland still has not recovered.

There were widespread speculations if the weather was the cause behind the blight epidemic. A Belgian mycologist named Marie-Anne Libert was the first to describe the cause of the blight as a fungus and proposed the name *Botrytis vastatrix* (Zadoks, 2008). About the same time another scientist, Jean Francis Camille Montagne, described the fungus and it was agreed to name it *Botrytis infestans*. At that time, however, it was a general belief that spores, germs and bacteria were not a cause but a consequence of diseases. 'The potato murrain' remained a mystery, and it was only when Anton de Bary described the life cycle of the potato blight pathogen in 1861 it was accepted and established that

a fungus was responsible for the disease. It was also de Bary who named the pathogen *Phytophthora infestans,* which means 'infectious plant destroyer' (Large, 1946).

Historically, there has been a longstanding controversy if potato late blight has its origin in Mexico or in the Andes (Andrivon, 1996). Due to the high genotypic and phenotypic variation and the presence of both mating types of *P. infestans*, central Mexico has been proposed as the centre of origin for the late blight pathogen. However, recently another theory has been suggested with the Andes as the centre of origin based on the mitochondrial and nuclear loci in *P. infestans* and its close relative *P. andina* (Gómez-Alpizar *et al.*, 2007).

2.1.1 Migrations

The first known migration of late blight took place from the U.S to Europe, which caused the Irish famine in the 1840's. Once the pathogen was introduced in Europe it was distributed by international seed trade to the rest of the world (Fry *et al.*, 1993). Between the 1840's and 1970's there is no clear evidence of any migrations of *P. infestans* taking place. The populations of *P. infestans* in the world (except Mexico) before 1970 consisted of only the A1 mating type and were dominated by a single clonal lineage, US-1 (Goodwin *et al.*, 1994).The second global migration of *P. infestans* can be divided in two parts and this is also the event that changed the population structure of this pathogen since this migration carried both mating types.

The first part of this second migration occurred in 1976 via a shipment of potato tubers from Mexico to Europe (Niederhauser, 1991). This shipment was meant to cover a shortage of potatoes in Europe. Import of potato tubers from Mexico was normally not allowed because of the fear of the A2 mating type (Fry *et al.*, 2008). However, this shipment probably carried both the A2 mating type and also other novel alleles. This relocation was not discovered in Europe until 1984 when the A2 mating type was found in Switzerland (Hohl & Iselin, 1984). The "new" population displaced the old one (Spielman *et al.*, 1991, Drenth *et al.*, 1994, Fry & Goodwin, 1997) and was spread to the rest of Europe and other parts of the world.

The second migration event of *P. infestans* brought new genotypes of *P. infestans* from Mexico to the U.S and Canada in the 1980's (Fry *et al.*, 1993). It has been confirmed that the genotypes found in the U.S correspond to genotypes found in northwest Mexico. Both mating types are present in the U.S and Canada but the population consists of only a few dominating genotypes (Fry & Goodwin, 1997).

2.2 The life cycle

The late blight pathogen *P. infestans* belongs to the group oomycetes and the kingdom of Straminopila. The cell walls of oomycetes mainly consist of cellulose in contrast to true fungi whose cell walls contain chitin. Taxonomically, *P. infestans* is more closely related to brown algae than to true fungi even if late blight often is classified as a fungal disease (Erwin & Ribeiro, 1996). The pathogen can attack all parts of the potato plant; foliage, stem and tubers, but not roots (Fehrmann & Dimond, 1967), as illustrated in figure 2. The life cycle of the pathogen (figure 1) can be divided into an asexual part, and if both mating types are present, a sexual part. The oomycetes are diploid for the major part of their life cycle.

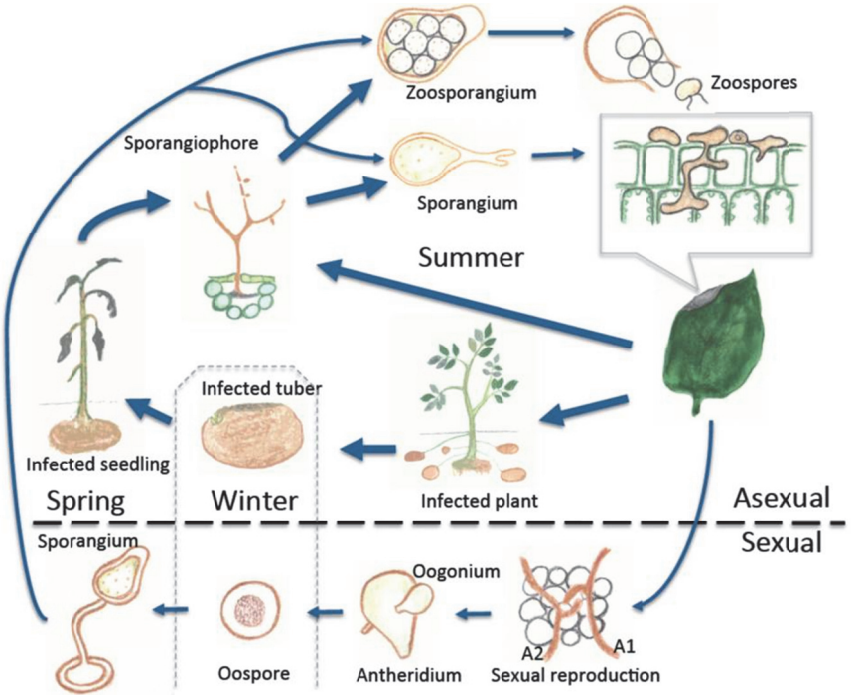

Figure 1. The life cycle of *Phytophthora infestans*. Illustration made by Alvar Grönberg.

2.2.1 The asexual part of the life cycle

If one mating type is present, only the asexual life cycle is possible. Shoots emerge with living mycelium of *P. infestans* from the infected tubers. From the mycelium sporangiophores emerge through stomata of stems and leaves, and produce sporangia. The sporangia are often released in the morning when the

temperature is rising and the air humidity is decreasing. The spores are dispersed by wind or rain to a nearby plant or a neighbouring canopy. The dispersal of airborne sporangia between fields and regions are limited by the effects of solar radiation and air humidity on the survival of these propagules. As a result, they can mostly only be dispersed over shorter distances (Mizubuti *et al.*, 2000). The sporangia can germinate directly or release three to eight zoospores. When in contact with leaf or stem the sporangia or zoospores form germ tubes and appressoria and penetrate the host. The mycelium grows intercellularly and haustoria are formed inside the cells. The cells in the potato plant die as the mycelium continues to colonise the host plant. New sporangiophores emerge through stomata and the sporangia produced can spread and give rise to new sporulating lesions after a few days. The zoospores have an optimum germination temperature of 10-15 ºC whereas the majority of sporangia germinate directly in temperatures around 20-25 ºC (Fry, 2008).

