Resistance to Early Blight in Potato and Genetic Structure of the Pathogen Population in Southeast Sweden

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Cover: Early blight symptoms on potato leaves.
(photo: F. Odilbekov)

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Abstract

Potato early blight caused by the necrotrophic fungus *Alternaria solani* is a common foliar disease in many potato-growing regions. Application of fungicides is commonly used to effectively control the disease, although they are undesirable due to environmental consequences. Use of resistant cultivars would be the most optimal solution, but there are no cultivars with high level of resistance available on the market. In the present thesis, assessments of early blight resistance both in leaves and tubers of potato cultivars/clones were performed by applying different screening methods (field and greenhouse). Plant defence signalling in response to *A. solani* infection with main emphasis on salicylic (SA) and jasmonic acid (JA) hormones, was also studied. Furthermore, the genetic variability in *A. solani* populations from different potato growing regions of southeast Sweden was investigated. The fungal isolates were analysed for the F129L substitutions, which are associated with loss of sensitivity to QoI fungicides. In addition, field experiments were conducted to determine the occurrence of the F129L substitution and genetic shifts in the population during one growing season in response to two different fungicide strategies.

Cultivars/clones revealed significant differences in resistance to *A. solani* both in leaves and tubers irrespective of screening method. Results from field and intact plant inoculation experiments were significantly correlated but there were no correlations observed between these two methods and detached leaf assays. Some cultivars/clones showed relatively higher level of resistance to the pathogen. Results from the data suggested that SA appears to be responsible for regulating symptom development while JA dependent COI1 defense signaling is important to inhibit fungal growth during early stages of infection. Microarray analysis showed rapid defense responses to *A. solani* infection mediated by partially overlapping SA and COI1 dependent jasmonic acid (JA) signaling. It was also observed that JA/ethylene signaling responses dominate at later time points.

The genetic variability was relatively high among isolates of *A. solani* and significant genetic differentiation was found among populations from different locations in southeast Sweden. Two mitochondrial genotypes (GI and GII) were found among the isolates but the F129L substitution was only detected in GII isolates. Results from the field experiment showed that application of azoxystrobin (QoI fungicide) alone did not control the disease; better disease control was achieved with boscalid combined with pyraclostrobin. Similar results were obtained for yield. Moreover, results of sensitivity tests showed that isolates with the F129L substitution were less sensitive to azoxystrobin. AFLP analysis indicated within season changes in the *A.solani* population, especially at the end of the season.

Keywords: Alternaria solani, field trial, resistance, genetic diversity, F129L substitution, QoI fungicides, salicylic acid, jasmonic acid, microarray analysis

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Dedication

To my family

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 **Odilbekov, F**., Carlson-Nilsson, U. & Liljeroth, E. (2014). Phenotyping early blight resistance in potato cultivars and breeding clones. *Euphytica*, 197(1), pp. 87-97.

II **Odilbekov, F**. & Liljeroth, E. Evaluation of foliar and tuber resistance to early blight in a (tetraploid) crossing population of potato (Manuscript).

III **Odilbekov, F**., Burra, D.D., Rosahl, S., Hedley, P.E., Morris, J., Ziegler, J., Liljeroth, E. & Andreasson, E. Positive role of SA and JA mediated COI1 signalling in early response to *Alternaria solani* in potato (Manuscript).

IV **Odilbekov, F.,** Edin, E., Garkava Gustavsson, L., Persson Hovmalm. H. & Liljeroth, E. Genetic diversity and occurrence of the F129L and G143A substitutions among isolates of *Alternaria solani* and *Alternaria alternata* in southeast Sweden (Manuscript).

V **Odilbekov, F.,** Edin, E. & Liljeroth, E. Within season changes in *Alternaria solani* populations in potato as response to fungicide application strategies (Manuscript).

Paper I is reproduced with the permission of the publisher.

The contribution of Firuz Odilbekov to the papers included in this thesis was as follows:

I Planned and performed the greenhouse and field experiments together with co-authors and participated in evaluation of the data and writing of the manuscript.

II Planned and performed most of the experimental work, evaluated the data and wrote the manuscript together with co-authors.

III Planned the experiments together with co-authors. Performed a large part of the experimental work and wrote the manuscript together with co-authors.

IV Planned the experiments together with co-authors. Performed a large part of the experimental work and wrote the manuscript together with co-authors.

V Planned and performed the experimental work together with co-authors and participated in the writing of the manuscript together with co-authors.

Abbreviations

ABA	Abscisic acid
AFLP	Amplified fragment length polymorphism
GI	Genotype 1
GII	Genotype 2
JA	Jasmonic acid
SA	Salicylic acid
PCoA	Principal coordinates analysis
PDA	Potato dextrose agar
QoIs	Quinone outside inhibitors
rAUDPC	Relative area under the disease progress curve

1 Introduction

Potato *(Solanum tuberosum* L.*)* is considered one of the most important global food crops and is also a promising source for non-food industry. It encounters a number of serious diseases, with one of the most significant being early blight, caused by the fungus *Alternaria solani.* It has been reported that early blight is on the increase in potato growing areas of Europe (Runno-Paurson *et al.*, 2015; Kapsa & Osowski, 2012; Leiminger & Hausladen, 2012). Early blight infections have also been detected with increasing frequency in potato fields in the northern countries of Europe (Edin & Andersson, 2014).

Currently, the main strategy to control early blight and other *Alternaria* diseases are repeated fungicide applications (Campo Arana *et al.*, 2007; Rodriguez *et al.*, 2006). However, repeated applications of fungicides lead to environmental concerns and may cause development of fungicide resistance strains in the target pathogen population (Rosenzweig *et al.*, 2008; Pasche *et al.*, 2005).

An optimal solution for restricting early blight disease in the field would be to grow resistant cultivars (Duarte *et al.*, 2014). Several studies evaluated potato germplasm for resistance to early blight (Duarte *et al.*, 2014; Boiteux *et al.*, 1995; Christ, 1991) and quantitative differences in levels of resistance were found. However, a cultivar with complete resistance has not yet been identified.

In the present thesis, potato cultivars/clones were evaluated for resistance to early blight both in foliage and tubers using different screening methods (greenhouse and field experiments). Plant defence signalling, with an emphasis on salicylic (SA) and jasmonic acid (JA) hormones in response to infection, was also studied. Furthermore, genetic variation in *A. solani* populations of southeast Sweden was investigated and collected isolates were analysed for F129L substitutions, associated with reduced sensitivity against QoI fungicides. In addition, a field trial was carried out to study genetic changes in the *A. solani* population in response to two different fungicide strategies during a single cropping season.

