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A tale of two blights

Studies of the interactions between *Solanum tuberosum*, *Alternaria solani*, and *Phytophthora infestans*

Sophie Brouwer

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A tale of two blights: Studies of the interactions between *Solanum tuberosum*, *Alternaria solani,* and *Phytophthora infestans*

Abstract

Potato is the third most important food crop for human consumption. However, the production is plagued by several pests and pathogens causing yield reducing diseases. Estimations indicate that 17% of the global potato yield is lost due to pests and pathogens. Considering the predicted growth of the human population and the already worrying prevalence of hunger, attenuation of these yield losses attributed to pests and pathogens is required. The major causal agents of disease in potato are the oomycete *Phytophthora infestans* and the fungus *Alternaria solani,* causing late blight and early blight respectively. The effective control of these two blights is achieved predominately by synthetic chemical fungicide treatments. However, even though currently effective, this reliance on chemical control is unsustainable and new knowledge is required for the development of future-proof control that takes the impact on the environment, human health and ecosystem dynamics into account. In this thesis, I studied the interactions between *A. solani, P. infestans,* and *Solanum tuberosum.* The main aim was to gain an increased understanding of the interactions between a single host plant and multiple pathogens, either alone or together. Using infection and transcriptomic studies of plant hormone deficient lines, I found that potato defences require intact salicylic acid signalling to limit *A. solani* infection. Additionally, I analysed the gene expression of both potato and *A. solani* during infection in more detail. Moreover, I analysed the spatial distribution of chemical elements in plants that were either susceptible or resistant to *P. infestans* and found several resistance specific redistribution patterns after inoculation with *P. infestans*. Finally, studies on the interactions between all three organisms, showed that the growth of *P. infestans* is limited in the presence of *A. solani* both *in vitro* and *in planta*. This holds true both in a controlled environment and in an agriculturally relevant setting. Overall, the work in this thesis has generated a deeper understanding of the interactions between potato and the two pathogens that cause the most significant losses in this crop. Furthermore, it highlights the importance of studying plant-pathogen interaction not solely as binary interactions and indicates potential new leads for the development of more sustainable control strategies.

Keywords: Solanum tuberosum, potato, *Phytophthora infestans*, late blight, *Alternaria solani*, early blight, ionomics, co-infection, tripartite interactions

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Dedication

In loving memory of my father,
who truly enjoyed having boiled potatoes with gravy for dinner

"It is a mistake to think you can solve any major problems just with potatoes."
Douglas Adams

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 Brouwer, S.M.*, Odilbekov, F.*, Burra, D.D., Lenman, M., Hedley, P.E., Grenville-Briggs, L., Alexandersson, E., Liljeroth, E. & Andreasson, E ¤. (2020). Intact salicylic acid signalling is required for potato defence against the necrotrophic fungus *Alternaria solani*. Plant Molecular Biology, vol. 104 (1), pp. 1–19

II Brouwer, S.M.¤, Lindqvist-Reis, P., Pergament Persson, D., Marttila, S., Grenville-Briggs, L.J., and Andreasson, E. Visualizing the ionome in resistant and susceptible plant-pathogen interactions (submitted)

III Brouwer, S.M.¤, Wolters, P.J., Liljeroth, E., Vleeshouwers, V.G.A.A., and Grenville-Briggs, L.J. Double trouble or a blessing in disguise? Co-infection of potato with the causal agents of late and early blight (manuscript)

IV Brouwer, S.M.¤, Brus-Skalej, M., Saripella, G.V., Liang, D., Liljeroth, E., and Grenville-Briggs, L.J. Transcriptome analysis of potato infected with the necrotrophic pathogen *Alternaria solani* (manuscript)

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¤ Corresponding author

The contribution of Sophie Brouwer to the papers included in this thesis was as follows:

I Contributed to parts of the study design, performed part of the laboratory experiments, analysed the data and wrote the final version of the manuscript with input of the co-authors.

II Designed the study together with co-authors. Planned and performed the sample preparations, co-responsible for μXRF data acquisition, analysed the data and wrote the manuscript with input of the co-authors.

III Designed the study together with co-authors. Planned and performed the experimental work together with co-authors. Analysed the data and wrote the manuscript with input of the co-authors.

IV Designed the study together with co-authors. Planned and performed the laboratory experiments. Analysed the data together with co-authors and wrote the manuscript with input of the co-authors.

List of figures

All illustrations, photographs and micrographs were created or captured by Sophie Brouwer, unless specifically mentioned otherwise.