The asexual part of the *P. infestans* life cycle enables rapid dispersal and a short generation time through the huge amount of sporangia released (Fry, 2008). With a latency period as short as three days (Flier & Turkensteen, 1999), all of the above ground part of the plant can be destroyed within a week. If the infected crop is left untreated, there is a risk of sporangia or zoospores reaching and infecting the tubers. In this way, the entire harvest can be destroyed. Furthermore, the infected tubers can serve as an inoculum source the following season.

Figure 2. Typical symptoms of late blight on leaves, stem and tubers. Photo: L. Sjöholm

2.2.2 The sexual part of the life cycle

Phytophthora infestans has two mating types designated A1 and A2. If both mating types co-exist the pathogen can undergo sexual reproduction. When the two mating types come in contact (infect the same leaflet), fertilization can

take place between the oogonium (female organ) and the antheridium (male organ) resulting in the formation of thick-walled and robust oospores. The oospores are formed within the plant tissue and when the infected plant debris fall to the ground, the oospores become incorporated into the soil where they can survive for years (Pittis & Shattock, 1994, Drenth *et al.*, 1995). When the oospores germinate a germ tube is formed which produces a sporangium, which will either produce infectious zoospores or infect the host directly analogous to asexual infection. The factors that govern the germination of oospores in the soil are still mostly unknown. The effect of oospores on the epidemiology of late blight is also to a large extent unexplored (Andrivon, 1995, Widmark *et al.*, 2011).

2.3 The hosts

The host range of *P. infestans* is mainly restricted to the family *Solanaceae*, of which potato (*Solanum tuberosum*) and tomato (*S. lycopersicum*) are the most important agricultural crops (Erwin & Ribeiro, 1996). In many places, potato and tomato are grown all year around. In colder climates, like, in Sweden, where only one growing season per year is possible, all commercial tomato cultivation is conducted in green houses which reduces the risks of late blight infections. There are a few wild *Solanum* spp. that are reported as hosts for *P. infestans* in Sweden. The most widespread are *S. nigrum* (black nightshade, a common weed in potato crops), *S. dulcamara* (bittersweet) and *S. physalifolium* (hairy nightshade). However, attacks by *P. infestans* on *S. dulcamara* and *S. nigrum* are very rare and must be considered to have no or very limited effect on late blight epidemics (Cooke *et al.*, 2002, Flier *et al.*, 2003). In contrast, another Solanum species, *S. physalifolium* has been found to be highly susceptible to *P. infestans* (Grönberg *et al.*, 2012).

2.3.1 Potato

The cultivation of potato has a long history. Potato originates from South and Central America. The Europeans first discovered the potato in 1537 when the Spanish conquistadors invaded the villages in the Andes (Robertson, 1991). The potato was probably brought to Sweden by the natural scientist Olof Rudbeck around 1655. The first years it was only used as an ornamental plant in different botanical gardens. During the 18th century, Jonas Alströmer started to cultivate potato on his farm in Alingsås. Despite his many efforts to establish the potato as a staple crop, the Swedish farmers were not too impressed. However, from the second half of the 18th century the potato increased in popularity. This was aided by soldiers returning from the

European continent bringing the habit of eating potato. Another contributing factor to the increased acceptance was the discovery of the possibility of producing alcohol from potato (Osvald, 1965).

Potato is not a major crop in Sweden today. Including both table and starch potato the total area of cultivated potato in Sweden in 2011 was 27 100 ha. This can be compared to the total area of cultivated land in Sweden which is 2.6 million ha. The average yield per hectare in Sweden is 30 tonnes. France and Belgium have an average yield of around 43 tonnes per hectare whereas Poland and Romania have yields of approximately 16 tonnes per hectare. In an international perspective the Swedish potato cultivation is efficient. The farmers have good knowledge, the potato is produced on suitable soils and most commonly with access to an adequate supply of water. However, more information to the customers linked to continuous product development is needed to maintain potato consumption (Rölin *et al.*, 2012).

2.3.2 Hairy nightshade

Hairy nightshade (*Solanum physalifolium* Rusby var. *nitidibaccatum* (Bitter) Edmonds (1986)) (in this thesis, referred to as *S. physalifolium* or hairy nightshade) is a summer annual plant native to South America, but has been widely introduced elsewhere in the world. Problems with hairy nightshade as a weed occur mostly in row crops with low competitive ability such as potato, sugar beet, and carrot (Edmonds, 1986). In South America it is a weed which is present all year around. However, it is important to be aware of the taxonomical uncertainty within the Solanaceae family. It is common with naturally occurring hybrids between different species of Solanum. For example, some studies report *Solanum sarrachoides* as a host for *P. infestans* (Deahl *et al.*, 2005). As hairy nightshade is used as the trivial name for both *S. physalifolium* and *S. sarrachoides* the confusion is a fact. Edmonds (1986) also mentions that *S. physalifolium* is often misidentified as *S. sarrachoides*.

Figure 3. Hairy nightshade (*Solanum physalifolium)* to the left, with late blight symptoms. Black nightshade (*Solanum nigrum*) to the right. Both found in a potato field. Photo: L. Sjöholm

In Sweden, *S. physalifolium* is a weed problem in row cultivated crops. The cultivation of row crops is concentrated to the southern parts of Sweden. In this kind of cultivation irrigation is a common practice which benefits the hairy nightshade with its shallow root system. Late planting also favour nightshades that require a relatively high germination temperature. Black nightshade (*S. nigrum*) shares the same habitat as hairy nightshade, whereas bittersweet (*S. dulcamara*) grows in wetter conditions. However, no infections by *P. infestans* on black nightshade or bittersweet have been reported from Sweden. It has been reported that *P. infestans* can infect *S. physalifolium* and that oospore production takes place in the host. This has been seen both under laboratory and field conditions (Andersson *et al.*, 2003) and attacks are observed every year under field conditions in Sweden. As a result, this alternative host plant might promote the spread of *P. infestans* during the growing season as well as between seasons. So far, *S. physalifolium* has mainly been restricted to the southernmost parts of Sweden but, with the predicted increases in temperature due to climate change, it has a potential to be established as a weed problem further north (Eckersten *et al.*, 2008). Interestingly enough, there are no reports of *P. infestans* infections on *S. physalifolium* under field conditions outside Sweden, which may be due to the availability of more effective herbicides in these regions or difficulties in host plant identification.

2.4 Population structure of *Phytophthora infestans* in the world

The coexistence of both mating types allows *P. infestans* to reproduce sexually. This will increase the genotypic diversity and affect the population structure (Shaw, 1991). In central Mexico, which is considered as the centre of origin for late blight, both mating types coexist and the genotypic diversity of *P. infestans* is high (Grünwald & Flier, 2005). One might suspect that the late blight population in the U.S would also be highly diverse due to the migration pattern, from Mexico to the U.S, and presence of both mating types. However, the populations of *P. infestans* in the U.S appear to be asexual and highly clonal (Hu *et al.*, 2012). Also in Central America the populations of the late blight pathogen are clonal (Blandón-Díaz *et al.*, 2012). The number of clonal lineages and common genotypes are limited enough to make it possible to give them individual names, i.e, US-8 and NI-1.