2 Background

2.1 Potato (*Solanum tuberosum* L.)

2.1.1 History and production

Potato (*Solanum tuberosum* L.) is one of the world's most important food crops, and is grown in more than 100 countries with different climate conditions including temperate, subtropical and tropical zones. Its origin is in the Andean region of Peru where wild relatives of the species with high genetic and morphological diversity still exist (Spooner *et al.*, 2005). At the end of the 16th century potato was transported to Spain from where it spread to the rest of the world (Hosaka & Hanneman Jr, 1988). During the 18th and 19th centuries potato was a predominant source of food in several European countries such as Ireland, Germany, Poland and Russia, but since then production of potato in these areas has generally decreased (Camire *et al.*, 2009). At the same time the production of potato has increased in developing countries in Africa and especially in Asia. In 2012 the world production of potato was close to 365 million tons and the area of potato cultivation was approximately 19.2 million hectares (FAO, 2012). The top three potato producing countries in the world are China, India and Russian Federation (Figure 1).

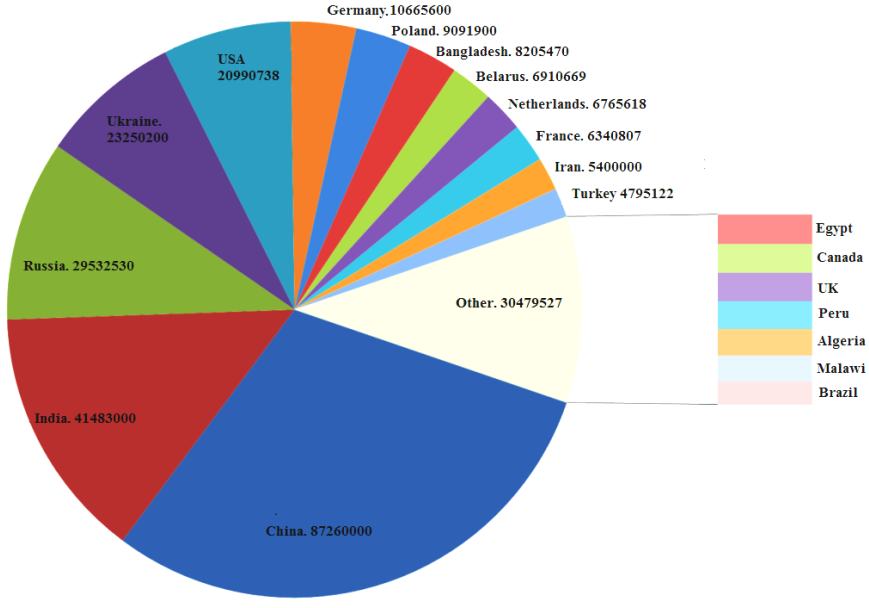

Figure 1. Potato production (ton) in the world (FAO, 2012)

2.1.2 Nutrition value

Potato is a highly popular carbohydrate food in many parts of the world, and can be used both for table consumption as well as in processed products. Fresh potato tubers contain around 80% water and 20% dry matter. More than 75% of the dry matter is starch but they also contain protein, fibre and small amounts of fatty acids (Figure 2) (Prokop & Albert, 2008). It is also rich in minerals such as potassium, phosphorus, magnesium and various vitamins like B1, B3 and B6 (Camire *et al.*, 2009). Potato contains high level of vitamin C. For instance, a single potato tuber of 150g can provide close to 50% of the daily adult requirement of vitamin C (Prokop & Albert, 2008).

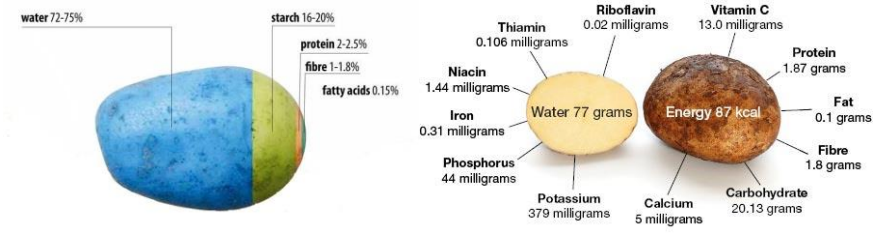

Figure 2. Chemical composition and nutrient content of potato (Prokop & Albert, 2008).

2.2 *Alternaria solani* as a pathogen

2.2.1 The pathogen

The genus *Alternaria* consists of a group of saprophytic and pathogenic fungal species (Thomma, 2003) often reported to be allergenic, food spoiling, mycotoxicogenic and opportunistic fungi associated with mycosis in animals and humans, and the cause of destructive plant diseases (Thomma, 2003; Rotem, 1994). There are about 60 known species within the genera *Alternaria*, and one of these species, *Alternaria solani* (E. & M.) Jones and Grout, is of economic importance in many potato and tomato producing areas worldwide since it causes large crop losses (Pelletier & Fry, 1989). First described by Ellis and Martin in 1882 *A. solani* was originally named *Macrosporium solani*. Galloway (1891) first described this fungus as parasitic and associated with potato blight in Australia. The name early blight was suggested by Jones (1893) to differentiate this disease from late blight. According to Jones (1893), early blight was prevalent in early maturing cultivars and less in medium or late maturing ones, whereas late blight was more serious in medium and late-maturing cultivars (Galloway, 1891). Jones & Grout (1897) found two different fungal species associated with potato leaves and identified that one of them, *A. solani*, was causing the symptoms of early blight. The other fungus, identified as *Alternaria alternata*, was considered as saprophytic and was found on decaying leaves (Jones & Grout, 1897).

A. solani is a mitosporic fungus, class Hyphomycetes, order Hypho-mycelium, family Dematiaceae and belongs to the large-spored group within the genus *Alternaria* (Agrios, 2005). The fungus has septate, branched, and melanised mycelia and the hyphae can be grey, black or olive in colour (Rotem, 1994). The conidia are brown or dark-coloured (melanized), have multinucleate cells and occur individually or in groups. Melanin has a protective function against unfavourable environmental conditions and is antagonistic towards microbes and their enzymes. The conidia (Figure 3A) are beaked with transverse and longitudinal septa, and their size vary from 12 to 20 µm in width and from 139 to 441 µm in length (Rotem, 1994).

2.2.2 Importance of early blight

A. solani causes early blight in several Solanaceous crops such as tomato (*Solanum lycopersicum* L.), potato (*Solanum tuberosum* L.) and eggplant (*Solanum melongena* L) (Rotem, 1981). In potato production areas in temperate regions this disease can decrease tuber yield by more than 20% (Shtienberg *et al.*, 1996; Johnson & Teng, 1990). However, losses up to 50% have been reported in Brazil (Campo *et al.*, 2001). In tomato, crop losses of up to 79% due to early blight were reported from Canada, India, USA, and

Nigeria (Gwary & Nahunnaro, 1998; Sherf & MacNab, 1986; Datarm & Mayee, 1981; Basu, 1974). The pathogen can also affect the quality of the harvested tubers during storage. In some countries this can cause serious problems and losses up to 30% have been reported in tubers (Al-Mughrabi, 2005).