Abbreviations

BF	Bright field
CCD	Charged Coupled Device
cDNA	complementary DNA
CLSM	Confocal Laser Scanning Microscopy
DAB	3,3'-Diaminobenzidine
DAMP	Damage Associated Molecular Pattern
DEG	Differentially Expressed Gene
DET	Differentially Expressed Transcript
DLA	Detached Leaf/Leaflet Assay
eGFP	enhanced Green Fluorescent Protein
ETI	Effector Triggered Immunity
ExIP	Extracellular Immunogenic Pattern
ExTI	Extracellular Triggered Immunity
ICP-OES	Inductively Coupled Plasma Optical Emission Spectrometry
InIP	Intracellular Immunogenic Pattern
InTI	Intracellular Triggered Immunity
JA	Jasmonic Acid
MAMP	Microbe Associated Molecular Pattern

NGS	Next Generation Sequencing
NLR	Nucleotide-binding Leucine-rich repeat Receptor
PAMP	Pathogen Associated Molecular Pattern
PMT	Photo Multiplier Tube
PR	Pathogenesis Related
PRR	PAMP Recognition Receptor
PTI	PAMP Triggered Immunity
QoI	Quinone outside Inhibitor
qPCR	quantitative Polymerase Chain Reaction
QTL	Quantitative Trait Locus
R-gene	Resistance gene
RH	Relative Humidity
RNAi	RNA interference
SA	Salicylic Acid
XRF	X-Ray Fluorescence

1. Introduction

"One cannot think well, love well, sleep well, if one has not dined well." [1]

Even though food production worldwide provides more than enough calories to feed the current world population (Holt-Gimémez et al., 2012), the sad truth remains that currently, 8.9% of the world population suffers from hunger with huge inequalities in the prevalence of hunger between world regions (FAO et al., 2019). In a recent report on the prospects of reaching the Sustainable Development Goal: Zero Hunger set for 2030, the UN reported that the outlook is bleak and an increase instead of a decrease of undernourishment is expected based on the current trends (FAO et al., 2020). The prevalence of hunger when enough food is produced is paradoxical, to say the least. Thus, tremendous efforts are required to ensure fairer redistributions of food and to curtail food waste.

However, food redistribution and a reduction of food waste alone are not enough to ensure future-proof sustainable food systems. The world population is predicted to increase to 10.9 billion by 2100 (United Nations Populations Division, 2019), thus increased production of food is also required. However, this increase brings an extra challenge, since it should be achieved without increasing land use for production. Food production currently takes up nearly 40% of the Earth's landmass, expansion of this percentage would lead to increased harm to ecosystems, loss of biodiversity and release of stored carbon, that should be avoided (World Resources Institute, 2019).

[1] Virginia Woolf, A Room of One's Own (1929)

Additionally, as the emergence of SARS-CoV-2 causing COVID-19 has made very evident for humans, new diseases can emerge quickly, and seemingly, suddenly. Plants, just like humans, are vulnerable to diseases and plagues caused by pests and pathogens. A plethora of plant pathogens are already known and require extensive control practices to mitigate their negative effects. However, current control strategies are often unsustainable and rely heavily on agrochemicals. Additionally, a changing climate, evolution of the pathogens and altered regulations regarding the use of agrochemicals, present extra risks for the occurrence and/or emergence of resistant or more aggressive pathogens, as well as the migration of pathogens to new habitats.

Ongoing research is required to find new ways to attenuate the risk for pathogen-induced crop losses as evidenced both by famous examples from history, e.g. the Great Irish Famine, and more current examples, e.g. the emergence of *Puccinia graminis* f. sp. *tritici* race Ug99, a race of the wheat stem rust-causing fungus to which 90% of the wheat cultivars grown were susceptible when this race first emerged (Singh et al., 2011).

In order to feed the growing world population, without increasing the land required for production, and with sustainable protection from disease, crop plants with high yield and nutritional value are essential. Potato is an excellent example of such a food crop. It is already a staple food crop with a high yield potential, high nutritional value and is adapted to a relatively wide range of environments. However, to ensure sustainable potato production in the future, adaption and new control strategies to mitigate the impact of abiotic and biotic stresses are required (Birch et al., 2012).

This study focuses on gaining a better understanding of the interactions of potato with two pathogens that lead to major crop losses, if uncontrolled.