During recent years there have been many attempts to characterise the population structure of *P. infestans* in the Asian countries. The population in Siberia belongs to a clonal lineage (Elansky *et al.*, 2001) and this lineage can also be found in China and Japan (Akino *et al.*, 2004). In a recent study from China, all isolates sampled belonged to the same unique SSR genotype (Guo *et al.*, 2009).

In northern Africa, both mating types of *P. infestans* have been reported (Baka, 1997, Shaat, 2002) and there is a potential for sexual reproduction in Morocco since the mating types have been found in the same field (Hammi *et al.*, 2001). This is in contrast to countries further south; Rwanda, Uganda, Kenya, Tanzania, Burundi and Ethiopia, which historically have had only the A1 mating type present, which was the clonal lineage US-1. More recent collections have shown substantial variation in Ethiopia (Daniel Shimelash, personal communication) and the presence of new genotypes on potato in Uganda and Kenya (Annie Njoroge, personal communication). In South Africa, the US-1 clonal lineage is predominant (McLeod *et al.*, 2001, Pule *et al.*, 2008).

An infamous clonal lineage is the so called “blue_13” or “13_A2”, which has been dominant in the U.K population of *P. infestans*. During the mid-1990s, A1 was the most common mating type. In 2005/2006 the population switched to a dominance of the A2 mating type, and the isolates sampled were mainly of the specific clonal lineage “blue_13”. This clonal lineage has been shown to persist between seasons, dominate the pathogen population throughout the epidemic, and has spread to several other European countries (Lees *et al.*, 2008, Montarry *et al.*, 2010). In a report from 2011 (Gisi *et al.*, 2011) it is stated that the population structure of *P. infestans* in Europe today is

largely clonal with a few dominating genotypes. It is further assumed that sexual reproduction is rare.

However, in the Nordic countries the late blight pathogen has a different population structure compared to in most other parts of the world. Several reports indicate that sexual reproduction and production of oospores occurs and that the oospores can survive for several years in the soil and cause early infections (Andersson *et al.*, 1998, Brurberg *et al.*, 1999, Lehtinen & Hannukkala, 2004, Widmark *et al.*, 2007, Brurberg *et al.*, 2011).

In conclusion, it seems that the population of the late blight pathogen in the Nordic countries is rather unique in the world with a level of genotypic variation shared only with the centre of origin for this pathogen.

2.5 Control methods

There have been made and still are many attempts to control the late blight disease. Many breeders have been occupied with trying to obtain the perfect cross, in order to breed the perfect resistant potato, but with this fast evolving pathogen it has proven difficult to control it without the use of fungicides.

2.5.1 The use of fungicides

Preventive application of fungicides is a main approach to control potato late blight (Fry & Doster, 1991). In Sweden, approximately one third of the fungicides used in agriculture are applied against this disease. Seen in relation to the small proportion of arable land used for potato production (~1%) this reflects the scale of the problems with late blight. Late blight fungicides are normally applied according to a routine based schedule, but efforts to achieve a more need-based control of late blight by using forecasting systems have been made. However, these systems are often met with scepticism by the growers due to the aggressive behaviour of the disease in the field.

Even though spraying with fungicides is still the main control strategy against late blight, a more desirable way is to use resistant potato cultivars. Resistant cultivars will delay the onset of the disease and/or reduce the rate of disease development, resulting in a reduced need for fungicide applications.

2.5.2 Breeding for resistance

The breeding of disease resistance in potato has a long history, and one of the most important diseases to breed against is late blight caused by *P. infestans*. The breeders usually divide the resistance of *P. infestans* into general resistance (also known as field or horizontal resistance) and race-specific resistance (vertical resistance). Most breeding efforts have been made to

develop cultivars with race-specific resistance against *P. infestans*. Breeding for race-specific resistance started in the beginning of the 20th century with crossing potato (*S. tuberosum*) with a wild Solanum species, *S. demissum*. Eleven different resistance genes from *S. demissum* were recognised, and a system of so called R-genes was set up by Black (1953). For example, if a cultivar possesses the resistance gene R4, race 4 of *P. infestans* can infect the plant. This is a gene-for gene relationship (Van der Plank, 1968) where the avirulence gene product is recognised by a dominant R-gene in the host, which triggers the hyper-sensitive response (HR) (Kamoun *et al.*, 1999). Today, *P. infestans* has managed to overcome all of the resistance genes that have been introduced by breeders (Fry, 2008).

Compared to race-specific resistance, general resistance is assumed to be more stable since the pathogen has to change in several loci to be able to infect the host (Umaerus *et al.*, 1983). The plant is not totally immune and the pathogen is exposed to less selection pressure. However, breeding for general resistance is a method which has been proven to be challenging since the basic chemical and physical factors leading to this type of resistance are unknown.

New breeding efforts have again focused on wild Solanum species and new resistance genes found in *Solanum bulbocastanum*. The R genes found ($R_{pi\text{-}blb1}$, $R_{pi\text{-}blb2}$, $R_{pi\text{-}blb3}$) have shown to confer resistance against all of the genotypes tested. It is assumed that these R genes will be more durable since they seem to be effective against the population of *P. infestans* from central Mexico (Song *et al.*, 2003, van der Vossen *et al.*, 2003). However, there are some strains today that have overcome this resistance, but these strains have not yet appeared in field tests so optimism and hope about these R genes are still retained (Fry, 2008).

2.6 The genome of *P. infestans* and its effectors

The genome of *Phytophthora infestans* was recently sequenced (Haas *et al.*, 2009). Compared to other related *Phytophthora* pathogens such as, *P. sojaea* and *P. ramorum*, *P. infestans* has by far the largest genome (240 Mb compared to 65-95 Mb for the other *Phytophthora* species). The big difference in genome sizes is probably due to the large amount of repetitive DNA and transposons in *P. infestans*. The genome also consists of an extensive expansion of specific families of secreted disease pathogenicity effector proteins which are coded in the mobile element of the genome. The genome of *P. infestans* codes for a large number of effector proteins (>700) (Haas *et al.*, 2009).

Effectors are proteins that can be seen as the pathogens key weapon to defeat the defence mechanisms of the host (Tyler, 2008). However, effector

proteins can both facilitate the infection (virulence factors or toxins) of a host and/or trigger defence responses (avirulence factors). The pathogen effector which is a product of an avirulence (Avr) gene interacts with the corresponding R protein in the plant. If either the Avr gene or the R is absent or non-functional the interaction is compatible and the host susceptible (Vleeshouwers *et al.*, 2011). If the plant is resistant a HR response occurs. Some of the effectors act in the apoplast while others act in the host cell. The effector proteins can target different sites in infected host plant tissue (Kamoun, 2006).