Early blight of potato is typically controlled with fungicides and a precise estimation of the economic costs of early blight control is difficult to make. However, estimated cost of fungicide usage to control early blight exceeded $44 million during a normal potato-growing season in North America (Stevenson, 1994).

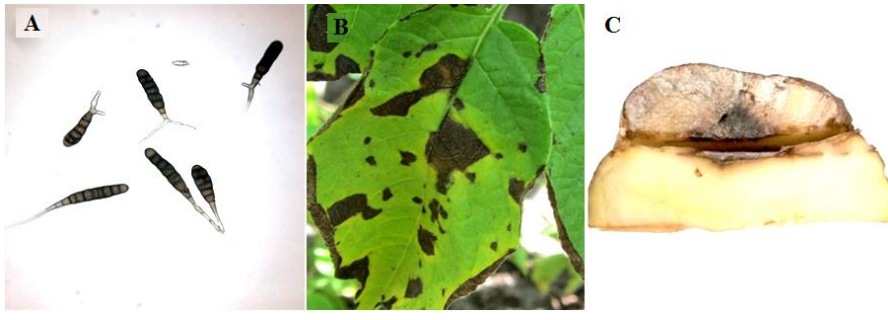

Figure 3. Alternaria solani spores (A), infection on leaves (B) and tubers (C). Photo: E. Liljeroth and F.Odilbekov

2.2.3 Biology of the pathogen

At a wide temperature range (8-32^{o}C) under free moisture the conidia start to produce germ tubes that penetrate the epidermial cells of the host. Penetration is facilitated by enzymes which degrade and kill host cells and enable the pathogen to obtain nutrients from the dead cells (Agrios, 2005; Rotem, 1994). Under appropriate environmental conditions the disease can cause defoliation of the plant. The loss of tuber yields is primarily due to this defoliation (Herriott *et al.*, 1990). During the growing season early blight epidemics develop via secondary spread of conidia. The pathogen has many asexual cycles per season where conidia are produced on infected leaves and spread by wind, running water, potato beetles and probably also by other insects to neighbouring leaves and plants (Figure 4). The fungus can survive as mycelia or conidia in soil or on plant debris and tubers (Sherf & MacNab, 1986). *Alternaria solani* can also survive as chlamydospores and overwinters at least one season or probably several years. According to Robert and Boothroyd (1972), this pathogen is able to survive around 18 months in dry diseased leaves. Consequently the life cycle of *A. solani* includes soil, seed, as well as

air-borne stages, which in turn makes the infection difficult to control (Patterson, 1991).

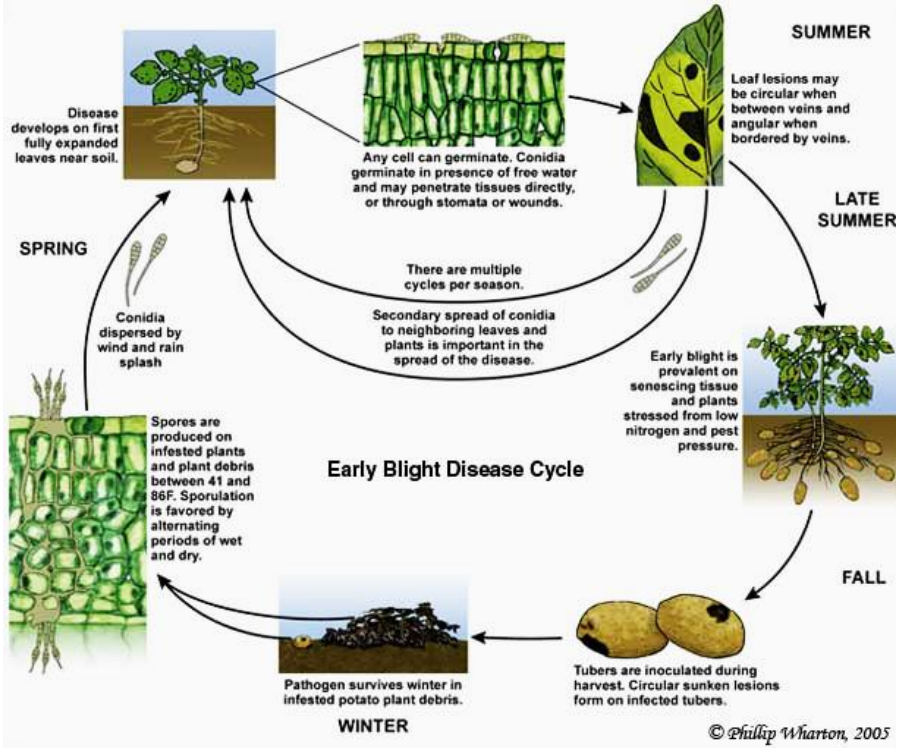

Figure 4. The disease cycle of early blight pathogen (Wharton & Kirk, 2012)

2.2.4 Disease symptoms

The first symptoms of early blight can be observed on older leaves as small, dark, necrotic lesions and later during the growing season the whole plant may be infected (Sherf & MacNab, 1986). The infections cause lesions that expand to a diameter of 9 to 10 mm, which easily can be detected by their target-like appearance (Figure 3B). Most of the time lesions coalesce, and cover a major part of the leaf surface. In severe epidemics the disease cause defoliation but sometimes drying leaves remain on the plants (Figure 5). Complete defoliation of a susceptible plant may occur within 6 weeks after initial infection.

A yellow zone often surrounds the lesions. Under dry conditions, the lesions may drop out leaving holes in the leaves. The fungus can also infect the stem where lesions are often sunken with concentric rings typically forming in the lesions, which can cause breaking of the stem. Tuber infections are characterized by sunken, irregular lesions with elevated borders and are often surrounded by raised purple borders (Figure 3C). The colour of the lesion

varies from grey to brown or purple black. The tissue beneath the lesion is dark brown, solid, leathery or corky with a brown discoloration and extends from a few mm up to 2-3 cm into the tuber (Sherf & MacNab, 1986).

Figure 5. Early blight infection in potato field, including a close up of the symptoms. Photo: E. Liljeroth

2.2.5 Genetic diversity

A. solani has an asexual life cycle but still the morphological and genetic diversity is comparatively high and there is also variation in pathogenicity (Meng *et al.*, 2015; Lourenco *et al.*, 2011; van der Waals *et al.*, 2004; Rotem, 1966; Henning & Alexander, 1959; Neergaard, 1945). Van der Waals (2004) detected a high genetic diversity among isolates from the United States, South Africa, Cuba, Brazil, Turkey, Greece, Canada, China and Russia based on vegetative compatibility groups (VCG). Also molecular markers such as random amplified polymorphic DNA markers (RAPDs) (Leiminger *et al.*, 2013; Weir *et al.*, 1998), random amplified microsatellite markers (RAMs) (van der Waals *et al.*, 2004), amplified fragment length polymorphisms (AFLPs) (Lourenco *et al.*, 2011; Martinez *et al.*, 2004) and SSR markers (Meng *et al.*, 2015) have revealed high diversity among isolates. Leiminger *et al.* (2013) found distinct genetic diversity among isolates collected from the same field and also observed clear genetic variability among isolates from

different years, indicating high genetic heterogeneity within populations. Isolates also differ in cultural characteristics and many morphotypes can be discovered (Rotem, 1966). It has also been found that isolates obtained from the same lesion can be genetically different (Kumar *et al.*, 2008). Stall (1958) reported that heterokaryosis could occur in *A. solani* and found that nuclei may migrate through septal pores between cells of hypha or conidia. Therefore, even isolates derived from single conidia or hypha can be genetically different.

2.3 Control of early blight

2.3.1 Cultural practices

Application of cultural practices like disease-free seed, crop rotation with non-host crops, resistant cultivars, sanitation of fields, supplying proper plant nutrition and water can be efficient against early blight (Madden *et al.*, 1978). Usually forage crops and grains, including maize (*Zea mays* L.) are the most applicable crops for crop rotation in potato fields. A high cropping frequency of potato or other members of the Solanaceae family in a field can be associated with an earlier appearance of early blight in the potato crop (Shtienberg & Fry, 1990).

Optimal host nutrition can slow down the disease progress in the field. Potato plants treated with high levels of nitrogen with low phosphorus and medium to high potassium have a higher level of resistance (Lambert *et al.*, 2005; Mackenzie, 1981). The explanation is that high nitrogen levels can extend the vegetative growth, which delays the ripening. Low levels of phosphorus reduce fruiting and medium to high potassium levels increase tuber formation. Thus, different types of cultural practices can decrease the disease severity, but under favourable environmental conditions with sufficient inoculum, complete control cannot be reached.

2.3.2 Chemical control

Currently, application of foliar fungicides is the most effective method to control early blight in the field (Wharton & Kirk, 2012; Horsfield *et al.*, 2010). Different types of protective and curative fungicides against early blight are available on the market. Disease management programs often involve protectant fungicides like mancozeb and chlorothalonil, which should be sprayed every seven to ten days to provide protection. These types of fungicides provide reliable efficacy and have multi-site mode of action, which decrease the chance of mutation for fungicide resistance. However, they have a negative environmental impact (Kemmitt, 2002).

Other types of fungicides that are used against early blight are the so called Quinone outside inhibitors (QoIs). These fungicides are important due to their broad-spectrum activity and low use rates (Bartlett *et al.*, 2002). In addition, these fungicides have positive effects on plants such as increasing chlorophyll content (Butkute *et al.*, 2008) and delaying leaf senescence (Bertelsen *et al.*, 2001). The fungicides prevent electron transport in mitochondrial respiration by binding to the Qo site of the cytochrome *b* (cytb) complex and inhibiting the ATP synthesis (Bartlett *et al.*, 2002). However, due to specific single-site mode of action there is a high risk for the evolution of resistance in the pathogen, which has been shown in different studies (Leiminger *et al.*, 2014; Ishii, 2009; Markoglou *et al.*, 2006; Pasche *et al.*, 2005). In many plant pathogenic fungi the most important resistance to QoI fungicides is the G143A mutation, which is due to a substitution of glycine (G) by alanine (A) at the amino acid position 143 in the cyt*b* protein (Kim *et al.*, 2003; Ishii *et al.*, 2001; Sierotzki *et al.*, 2000). In *A. solani*, G143A has not been found, but a decreased Qol fungicide sensitivity is instead related to the F129L amino acid substitution of phenylalanine (F) to leucine (L) at position 129 (Leiminger *et al.*, 2014; Pasche *et al.*, 2005; Pasche *et al.*, 2004). While the G143A substitution causes complete loss of sensitivity to QoI fungicides the F129L substitution causes reduced QoI sensitivity (Yamada & Sonoda, 2012; Bartlett *et al.*, 2002).

2.4 Breeding for resistance to early blight

Potato cultivars with a higher level of resistance to early blight would be important in reducing losses in potato fields for practical, economic and environmental reasons (Boiteux *et al.*, 1995). Cultivars with high levels of resistance have already been found in potato germplasm (Duarte *et al.*, 2014; Boiteux *et al.*, 1995; Christ, 1991), but the resistance that has been identified so far is distinguished by a typical rate-reducing reaction. In contrast to late blight there are no varietal differences that point to specific R-genes for resistance against early blight in potato and the resistance trait to early blight displays a quantitative inheritance pattern (Christ & Haynes, 2001; Herriott *et al.*, 1990) Therefore, it is hard to identify potato germplasm with race-specific resistance (Zachman, 1982).

The other main factor in resistance to early blight is cultivar maturity. It has been shown that early maturing cultivars are more susceptible than late maturing ones (Duarte *et al.*, 2014; Rodriguez *et al.*, 2006; Holley *et al.*, 1983). However, in some studies it has been observed that early maturing clones can have high levels of resistance (Zhang, 2004; Boiteux *et al.*, 1995). Zhang (2004) found a number of QTLs for early blight resistance in diploid potato and identified two of them as being closely linked to regions of QTLs linked to

foliage maturity. Zhang (2004) also suggested pleiotropic effects from both traits.

Another important aspect for future development of resistant cultivars is an understanding of plant defence mechanisms and their interactions with the pathogen (Zhang *et al.*, 2013). Plants have evolved different physical and chemical barriers as well as inducible defence mechanisms that prevent various pathogen invasions. During pathogen infection plants activate complex signalling networks where plant hormones play a crucial role. Plant hormones such as salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) are considered as the backbone of this network but other hormones like abscisic acid (ABA), auxins and gibberellins (GA) are also involved in defence signalling networks (Novakova *et al.*, 2014; Pieterse *et al.*, 2012). These complex signalling cascades and their activation are dependent on the type of the invading pathogen. Plant-pathogenic fungi can be biotrophs, necrotrophs or hemi-biotrophs. Biotrophs are pathogens which create a long-term feeding relationship with the host plant without killing it. Necrotrophs are pathogens that kill the living host cells and feed on the dead matter. Other fungi that were previously believed to be necrotrophs are in fact hemi-biotrophs, due to a biotrophic stage in beginning of the infection process (Oliver & Solomon, 2010).

Salicylic acid (SA) and jasmonic acid (JA)

The phenolic compound salicylic acid (SA) is a plant hormone that is important both in abiotic and biotic stress responses. Several studies indicate that SA regulates and facilitates plant responses to abiotic stresses like heat (Larkindale & Knight, 2002) heavy metals (Metwally *et al.*, 2003), drought (Chini *et al.*, 2004) and osmotic stress (Borsani *et al.*, 2001). However, the majority of studies have focused on its role in plant defence responses to biotic stress. SA plays an important role in the induction of plant defence against biotrophic and hemi-biotrophic pathogens and is also involved in the formation of systemic acquired resistance (SAR) that forms broad-spectrum resistance after pathogen invasion (Fu & Dong, 2013; Wang *et al.*, 2006).