These protein-effector interactions are studied to better understand the underlying mechanism of late blight resistance on a molecular basis. All oomycete avirulence genes discovered today have an RXLR-motif (RXLR=arginine, any amino-acid, leucine, arginine) (Poppel *et al.*, 2008). The *P. infestans* effectors Avr3a, Avr4 and Avr-blb1 are the most studied effectors and belong to the RXLR group (Armstrong *et al.*, 2005, Poppel *et al.*, 2008, Vleeshouwers *et al.*, 2008). The RXLR motif defines a domain that enables translocation of the effector proteins into the host cell (Whisson *et al.*, 2007). The discovery of the effector proteins is a major breakthrough in the understanding of the *P. infestans* - *S. solanum* interaction, but more knowledge is needed to fully understand the underlying mechanisms.

3 Objectives

The main goal and everyone's dream when working with late blight is to find a solution that will control the disease and thereby decrease the amount of fungicides used. After having worked with this pathogen for a while you discover that this organism is quite complex and the main goal feels further and further away. However, with small steps and more knowledge of the underlying mechanisms of this pathogen and disease the goal might eventually be reached. The objectives for this thesis are to study how sexual reproduction affects the population biology of *Phytophthora infestans*. This was done by answering the questions:

- How is the population of the late blight pathogen in the Nordic countries structured? Is a predominant clonal lineage present as in other European countries? Is there a migration pattern between the Nordic countries? Is the reproductive mode primarily sexual or asexual?

- Do the first genotypes of *P. infestans* in a field persist during the season and do they have any epidemiological advantage? Can the first genotypes compete against the genotypes coming in from outside?

- Is the Swedish population of *P. infestans* differentiated with respect to the two different hosts, hairy nightshade and potato? Can the pathogen be more aggressive on any of the hosts?

- Are there any differences in effector variation in a clonal population of *P. infestans* compared to a sexually reproducing one?

4 Methods for studying population biology of plant pathogens

With a more intensive agriculture, more environmental awareness and the goals to feed the world, new strategies must be developed to understand more about plant-pathogen interaction and population biology of plant pathogens. Many of these are fast evolving organisms that easily adapt to environmental changes (McDonald, 1997) and it is therefore important to study and monitor their population genetics in order to develop good control strategies.

Plant pathogenic organisms can have several stages, a sexual or/and asexual life cycle and a diploid or haploid phase. Knowledge about these traits is a prerequisite when studying many basic processes in population biology (Stukenbrock & McDonald, 2008). Samples of plant pathogens are easy to collect in large numbers and relatively easy to culture and maintain in the laboratory. When studying the genetics of plant pathogens it is important to have genetic markers that give relevant information. Traditionally, plant pathologists have used fungicide resistance and virulence as markers. These functional markers have of course been important in agriculture and very useful for chemical companies and breeders. However, virulence and fungicide resistance factors are under strong selection pressure in agricultural systems and the results might be biased if using only these factors. It is sometimes problematic to collect a representative sample of the population in question, and choose the right method of analysis. What is observed in the field can sometimes be hard to study with laboratory methods and analyses. Especially when studying the population biology of plant pathogens, the definition of a population can be difficult to interpret. In the case of potato and *P. infestans,* the host plant is usually cultivated only during a certain period of the year and the pathogen is subjected to man-mediated movement of inocula, both of which complicates the definition of the pathogen population.

4.1 Field work

In population biology, it is important to start with an appropriate sampling of the material in question when studying allele frequencies. The sampling depends on the question you want to answer and what is known about the population beforehand. A stratified sampling (paper I) is considered to be a good strategy since it can deal with several aspects and levels of the pathogen population in question (McDonald, 1997). With this method, all levels from single leaflets, plants, disease foci, field, region and country can be covered. The sampling of a plant pathogen can be challenging since the life cycle and dispersal of the pathogen is highly influenced by the weather conditions.

The traditional way of sampling plant pathogens is to collect living material and then transfer it to a suitable artificial medium. This method is time consuming and limits the sample size. It is moreover a selective method since some isolates might be lost during culturing. During the last years, FTA-cards used in forensic science have become popular also in plant pathology. The method is easy to use and simplifies transport of material between labs and across borders. Another method that is simple and inexpensive is to dry infected leaflets and extract the pathogen DNA directly from them. With this method it is possible to collect and analyse a large number of samples. This approach also reduces the risks of getting a biased result due to loss of isolates.

4.2 Characterising phenotypes

There are important traits for plant pathogens that still cannot be measured with genetic markers. These traits are often put together in the term aggressiveness. Originally, Vanderplank (1963) was first to define the term aggressiveness as the quantity of disease caused by a pathogen on a compatible host. Traits commonly used as components of aggressiveness are latency period (the time between infection and spore production), lesion growth (how fast the lesion expands) and sporulation capacity (number of spores formed per lesion). In the interaction between host and pathogen the environment is an important factor which also influences the disease severity. Usually, when assessing aggressiveness the environmental and host traits are kept constant when determining and measuring the pathogen dependent characters. Aggressiveness of *P. infestans* has been extensively studied by comparing different potato cultivars (Carlisle *et al.*, 2002), various growing regions (Day & Shattock, 1997, Flier & Turkensteen, 1999) and alternative crops (Lebreton *et al.*, 1999). However, less work has been done on aggressiveness of this pathogen on non-crop Solanaceae species (Platt, 1999, Fontem *et al.*, 2004,

Olanya *et al.*, 2005). In paper III the aggressiveness of *P. infestans* is compared on potato and the weed hairy nightshade (*S. physalifolium*) (Grönberg *et al.*, 2012).

When assessing aggressiveness, the size of the experiment depends on if it is performed on detached leaflets or whole plants, in growth chambers or in the field. In order to have a representative sample of the natural variation in a population it is necessary to avoid selection caused by loss of "weak" isolates. This can be a potential risk during isolation on artificial media and can cause biased results from aggressiveness tests. Also, long term storage on artificial media will affect the aggressiveness of individual isolates. Usually, the aggressiveness tends to decrease when a plant pathogen is continuously cultured in the laboratory. Another important source of error is the condition of the plant material used for inoculation (Lehtinen *et al.*, 2009). To improve the validity of the results in paper III, sporangia from all isolates used in the aggressiveness tests were harvested directly from the sampled leaves, and all test-leaves used for inoculation were taken from field grown plants of potato and hairy nightshade. *Phytophthora infestans* can infect both potato tubers and foliage. However, the resistance levels in foliage and tubers within a cultivar are usually not correlated and therefore the aggressiveness is best tested separately for tubers and foliage.

There can sometimes be confusion between the term aggressiveness and virulence. Virulence is another phenotypic characteristic in plant pathogens and is defined as the ability of a pathogen to infect a particular host genotype (Van der Plank, 1968). For *P. infestans* the virulence refers to the ability of a specific genotype to overcome specific R-genes (resistance genes) in the plant. There are differential potato lines where each clone contains one of the eleven R-genes. These can be used to define the virulence/avirulence of isolates from a population. The virulence is not correlated to the aggressiveness. For example, populations of *P. infestans* can display different unlinked levels of variation in genotypes, aggressiveness and virulence spectra.

4.3 Characterising genotypes

During the last decade the methods for studying population biology of plant pathogens have developed tremendously. Monitoring of late blight is common today and changes in population structure and biology occur since *P. infestans* is a fast evolving pathogen. Detecting and identifying genotypes will answer the questions about genetic drift, migration and recombination, which will give a good overview of the population biology. However, no single marker system is perfect (Milbourne *et al.*, 1997) and can fulfil all the requirements and

aspects of *P. infestans* research. Before development of the DNA-based molecular methods isozyme variation was used. The isozymes give a low resolution and the analysis is very time consuming. Another time consuming method is RFLP (restriction fragment length polymorphism) with the probe RG57 (Goodwin *et al.*, 1992). This gives a fingerprint and has been a valuable tool for detecting genetic variation. The disadvantage is that the data is difficult to interpret and a large amount of DNA is required. After the mitochondrial genome of *P. infestans* was sequenced, mitochondrial DNA markers were developed. However, the variation in mitochondrial haplotypes for *P. infestans* is very limited and the resolution is also for these markers low. Furthermore, to address questions with respect to population genetics such as recombination, genetic drift etc., markers from the nuclear genome are required. AFLP markers (amplified fragment length polymorphism) have a high resolution since they yield many loci per primer combination (Van der Lee *et al.*, 1997). This method is however also time-consuming and it is difficult to compare results between laboratories. In addition, these markers are dominant, i.e. cannot distinguish between heterozygotes and homozygotes, and therefore not very suitable for a diploid organism like *P. infestans.*

At present, the most used markers are SSRs (simple sequence repeats) also called microsatellites. The SSR-markers are ideal for studying population structure since they are highly polymorphic, well-defined and easy to score (Cooke & Lees, 2004). Additionally, the markers are co-dominant which gives the possibility to track both alleles at the same locus. Another benefit is that only small amounts of DNA are required. In this thesis all the genotyping (paper I, II and III) has been done with SSRs developed by Knapova *et al* (2001) and Lees *et al* (2006). In paper I, six SSRs were used to identify the population structure in the Nordic countries. The number of unique multilocus genotypes found (paper I) was high compared to the total number of isolates sampled. This indicates that the number of SSRs was sufficient since the genotype saturation was reached with a low number or markers. However, neutral markers like SSRs cannot reveal the race structure of an isolate. A marker which shares most of its advantages with SSRs is SNPs (single nucleotide polymorphism). Mutation rates are easier to score with SNPs compared with SSRs. However, the SNPs have so far not been used in population studies of *P. infestans* but with developing techniques and more investigation from the genome this could be markers to use in the future.

Due to the wide range of markers used in the different laboratories it is difficult to compare population genetic data of *P. infestans* between research groups.

4.4 Sequencing

With the genome of *P. infestans* sequenced, new possibilities of studying population biology has arrived. The sequencing of different species and isolates makes it possible to perform powerful phylogenetic studies. With a high throughput method it will be possible to estimate the type and extent of selection pressure on functional genes.

Population studies can be taken to new levels by sequencing and comparing the whole genomes instead of specific regions. With the discovery of effector genes the evolutionary drivers of pathogenicity can now be explored. Hopefully, these methods will increase the knowledge of observed phenotypes and available resistance genes.

4.5 Analysing population genetic data

When studying population biology one of the most important attributes to investigate is the genetic diversity. With constantly changing environments the genetic diversity is necessary for a population to continuously evolve and adapt to new situations. The majority of the estimates for genetic diversity are based on the allele frequencies. Most of the population genetic analyses are designed for a diploid or haploid organism. However, for *P. infestans* the occurrence of more than two alleles in a locus has been reported (Brurberg *et al.*, 2011). It has proven to be challenging to analyse populations comprised of a mixture of isolates with different ploidies (Cooke *et al.*, 2011). However, there is a recent method (Bruvo *et al.*, 2004) for dealing with the problem of ploidy mixture in a population which has been further developed and implemented in the statistical program R as the package POLYSAT (Clark & Jasieniuk, 2011). This will hopefully improve the genetic analyses and interpretation of *P. infestans* populations.

Moreover, when studying population genetic data most analyses are developed for populations at Hardy-Weinberg equilibrium. However, plant pathogens under agricultural conditions are often not likely to be at evolutionary equilibrium. The definition of a population for a plant pathogen can be challenging (McDonald & McDermott, 1993). For *P. infestans*, it is the host (potato) which sets the boundaries for the definition of a population. In addition, the man-mediated movement of inoculum of *P. infestans* (seed tubers) and introduced selection (e.g. fungicide use), means that common analyses for population genetic data are not fully applicable for *P. infestans* on potato. In order to test for sexual recombination, it may be sufficient to check for the presence of sexual structures of the pathogen, if mating types are in equal proportions and if there is high genotypic diversity. A simple and fast

method to check for genotypic diversity is to divide the number of unique multilocus genotypes with the total number of samples (Paper I and II). A population with high genotypic variation results in a ratio close to one which would indicate that sexual recombination takes place.

5 How sexual reproduction affects the population biology of *Phytophthora infestans*

5.1 Population structure and migration patterns of *P. infestans* in the Nordic countries (paper I)

The late blight agent *Phytophthora infestans* has been extensively studied throughout the world and many population studies have been conducted. During the past several decades there have been major changes in the population structures of *P. infestans*. Sexual reproduction occurs outside central Mexico but still most populations are dominated by asexual reproduction. These changes are probably due to migration, sexual reproduction and mutation/selection. The clonal lineage "blue_13", which is dominant in Europe seems likely to have resulted from sexual reproduction followed by selection (Fry *et al.*, 2009).

The population of the late blight pathogen in the Nordic countries does not have this dominant clonal lineage and is one of the regions outside Mexico where sexual reproduction and production of oospores occur (Andersson *et al.*, 1998, Brurberg *et al.*, 2011, Widmark *et al.*, 2011). These studies suggest that sexual reproduction in *P. infestans* does take place in the Nordic countries but the question of how common it is still remains open. The migration patterns between the different Nordic countries have also been unknown but it is hypothesized that the migration rates are linked to the geographical distances.