Mutants of *Arabidopsis thaliana*, that are compromised in the production of SA (e.g. enhanced disease susceptibility1 (eds1), phytoalexin deficient 4 (pad4) and SA induction deficient 2 (sid2)) as well as transgenic lines (NahG) that are unable to accumulate SA, showed higher susceptibility to biotrophic and hemi-biotrophic pathogens (Kunkel & Brooks, 2002). Although, SA activates defence responses against biotrophic or hemibiotrophic pathogens, there are reports also indicating the importance of SA signalling in response to necrotrophic pathogens (Novakova *et al.*, 2014; Jia *et al.*, 2013; Wang *et al.*, 2012). Novakova *et al* (2014) found that levels of SA and expression of SA

marker genes incresed in *Brassica napus* infected with the necrotrophic fungus *Sclerotinia sclerotiorum.* This result is in accordance with results of Jia *et al* (2013), which showed that tomato NahG transgenic lines exhibit susceptibility to *Alternaria alternata.*

In contrast to SA, JA is involved in different processes like leaf senescence, germination, tuber formation and fruit ripening. It plays a crucial role in defence responses and previous works showed that JA is involved in host resistance against various necrotrophic fungi (Jia *et al.*, 2013; Thaler *et al.*, 2004; Stintzi *et al.*, 2001; Penninckx *et al.*, 1998). For example, the JA mutant fad3/fad7/fad8 showed an increased susceptibility to *Alternaria brassicicola* (Stintzi *et al.*, 2001) and the *Arabidopsis* JA signalling mutant coronatine insensitive1 (COI1) that causes JA insensitivity, exhibited an increased susceptibility to the necrotrophic fungi *Botrytis cinerea, Alternaria brassicicola, Fusarium oxysporum* and *Plectosphaerella* (Thatcher *et al.*, 2009; Thomma *et al.*, 1998). It has been reported in a number of studies that levels of JA increased locally during tissue damage or pathogen penetration as well as exogenous application of JA induced defence related genes (Wasternack, 2007; Lorenzo & Solano, 2005).

Many studies have indicated that SA and JA defence pathways are mostly antagonistic, however, some reports show evidence of synergistic interactions between these two hormones (Beckers & Spoel, 2006; Kunkel & Brooks, 2002; Schenk *et al.*, 2000). It is suggested that activation of the defence signalling pathways is dependent on the nature of the pathogen. However, lifestyles of plant pathogens are not properly classified and many pathogens are not purely biotrophic or necrotrophic. Therefore, the cross talk between SA and JA/ET pathways may be adjusted to each specific pathogen (Adie *et al.*, 2007).

3 Aims and objectives

The main objective of this thesis is to improve the knowledge basis of early blight in potato, which could be implemented in future development of resistant cultivars. Therefore, disease resistance/susceptibility in different potato genotypes was quantified and plant defence signalling with main emphasis on hormones was studied. A further aim was to study the genetic diversity of *Alternaria solani* populations and to investigate the occurrence of the F129L substitution, which is associated with reduced sensitivity to QoI fungicides. The more specific objectives of this study were:

- To establish a reliable greenhouse screening method for identification of resistant/susceptible potato clones that could speed up the breeding process (Paper I).

- To characterize the segregation of resistance to early blight among the progenies of a cross between resistant and susceptible potato cultivars both in leaves and tubers (Paper II).

- To elucidate the roles of SA and JA hormone signalling pathways in potato defence responses to *A. solani* (Paper III).

- To characterize genetic variation within and among populations of *A. solani* from two potato growing regions in southeast Sweden and to examine the occurrence of F129L substitutions among isolates (Paper IV).

- To determine changes in the occurrence of the F129L substitution and genetic shifts in *A. solani* populations within a field during one growing season as response to two different fungicide strategies (Paper V).

4 Evaluating resistance in potato germplasm and understanding SA and JA signalling in responses to early blight

One of the most important issues for early blight resistance breeding in potato is developing efficient screening methods. Field screening has been recognized as the standard procedure for achieving reliable information. However, there are many uncontrollable environmental conditions like temperature, humidity, soil and pathogen inoculum that can affect the result of field screening. Therefore, a reliable greenhouse screening method for early blight resistance in potato was developed and the results were compared with the data from field experiments (Paper I). Resistant and susceptible potato clones were identified. Among them resistant and susceptible genotypes were selected as parents and were crossed. The progeny of the cross were evaluated both for leaf and tuber resistance (Paper II).

It is also important to understand the molecular nature of defence responses to plant pathogen infection, since that can give valuable information for future resistance breeding. Therefore the roles of salicylic acid (SA) and jasmonic acid (JA) hormone signalling in defence responses to *A. solani* were investigated. Pathogenicity assays, quantification of fungal biomass, hormone level analysis and time course microarray analysis were performed (Paper III).

4.1 Materials and methods

Plant materials (Paper I, II & III)

Thirty-four potato genotypes, including cultivars and breeding clones were used in the experiments (Table 1, Paper I). The varieties were obtained from different companies in Sweden and the breeding clones were provided by the potato breeding program at SLU, Alnarp. For Paper II tubers from 80 progeny were used and for Paper III the cultivar Desiree (wild type), two SA deficient

transgenic NahG clones (A, D2) and two JA insensitive coi1 mutants (H1, X5) were used (Halim *et al.*, 2009; Halim *et al.*, 2004).

Inoculum preparation and greenhouse experiments (Paper I)

Isolates of *A. solani* were obtained from naturally infected potato plants in Sweden. The fungus was grown on potato dextrose agar (PDA) with reduced strength (1/5 strength of PDA) and in order to induce sporulation the cultures were treated with UV light. After incubation, conidia were harvested by washing the agar surface with ddH_2O. Spore suspensions at a concentration of 10^4 conidia mL^{-1} were used for inoculations in all greenhouse experiments.

Greenhouse experiments were carried out in 2010 and 2011. Two different methods, detached leaf and intact plant inoculation method were used to investigate early blight resistance. In the intact plant inoculation method resistance in leaves at different positions of the plant (lower, middle and upper) was investigated. The experiments were performed in three randomized complete blocks and repeated twice. The details of inoculum preparation and greenhouse experiments are described in Paper I.

For Paper III the plants were grown in tissue culture in a phytochamber for three weeks and were then transferred to a greenhouse chamber. The experimental conditions for plant growth are described in Paper III. 15 µL drops of conidial suspensions (2×10^4 conidia mL^{-1}) were placed on the surface of 10 randomly chosen middle leaflets. Mock-treated plants were inoculated with ddH2O. The experiment was performed in three randomized complete blocks and four leaflets per plant were collected at 0, 24, 72 and 120 h after inoculation. Disease development was measured as diameter of the lesions ten days after inoculation.

Field and tuber test experiments (Paper I & II)

Field experiments were performed in 2011, 2012 (Paper I) and 2014 (Paper II) at the Swedish Rural Economy and Agricultural Societies experimental farm in Kristianstad, Sweden. The field sites were chosen particularly for their natural sources of *A. solani*. The fields were fertilized and treated with insecticides and fungicides against other pathogens according to standard practice for that area. Tubers were harvested from the field experiments in 2012 (Paper I) and 2014 (Paper II). Harvested tubers were kept in storage at the $4^{\circ}C$ with a relative humidity of 70-75%. After storage tubers were inoculated and tuber lesion diameter and lesion depth were measured. The details of field and tuber test experiments are described in Paper I and II.

Fungal biomass and hormone measurements (Paper III)

Five days after inoculation, four leaves per plants were sampled and powdered. Genomic DNA was extracted and the concentration was adjusted to 50 ng/µl.

For qPCR *A. solani* species-specific primer pairs were used (Gannibal *et al.*, 2014). Plant hormones, such as jasmonic acid (JA), abscisic acid (ABA) and auxin (IAA), were measured as previously described (Ziegler *et al.*, 2014) and quantification of SA was done according to Verberne *et al,* (2002). All the details are described in Paper III.

Microarray analysis (Paper III)
RNA was extracted from 4 leaflets per plant using Qiagen RNeasy mini kits (Qiagen, Hilden, Germany). RNA concentration and purity was checked by a NanoDrop analysis (Wilmington, USA), and Experion™ Automated Electrophoresis System (Bio-Rad Laboratories, Hercules, USA) was applied to determine the integrity of the samples. Expression analysis of mRNA was performed by applying a custom-made expression array (Agilent JHI *Solanum tuberosum* 60 k v1) and analysed using the predicted transcripts in the *Solanum phureja* genome (version 3.4). All the statistical details of the microarray analysis are described in Paper III.

Data analysis (Paper I & II)
Differences in lesion sizes were analysed with ANOVA (PROC GLM). Mean comparisons were performed using Tukey's test and Pearson's correlation coefficient was used to analyse correlations between different parameters. For field experiment data relative area under the disease progress curve (rAUDPC) and area under the defoliation development curve were calculated.

4.2 Results and Discussion

Plant and tissue age are typical factors important for the degree of infection by *Alternaria* species. Several studies have reported that older leaves are more susceptible compared to young leaves (Green & Bailey, 2000; Hong & Fitt, 1995; Aveling *et al.*, 1994). Similar results were obtained in our study where lower/older leaves had larger lesions than upper/younger leaves. We did not find any correlation in lesion size between lower and upper leaves. However, there was a significant correlation (Paper I, Figure 1) in lesion size between lower and middle as well as between middle and upper leaves. These findings suggest that the middle leaves are most suitable for resistance screening of potato in greenhouse.

Another main objective for this part of the project was to find the most reliable greenhouse screening method for *A.solani* resistance. Results from our data showed a significant correlation between the field results and inoculated intact plants in greenhouse experiments (Paper I, Figure 2). Therefore, we can conclude that the intact plant inoculation method is reliable for screening

potato cultivars for early blight resistance. However, the results from field experiments and inoculated intact greenhouse plants had poor correlation with the results from inoculated detached leaves, indicating that the detached leaf method is not an accurate screening method for evaluation of potato material for resistance to early blight.

Growing cultivars with a high level of resistance is important for integrated disease control and management of all crops. Potato cultivars having an efficient high level of resistance to early blight would be the best management practice giving the low cost and low risk for the environment. However, early blight disease resistant cultivars are not available yet. Therefore, we screened potato cultivars and breeding clones in order to obtain information about the level of resistance/susceptibility to early blight. Our results revealed significant differences in resistance to early blight among cultivars/clones in both intact plant and field experiments (Paper I, Table 2, Table 3). Many of the cultivars/clones from the intact plant inoculation and field experiment showed approximately the same level of infection and were considered moderately resistant. However, we also found that some cultivars/clones revealed higher level of resistance or susceptibility. In the field experiments we investigated relative area under the disease progress curve (rAUDPC) in addition to the relative area under the defoliation progress curve. In both years, there was a positive correlation between degree of infection (rAUDPC) and defoliation (Paper I, Figure 4), which is in accordance with Foolad (2000).

In paper II we investigated the levels of resistance to early blight in the progeny population of a cross between tetraploid potato cultivars. The parents were chosen based on their level of resistance (Magnum Bonum as resistant and Matilda as susceptible) (Paper I). There were significant differences in resistance among the progeny clones. Some clones exhibited higher levels of resistance whilst the others showed susceptibility (Paper II, Figure 1). The normal distribution of resistance indicates a typical quantitatively inherited trait (Paper II, Figure 2A, 2B).

In addition to leaf resistance we also evaluated tuber resistance (Paper II). We found significant differences in tuber resistance among the progeny clones using both lesion diameter and lesion depth measurements. A positive correlation was found between these two measurements, indicating that both could be used for evaluation of early blight resistance in tubers (Paper II, Figure 3). However, to perform a more accurate assessment the volume of the infected area was calculated based on both lesion diameter and depth. Based on the volume of infected area we found significant differences among the progeny clones of the crossing population (Paper II, Figure1). The degree of tuber resistance did not show the same pattern as resistance in the leaves since no correlation between resistance in leaves and tubers was found. For example,

Magnum Bonum, which is considered as a resistant cultivar based on the leaf resistance, was one of the most susceptible cultivars when assessing tuber infection. This indicates that leaf and tuber resistance may be determined by different genes. However, some clones showed good resistance both in leaves and tubers (Paper II, Figure 1) and they might be used as potential candidates in breeding programs.

Results from this study (Paper III) revealed that salicylic acid (SA) has an important role in symptom development after *A. solani* infection since inoculated NahG plants had significantly larger lesions than the inoculated wild type (cv. Desiree) and JA insensitive coi1 plants 10 days post inoculation (dpi) (Paper III, Figures 1A, 1B). This role of SA in symptom development was further confirmed by SA level quantification in inoculated plants. While SA was induced in all other inoculated plant types, it was not induced in inoculated NahG plants (Paper III, Figure 2,). SA also appears to restrict fungal growth since inoculated NahG plants had a significantly higher fungal biomass 5 dpi compared to inoculated wild type (cv. Desiree) plants (Paper III, Figure 3). These results indicate that SA induction is important, allowing potato to mount an effective defense response against *A. solani.* In contrast, there were no significant differences in lesion size between inoculated jasmonic acid insensitive coi1 plants and inoculated wild type plants (cv. Desiree) 10 dpi (Paper III, Figure 1). Surprisingly, fungal biomass quantification 5 dpi showed that inoculated coi1 plants had a significantly higher fungal biomass than inoculated wild type plants (cv. Desiree) (Paper III, Figure 3), suggesting that also coi1 is involved in restricting *A. solani* growth, although it did not seem to have a role in restricting symptom development after inoculation. However, this difference in fungal biomass was not dependent on jasmonic acid (JA) levels, as there was no difference in JA levels, since inoculated coi1 and wild type plants (Paper III, Figure 4A, 4B and 4C). In fact, JA was induced in all inoculated plants, suggesting that JA production is part of the general defense response to *A. solani*. Furthermore, quantification of hormones such as abscisic acid (ABA) and auxin (IAA) revealed that they are not directly involved in defense response to *A. solani* as they were not induced after pathogen inoculation (Paper III, Figure 5).