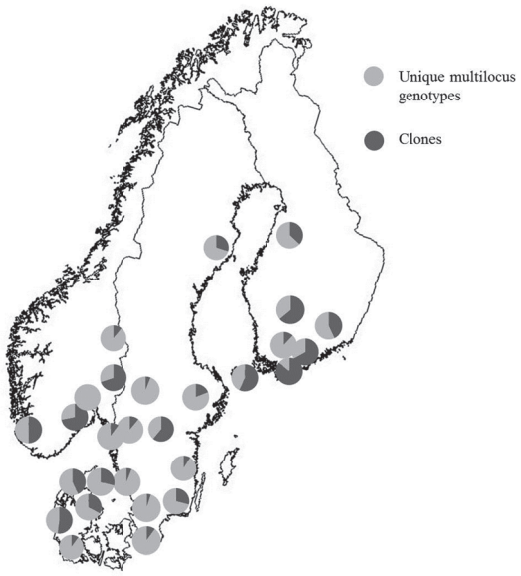

Figure 4. A map of the Nordic countries with circles representing the different sampling sites. The light grey colour corresponds to the proportion of unique multilocus genotypes found in each field (sampling site). The dark grey corresponds to the proportion of clones found in each field. The clones were unique for the field and could be found anywhere else.

The results from our study showed that the genotypic variation is very high in the Nordic region. From 836 isolates, 524 multilocus genotypes were detected and most of the variation was found within fields. The clones were only found in the lowest strata (foci within field or within field if no defined foci were found), figure 4. The high genotypic variation of *P. infestans* was expected but the lack of population differentiation was a surprise, however, this is in agreement with the presence of sexual reproduction, which is also supported by the low association among loci. As mentioned, previous reports show that sexual reproduction of *P. infestans* and oospore formation occurs in the Nordic countries. The oospores might have an advantage by resulting in earlier infections compared to tuber derived inoculum (Yuen, 2012). Our results indicate that oospores are an important inoculum source which will have a strong influence on the population structure within fields. A single mating between an A1- and an A2 *P. infestans* isolate can result in thousands of oospores in a single leaflet (Götz, 1991). According to earlier mating experiments not all crossings will produce infectious oospores (Smoot *et al.*,

1958, Fay & Fry, 1997). However, as infections from tubers are a rare event, even a limited inoculum load from the oospores could have a considerable effect on the population biology of *P. infestans*. Oospores can infect all through the season, and in this way constantly introduce new genotypes, reduce the limiting effect of selection on the genotypic variation during the season. It is also possible that the population of *P. infestans* in the Nordic countries has adopted another strategy of survival than in other regions. With distinct growing seasons with cold winters in between and no other host present than potato tubers, the pathogen increases the chances of survival with the ability to form oospores instead of only clonal overwintering in tubers.

It is, however, unlikely that infections originating from oospores cause the high genotypic variation in all fields. Sometime in the past there must have been a change in the population from solely clonal to more sexual reproduction of *P. infestans* in the Nordic countries, but it is not known how long sexual reproduction has been present. It can be assumed that the high genotypic variation resulting from sexual reproduction will be reflected and accumulated in the population that overwinters in infected tubers. Consequently, planted infected seed tubers may already have a high genotypic diversity of *P. infestans* which later will also be seen in the foliage.

As seen from our results, the export-import of seed tubers between the Nordic countries was not correlated to the migration patterns. It was believed that the migration rates between closely related countries would be high, and a low migration rate between countries situated further away from each other. However, the differences in migration rates are most likely caused by a higher degree of genetic similarity between the pathogen populations as can be seen for the populations in Sweden and Denmark compared to Denmark and Finland. The analysis of the maximum likelihood indicates that the populations of *P. infestans* in Sweden and Denmark are genotypically very similar when compared to the rest of the Nordic countries. Moreover, the exchange of airborne inoculum between Sweden and Denmark is possible since the cultivation of potato is concentrated to two geographical areas situated close to each other.

The sexual reproduction of the late blight pathogen in the Nordic countries affects the population biology in different ways. The additional source of inoculum makes disease control based on crop rotation problematic and high genotypic variation gives the pathogen increased adaptive capacity to new conditions, e.g. climate, fungicides and cultivars.

5.2 Immigration and persistence of *P. infestans* in a single field (paper II)

In Sweden, the population of *P. infestans* is highly diverse and oospores act as an important source of inoculum, however, it is difficult to predict the epidemiological consequences of such a population. Infected tubers have been considered as the most common way for the pathogen to overwinter (Zwankhuizen *et al.*, 1998), however it is important, to be aware of that only a low proportion of infected tubers will produce infected plants (van der Zaag, 1956, Hirst & Stedman, 1960, Inglis *et al.*, 1999). As mentioned earlier, only a small proportion of all crossings between an A1- and an A2 *P. infestans* isolate will produce infectious oospores. However, even if very few oospores infect the crop they could be of importance as primary inoculum since a restricted number of successful infections could have a big impact on the epidemiology of late blight.

In this study, tubers were inoculated with known genotypes of *P. infestans* (originating from foliage or tubers) and planted in a field trial. The population of the late blight pathogen was monitored during the season to investigate if the isolates causing the first infections will affect the population structure throughout the season. The field trial was situated on an experimental farm and was surrounded by potato fields treated with fungicides. The hypothesis was that the isolates used for the inoculation of the tubers would give the first symptoms and have an advantage compared to airborne immigrating genotypes from the surrounding fields.

In contrast to the hypothesis, the immigrant genotypes dominated the population of the late blight pathogen and were continuously introduced into the field. As a result, the isolates used to inoculate the seed tubers had very little impact on the *P. infestans* population in the trial. The genotypic variation was high and the population gradually shifted during the season. This indicates the lack of dominating clonal lineages of *P. infestans* in the infected fields in the region, unlike what can be seen in other European countries (Lees *et al.*, 2008, Montarry *et al.*, 2010). Genotypes from the seed tubers do not necessarily start the epidemic of late blight. In Sweden, oospores act as an additional inoculum source. In this particular field trial it is unlikely that the first infections originated from oospores due to the stringent late blight control on the research farm. Therefore, it can be assumed that the first infections were caused by immigrant inocula from surrounding fields. This inoculum load must have a high genotypic diversity, which probably originates from germinating oospores and/or infected tubers carrying of a variety of genotypes. The first observed infections in the field came relatively early in the season and showed a high diversity, implicating oospores as inoculum source. The oospores must

originate from other fields and are a result of previous years random mating in the crop. From a population biology perspective, this observation is important since it may explain the low genetic differentiation observed among populations (paper I). The genotypes originating from the tubers will be moved between farms and may only have a small immediate effect on the population. However, the genotypes from the tubers can mate with other genotypes and produce oospores. These oospores would then be the source of *P. infestans* infections the following year, and this would be enough to counteract genetic drift.

All of the isolates gave tuber infection after incubation and there was a strong correlation between the proportions of emerged plants and infected tubers. Since the isolates differed in the ability to infect tubers the plots also differed in plant emergence, fig 5. This indicates that almost none of the infected tubers were able to develop emerging plants, resulting in the low recovery rate of the isolates used to infect the tubers. All genotypes used in the inoculation were recovered but only once at the third sampling time. The difference in crop emergence and plant density could have an effect on the micro climate in the plots. It might have been better to dilute the amount of infected tubers with healthy ones before planting so that all plots had the same proportion of infected tubers. However, in our field trial the two plots with the highest crop density were the last to show symptoms of late blight. A possible explanation for this is that the weather during the sampling period was very conducive for late blight, compensating for any differences in crop microclimate.

Figure 5. The field trial with seed tubers inoculated with known genotypes of *P. infestans*. To the left a plot planted with healthy seed tubers, to the right a plot with heavily infected seed tubers. Photo: L. Sjöholm

It has been reported that different strains of *P. infestans* vary in their ability to infect tubers (Lambert & Currier, 1997) and possibly also have different abilities to start new epidemics. The origin of the isolates used for inoculation of the seed tubers could have played a role in the infection rate. Genotypes originating from tubers must have had the ability to infect the plant and persist in the foliage to sporulate and infect the tubers. The *P. infestans* isolates originating from tubers have therefore been selected for epidemiological fitness (the ability to compete with other genotypes). In the field trial, however, no effect of isolate origin was detected.

In a clonal population of *P. infestans* a high aggressiveness is not necessarily the best trait for an isolate to be able to overwinter in tubers (Montarry *et al.*, 2007). Genotypes with less aggressiveness sporulate for a longer period during the season and the chances of infecting tubers increase. With high aggressiveness the genotype can dominate the epidemic during the season. However, highly aggressive genotypes present in the tubers will increase the risk of rotting in the soil before emergence. This will result in a bottleneck giving a negative selection for aggressive isolates of *P. infestans* (Shattock, 1976).

In an earlier report, the aggressiveness of *P. infestans* in the sexually reproducing population in the Nordic countries was analysed, and only small differences were found in isolates from Sweden, Denmark, Norway and Finland. This could be explained by sexual reproduction breaking up favourable sets of alleles in more aggressive genotypes and "diluting" the overall aggressiveness in the population (Lehtinen *et al.*, 2009). However, in our field trial one genotype showed higher competitiveness indicating that some variation in epidemic fitness exists in the Swedish population of *P. infestans*. With a sexually reproducing population the additional source of inoculum in the form of oospores plays an important part in the epidemiology of the late blight. The oospores can survive in the soil between seasons without any host present. Also, unlike infections originating from tubers, oospores can germinate and infect the crop all through the growing season. Sexually formed oospores can introduce new genotypes with a higher epidemic fitness compared to old genotypes (Day & Shattock, 1997). This can result in increased epidemic growth rates (Zwankhuizen *et al.*, 2000) and add to the already immense problems of controlling late blight in potato.

5.3 Population differentiation of *P. infestans* on two different hosts (paper III)

In the south of Sweden two hosts of late blight, hairy nightshade (*Solanum physalifolium*) and potato (*Solanum tuberosum*) are present. These two hosts co-exist with a sexually reproducing population of *P. infestans* present, which led to the hypothesis that there is a population differentiation of the late blight pathogen on the two hosts. The genotypic variation of *P. infestans* was high on both hosts, however no genetic differentiation using the SSR-markers could be found between the two hosts. It was assumed that if the two hosts would have been in allopatry, the populations would have been more differentiated since other studies have shown genetic differentiation of *P. infestans* on different hosts (Lebreton & Andrivon, 1998, Erselius *et al.*, 1999, Knapova *et al.*, 2001).

However, the phenotypic data showed differentiation of the pathogen on hairy nightshade and potato. Isolates originating from hairy nightshade had a shorter latency period and higher sporulation capacity when inoculated on potato compared to isolates originating from potato. Additionally, the nightshade isolates could more easily infect nightshade plants compared with the potato isolates but this difference was not seen on potato plants. The results suggest that only parts of the *P. infestans* population in the sampled field were able to infect the hairy nightshade and also that it is easier for the pathogen to infect potato than hairy nightshade. This will mean that "weaker" isolates restricted to potato will be outcompeted by the isolates coming from nightshade. In this way, the population of *P. infestans* will be filtered towards an increased aggressiveness on the potato crop.

One can speculate why *P. infestans* will infect the hairy nightshade at all? During the sampling, all of the hairy nightshade plants observed in the region were infected even though the late blight epidemic probably starts on the potato since it would be established before the weed. One explanation could be that the sexually reproducing population of *P. infestans* in this region (Widmark *et al.*, 2007, Brurberg *et al.*, 2011) generates new genotypes that can infect new hosts at a higher rate compared to in a clonal population. However, it is not only the pathogen which has a sexual reproduction but also the nightshade, which can increase the selection pressure towards more aggressive genotypes of the pathogen.

Oospore formation of *P. infestans* has been reported in *S. physalifolium* (Andersson *et al.*, 2003). In an additional study (Björling, 2012) the same isolates of *P. infestans* collected from hairy nightshade and potato were used to investigate if the isolate origin could affect the ability to produce oospores. The group where both isolates of *P. infestans* originated from hairy nightshade (NN) produced significantly more oospores per cm^2 on potato compared to the

group where both isolates originated from potato (PP), figure 6. Moreover, the results show that the group with one isolate originating from hairy nightshade and one from potato (NP) also produced significantly more oospores than the PP group. This indicates that as soon as isolates of *P. infestans* originating from hairy nightshade are involved in mating type crosses they produce more oospores than isolates originating from potato. These crosses were also tested on nightshade leaves and the same pattern could be seen with the NN-group that produced more oospores per cm^2 compared to the other groups. However, these results were not statistically significant.

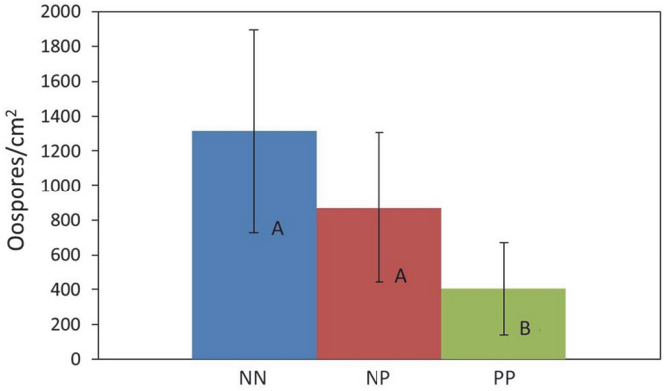

Figure 6. The mean number of oospores per cm^2 produced by crosses between isolates of *P. infestans* from hairy nightshade-hairy nightshade (NN), hairy nightshade-potato (NP) and potato-potato (PP). Error bars indicate the 95% confidence interval. Means with the same letter are not significantly different (Students T, $p \leq 0.05$).

The genotypes from hairy nightshade might have undergone more selection for oospore production compared to isolates originating from potato since the hairy nightshade does not produce tubers and the pathogen cannot overwinter except in the form of oospores. Oospores acting as inoculum will increase the genotypic variation and, thereby, the adaptability of the pathogen population.