Transcriptomic analysis also showed that rapid (i.e. within 24 hpi) defence responses to *A. solani* inoculation are characterized by SA and COI1 dependent defence activation. A number of SA and COI dependent transcripts (2134) related to wound, immune and oxidative stress responses were differentially expressed in inoculated wild type (cv. Desiree) plants, while no change was detected in inoculated NahG and coi1 plants (Paper III, Figure 6A-6D). Thus, rapid activation of SA and COI1 dependent gene expression is important for resistance to *A. solani* (Paper III, Figure 6, Table 1). Response at later stages

(i.e. 72 and 120 hpi) were characterized by a larger overlap in transcripts that were either induced or repressed in inoculated wild type (cv. Desiree), NahG and coi1 plants (Paper III, Figure 7 and 8), suggesting that responses at later time points are not dependent on SA and COI1 signalling. In fact, further analysis revealed that defence responses at 72 and 120 hpi are dominated by transcripts related to jasmonic acid (JA) and ethylene signalling (Paper III, Figure 7 and 8, Table 1). Furthermore, transcripts related to cyclopentenone responses and auxin repression were observed only in inoculated NahG and coi1 plants 72 and 120 hpi. Therefore, while JA/Ethylene signalling dominates responses at later time points in all inoculated plants, enhanced susceptibility in inoculated NahG and coi1 plants, as observed from lesion size measurements and fungal biomass quantification, could also be attributed to transcripts related to cyclopentenone response and auxin repression (Paper III, Table 1).

In conclusion, SA hormone levels and rapid SA dependent signalling is necessary to restrict fungal growth and symptom development after *A. solani* inoculation. In contrast, JA hormone levels/signalling and ethylene signalling contributes to a general response to *A. solani* during later stage of infection. Rapid and early response is also characterized by COI1 dependent defence signalling. However, COI1 does not seem to influence symptom development. Finally, in addition to the absence of functional rapid NahG/COI1 dependent defence signalling, transcriptional activity related to cyclopentenone response and auxin repression at later time points during infection may also contribute to enhanced susceptibility in inoculated NahG and coi1 plants.

5 Genetic variability and occurrence of the F129L substitution in the *Alternaria solani* population in Southeast Sweden

An understanding of the genetic variability in plant pathogen populations is important for disease management strategies. It helps us to better understand the epidemiology, host pathogen co-evolution and also the potential for change in the pathogen population. Therefore, the genetic diversity of populations of *A. solani* from two potato-growing regions of southeast Sweden was studied. The collected isolates were also examined for substitutions in the gene encoding cytochrome *b* associated with resistance to Qol fungicides (Paper IV, & V). In addition, the occurrence of the F129L substitution and general genetic shifts in the *A. solani* population within a field in response to two different fungicide application strategies over a single growing season was investigated (Paper V).

5.1 Materials and methods

Collection, isolation and identification of fungal cultures (Paper IV & V)
Leaflets with symptoms were collected from potato fields in two main regions i.e. Kalmar/Öland (two fields) and Kristianstad (three fields) in 2011 (Paper IV, Figure 1) and in Kristianstad (one field) in 2014 (Paper V). In total, 55 isolates of *A. solani* and 19 isolates of *A. alternata* were investigated in Paper IV and 143 isolates of *A. solani* were studied in Paper V.

Small sections from the edge of typical leaf lesions were cut, washed, and placed on water agar containing antibiotics and incubated for 3-4 days. Single conidia microscopically harvested and placed on new PDA for germination. Morphological identification of the species was done based on Simmons, (2007) and identifications of *A. solani* (Edin, 2012) and *A. alternata* (Vega & Dewdney, 2014) were verified with PCR based methods using species specific

primers. Species specific primers for *A. tomatophila* were also used (Gannibal *et al.*, 2014). The details of these procedures are described in Paper IV & V.

Field experiment (Paper V)

The field experiment was carried out in 2014. The location and growing conditions are described in section 4.1. Two different fungicide treatments were compared with an untreated control. In treatment 1 a spraying regime comprising two application of Revus top (active ingredients: Mandipropamid and Difenokonazol) followed by four applications of Signum (active ingredients: boscalid and pyraclostrobin) was used. In treatment 2 Amistar (active ingredient: azoxystrobin) was applied twice. All treatments were performed based on the manufacturer's recommendations.

DNA extraction and AFLP analysis (Paper IV & V)

For DNA extraction, isolates were grown in liquid medium according to Zur *et al*, (2002). DNA extraction was performed using the Genomic DNA Purification Kit (Fermentas Lithuania). The AFLP analysis was carried out using the AFLP Microbial Fingerprinting Kit (Applied Biosystems, CA USA) according to the manufacturer's protocol. Seven primer combinations in Paper IV and six primer combinations in Paper V were used for selective amplification. The amplified products were analyzed on ABI 3730 capillary DNA analyzers (Applied Biosystems) and the results were analyzed using Genemarker (Softgenetics®, PA, USA). All the details are described in Paper IV & V.

Cytochrome b mutations (Paper IV & V)

Detection of substitutions in the gene encoding cytochrome *b* for the GI genotype was conducted according to Edin (2012) and for the GII genotype the analysis was based on Pasche *et al*. (2005). For analysis of *A. alternata* an adopted method developed in citrus was applied (Vega & Dewdney, 2014).

Data analysis

The AFLP data were analysed with POPGENE software version 1.32 to calculate Shannon's information index (I) and Nei's gene diversity (H). Cluster analysis and principal coordinates analysis (PCoA) were carried out according to Nei and Li (1979). Similarity coefficients were calculated using NTSYS pc 2.2 statistical packages. For statistical support of dendrogram branches in the cluster analysis the FreeTree software was applied. Analysis of molecular variance (AMOVA) was conducted according to Excoffier et al. (1992) using Arlequin 3.0. STRUCTURE software version 2.3.4, STRUCTURE HARVESTER and DISTRACT version 1.1 were used to visualize the population structure.

5.2 Results and Discussion

A lower level of diversity was observed among *A. solani* isolates in Kristianstad compared to isolates in the Kalmar region (Paper IV, Table 4). A similar level of gene diversity was also found in Kristianstad in Paper V. The same level of diversity among *A. alternata* isolates was found in Sweden and in Tajikistan (Paper IV, Table 4). However, cluster analysis indicted that the *A. alternata* populations from Sweden and Tajikistan are genetically different from each other (Paper IV, Figure 2). In addition, the AFLP analysis showed a clear differentiation between the two species, which is in agreement with Perez Martinez *et al*. (2004). Two distinct sub-clusters of *A. solani* isolates, supported with high bootstrap value, were found. Most of the isolates which represented the island in the Kalmar location deviated from the other isolates, suggesting that part of the population at this location is genetically different (Paper IV, Figure 2). In general, we found a significant genetic differentiation (Paper IV, Table 5) among the *A. solani* populations from different locations. Both studies showed that the within population variation was higher than among population variation (Paper IV, Table 5, Paper V, Table 3), suggesting gene flow, probably caused by movements of infected host material.

The occurrence of F129L and G143A substitutions among the isolates was also evaluated. Results from Paper IV (Table 1) and Paper V (Table 1) confirm the presence of both genotypes (Genotype I, Genotype II) in the *A. solani* population. The majority of Swedish GII isolates carried the F129L substitution but the substitution was not detected in GI isolates. These results are in accordance with the results of Leiminger *et al*. (2014). None of the *A. alternata* isolates from Tajikistan carried the G143A substitution while it was detected in most of Swedish isolates, which may indicate that the G143A substitutions could be present also in other parts of the country. However, further investigations are needed to confirm that. In addition a field experiment to determine the occurrence of F129L substitution as well as general genetic changes in the *A. solani* population over a season after applying two different fungicide strategies was carried out (Paper V). Only two GI isolates (wild type) were found in the starting population before fungicide applications, whereas the remaining isolates were GII and all of them carried the F129L substitution. Therefore, we were not able to examine changes in frequency of F129L in the *A. solani* population over the season.

This study indicated that the F129L substitution in *A. solani* also occurs in Sweden and results from field data (Paper V, Figure 1) showed a low efficacy of azoxystrobin. The same tendency was found in tuber yield (Paper V, Table 2) where treatment with azoxystrobin alone gave significantly lower yield compared to plots treated with difenokonazol, boscalid and pyraclostrobin.

Moreover, results from the sensitivity test demonstrated that isolates with the F129L substitution were less sensitive to azoxystrobin (Paper IV, Table 6). Altogether, these results suggest a low efficacy of the fungicide due to a strong influence of the F129L substitution. In Sweden, the substitutions generally associated with fungicide resistance are still found at lower frequencies in most areas (Edin & Andersson, 2014) but we hypothesise that the frequency of these substitutions will rapidly increase as the frequency of QoI fungicides applications increase.

The results of population structure analysis and PCoA (Paper V, Figure 4, 6) based on AFLP data confirmed the existence of two groups with a mixed pattern of isolates. Overall, the population of isolates at the last time point (Paper V, Figure 6A, B, C) had a lower diversity index, indicating that changes occurred in the population over time, mostly at the end of the season. Interestingly, isolates from treatment 2 (Amistar) showed a different pattern at the last time point with lower genetic diversity index and the isolates belonged to one cluster only (Paper V, Figure 6C). This indicates that using a single fungicide (azoxystrobin) may result in changes within the population of *A. solani* during a cropping season. However, further research is needed to confirm this hypothesis.

6 Conclusions

- This study supports the use of the intact plant screening method for *A. solani* resistance screening in greenhouse tests, since high correlation with results from field experiments was found. Detached leaf assays are not reliable. In addition, inoculating leaves from the middle part of the plant is more accurate than using lower or upper leaves.

- The present work reports differences in resistance response to early blight among potato genotypes both in leaves and tubers. However, there was no correlation between resistance in leaves and tubers. Some of the potato clones might be valuable candidates for future resistance breeding as they showed both foliar and tuber resistance.

- Hormone and microarray analysis revealed that symptom development after *A. solani* infection is dependent on SA levels and quick induction of SA-defence transcripts is needed to restrict infection.

- JA plays a significant role during infection as JA levels are induced after pathogen invasion. Moreover, JA dependent COI1 defence signalling seems to be necessary to restrict fungal growth during the early stages of infection.

- Results from transcriptomic data indicate that host resistance to *A. solani* is dependent on induction of both SA and COI1 dependent defence signalling at early stages of infection, whereas defence response at later stages is dominated by JA/ethylene signalling.
- Relatively high genetic diversity and a significant genetic differentiation among populations of *A. solani* from different locations in the southeast of Sweden were found.

- The presence of F129L substitutions in Swedish *A. solani* populations was confirmed. Two mitochondrial genotypes, GI and GII, were found and the F129L substitution was only identified in GII isolates. In some fields almost all isolates had the F129L substitution. Results of sensitivity tests revealed that isolates containing the F129L substitution were less sensitive to azoxystrobin.

- Our results from the field experiments clearly showed that a significant control of *A. solani* was not achievable using azoxystrobin alone. Therefore we can conclude that the presence, and sometimes domination, of isolates with F129L substitution within pathogen populations may have a strong influence on the efficacy of this fungicide.

7 Future perspectives

Results from a field trial indicated that population changes occurred within a season as a response to different fungicide treatments. However, further research is needed to confirm it. At present time, early blight control is dependent on fungicide applications. However, they have undesired environmental consequences and there is an increasing problem with development of fungicide resistance in the pathogen population. Therefore there is a need for cultivars with improved resistance and IPM strategies that involve optimized cultural practises, minimized use of fungicides and perhaps the use of elicitors or plant strengtheners that further improve plant defence.

To efficiently be able to breed for early blight resistance markers for maturity type independent resistance genes need to be developed. The present crossing population, preferably extended with a higher number of clones or combined with other populations, could be used for QTL mapping. The maturity type of all clones should also be phenotyped. This in combination with expression analysis and bioinformatics techniques could facilitate the detection of good candidate resistance genes that are independent of the maturity type.

There is also a need to develop sustainable IPM strategies for controlling early blight. To minimize environmental effects and the risk of fungicide resistance, fungicides have to be used with care. Greenhouse and field experiments involving combinations between cultural practices, partially resistant cultivars and combinations/alterations of fungicides with different modes of action should be carried out. In this context it would also be interesting to investigate the effect of elicitors and plant strengtheners that further may improve plant defence and if they can be combined with fungicides. Preliminary greenhouse results (not shown in this thesis) indicate that silicates may reduce infections by *A. solani* and this should be further investigated also under field conditions.

Another important aspect is to determine the levels of genetic variation of *A. solani* populations covering a wider geographic range of Sweden in order to get a better understanding of the population structure and migration patterns. It is also of great importance to carefully monitor the occurrence of the F129L substitution in a broader context as well as to determine isolate's sensitivity to QoI fungicides and changes over time.

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