If the weed hairy nightshade is present in a potato field it will increase the problems with late blight. Not only can isolates of *P. infestans* from hairy nightshade increase the aggressiveness on the potato but they also increase the production of oospores. In Swedish potato production, crop rotation is normally practised to manage problems with soil borne pathogens. With hairy nightshade present both as a weed and an alternative host to *P. infestans* the effect of a good crop rotation is reduced. Hairy nightshade will function as a reservoir for the pathogen by supporting oospore formation. Earlier epidemics, possibly caused by oospores, in combination with an increased aggressiveness will have serious consequences for the control of potato late blight.

5.4 Variation in effectors (paper IV)

Phytophthora infestans is notorious for overcoming and adapting in response to R genes (Wastie, 1991). To understand more about the pathogen-plant interaction more knowledge is needed regarding the function and variation in effector proteins. The durability of an R gene is highly dependent on the stability or the role of its corresponding effector. This means that if an Avr gene can mutate without decreasing the fitness, the pathogen can avoid recognition and overcome the resistance. However, there is no knowledge of how or if the effectors vary between different population of *P. infestans*. Most populations of the late blight pathogen in the world are clonal (Akino *et al.*, 2004, Guo *et al.*, 2009, Blandón-Díaz *et al.*, 2012, Hu *et al.*, 2012). Nicaragua is one example of a country where the population of *P. infestans* belongs to one dominant clonal lineage. Nevertheless, the population in Nicaragua is very variable with regard to functional characters such as the virulence spectra and fungicide resistance.

In this study, the diversity in the PiAvr4 effector gene in a clonally reproducing population of *P. infestans* was investigated with regard to any possible mutations or deletions. The sexually reproducing population of *P. infestans* from Sweden was used as an outgroup. Our hypothesis is that since the R4 resistance is rarely seen, *P. infestans* in Nicaragua has a mutation or deletion in the PiAvr4 locus. A second objective was to look for any indications of recent population expansion in the Nicaraguan population of *P. infestans*.

The results revealed that all isolates from the Nicaraguan and Swedish population of *P. infestans* had a frame shift at the same position resulting in that the PiAvr4 is not recognised and that all genotypes therefore can infect plants with the R4 gene. These results are in agreement with a previous study from Nicaragua where a high phenotypic variation was found. The population most frequently overcame resistance genes R1, R3, R4, R7 and R11 and the most complex races of the pathogen overcame eight and nine resistance genes, respectively. The high virulence diversity in a clonal population can be explained by the fact that the Avr genes encoding effectors are located in highly variable regions of the genome (Jiang *et al.*, 2008). The selection pressure is much higher on the PiAvr4 diversity compared to the neutral microsatellite diversity. The results from this study also indicate that the population of *P. infestans* in Nicaragua has undergone a population expansion and/or selection. This can be an explanation why the present genotype NI-1 has replaced the “old” genotype(s) and is now the dominating one as suggested by Blandón-Díaz (2012).

The sexually reproducing population of *P. infestans* in this study is only represented by three isolates. Further sequencing is needed to be able to draw any conclusions about the effector diversity in a recombining population. However, the results indicate that the reproduction system in a population of *P. infestans* does not necessarily reflect the variation in virulence factors. The phenotypic variation in the Swedish population of the late blight pathogen is high as could be expected in a recombining population. An earlier study showed that all virulence genes except the one corresponding to R9 could be found in the Swedish population. The lack of virulence to R9 can be explained by the absence of selection pressure since R9 has never been introduced in commercial cultivars (Lehtinen *et al.*, 2008). The use of cultivars in Sweden carrying the R4 gene is at present unknown.

Effectors are examples of good genes to study since they might increase the knowledge of the pathogen-plant interaction. Of course, further studies of other clonal and sexually reproducing populations of *P. infestans* need to be done to clarify the possible differences in effector variation. However, it is still important to observe how the pathogen behaves in field situations as well as pursuing functional studies of *P. infestans* effectors in order to increase the knowledge of control methods.

6 Conclusions

The population biology of *P. infestans* in the Nordic countries is complex and is similar to the pattern seen in the centre of origin in central Mexico.

- The late blight pathogen is reproducing sexually in the Nordic countries. As a result, the pathogen population has a high genotypic variation and no dominating clonal lineage. The lack of dominant clonal lineages might be explained by the cold winters and discrete growing season.
- The sexual reproduction of the pathogen also influences the migration patterns between the Nordic countries. The geographical distances, however, are not linked to the gene flow.
- Genotypes of *P. infestans* originating from tubers do not always start the epidemic and are not able to dominate the pathogen population during the season. New immigrants, which probably originate from oospore derived infections from outside, are more important for the epidemiology of the disease.
- The alternative host, hairy nightshade selects the *P. infestans* population towards increased aggressiveness on potato. Additionally, crosses between mating types of *P. infestans* collected from hairy nightshade produce more oospores than crosses between isolates collected from potato. The presence of an alternative host will have major consequences for the control of late blight in potato production.
- All isolates of *P. infestans* from Nicaragua and Sweden had a frame-shift mutation in the Avr4 locus resulting in that all isolates can infect R4 plants. This indicates the presence of a selection pressure for *P. infestans* to loose intact Avr4 genes. The population from Nicaragua has undergone a recent population expansion which can be explained by the replacement of the “old” genotype(s) to the new dominating NI-1.

7 Future perspectives

The late blight pathogen has been studied for more than 170 years and there are still many question marks to be straightened out. During this time there have been major changes in the population structure of *P. infestans*. Most populations are still dominated by asexual reproduction but in some parts of the world sexual reproduction is more common. This is probably due to migration, selection and local conditions. The changes in population structure have a huge impact on the epidemics and the control of late blight. However, there is still a knowledge gap in how important oospores are for the epidemiology of late blight and if the oospores can germinate during the season. The Swedish population of *P. infestans* is excellent for studying these questions. The genome of *P. infestans* has been sequenced and the discovery of the large collection of effector genes has increased the knowledge of the pathology of the late blight pathogen. In the Swedish population of *P. infestans* it would be interesting to investigate the effector variation in genotypes infecting potato and the alternative host, hairy nightshade. Will similar effectors have distinct function in different pathogen-host interactions?

Can the alternative host hairy nightshade, present as a weed in the South of Sweden select for a higher aggressiveness compared to newly introduced nightshades further north? Climate change can influence the population biology of *P. infestans* in the Nordic countries. That hairy nightshade is spreading further north is already a fact. Another effect from milder winters in Sweden could be an increased clonal survival of the pathogen. All this will have an effect on the population structure of *P. infestans*.

Phytophthora infestans has been shown to be evolving quickly and can easily overcome resistance genes. The genomes of both the host and the pathogen are sequenced, and this knowledge can be used in combination with high throughput methods to study potato late blight. However, in the future, the monitoring of genotypic and phenotypic changes in the pathogen will still be very important to increase the understanding of how the disease behaves in

field situations. This will hopefully lead to improved breeding of cultivars with durable resistance.

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