2. Background

2.1 *Solanum tuberosum*

2.1.1 Origin and production

Potato, *Solanum tuberosum*, is a cultivated plant species belonging to the nightshade, *Solanaceae*, family that produces starchy tubers. The potato resulted from a single domestication in the Andes region of South America up to 10000 years ago (Spooner et al., 2005; Hardigan et al., 2017). During the 16th century the potato travelled to other continents, arriving on the Canary Islands and was subsequently brought to Spain from where it spread all over Europe and beyond to Africa, Asia and North America. Due to rumours about dangers of potato consumption aplenty, the potato was not immediately accepted by everyone as the new 'superfood'. However, due to famines, a growing population, efforts such as those by King Frederick II of Prussia, nicknamed 'Der Kartoffelkönig' (the potato king), who ingeniously triggered interest in the potato by ordering soldiers to guard potato fields, the use of potato as a food crop steadily increased (Komlos, 1998, Niemann, 2012).

By the 19th century, the potato became an important food crop, especially for the lower-income classes. By the mid-19th century, for example, the livelihood of three million people in Ireland depended on the potato (Komlos, 1998). Thanks to a naturally high nutritional value, the introduction of the potato into Europe is credited with about one-fourth of the increase in population and urbanization occurring during the 18th and 19th century (Nunn and Qian, 2011). The potato has since become a staple food crop all over the

world, with over 368 million tonnes produced worldwide in 2018 (Figure 1A) (data source: FAOSTAT, 2020). The current largest producers of potato are China, India and the Russian Federation (Figure 1B). However, as for all food crop productions, the production of potato is not without problems. The production is plagued by several diseases that require control and management to mitigate yield losses.

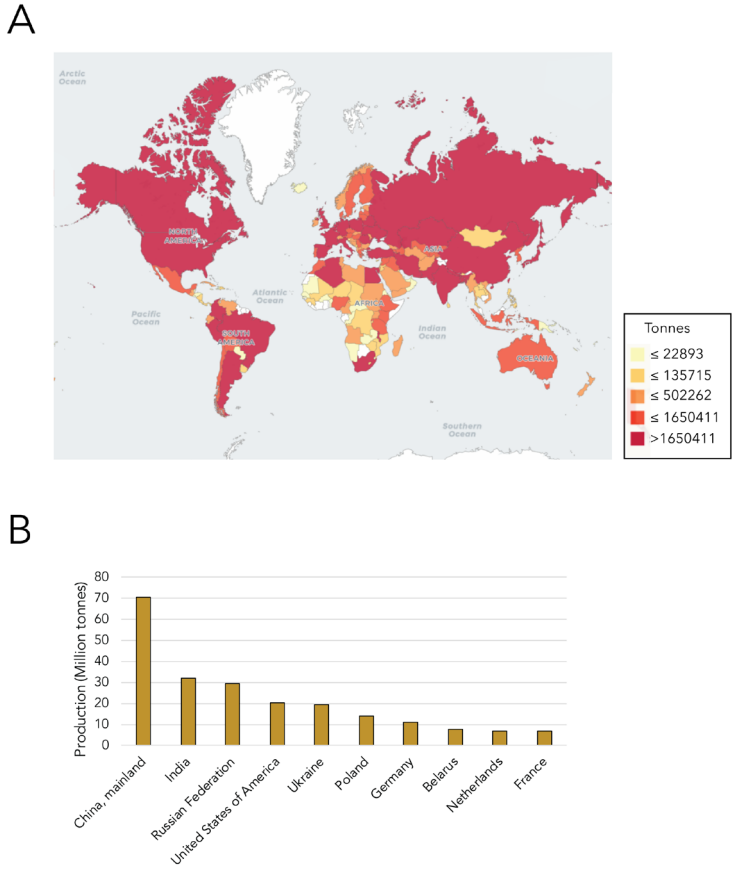

Figure 1 potato production worldwide (A) and the top 10 producers of potato over a period from 1994-2018 (B) (source: FAOSTAT, 2020)

2.1.2 Plant morphology and desired commercial product

In its native form, the potato plant is a perennial plant. However, in cultivated potato production it is treated as an annual plant, since the desired product consists of the nutrient-rich tubers. Even though the plant produces fruits with seeds, to ensure a genetically uniform crop, commercial potato production utilizes vegetative reproduction by using 'seed' tubers to start a new cycle of production. The above soil part of the potato plants consists of one or several main stems from which compound leaves branch. The leaves of the plant, also referred to as foliage, are essential for the starch production that is transferred for storage to the below soil stems, the stolons, and subsequently stored in the tubers (Lorenzen and Ewing, 1992) (Figure 2). Potatoes are commercially produced both for table potatoes, including processed forms such as fries and crisps, and for industrial starch production. Different cultivars of potatoes have been developed to possess ideal characteristics for these different uses (Zaheer and Akhtar, 2016).

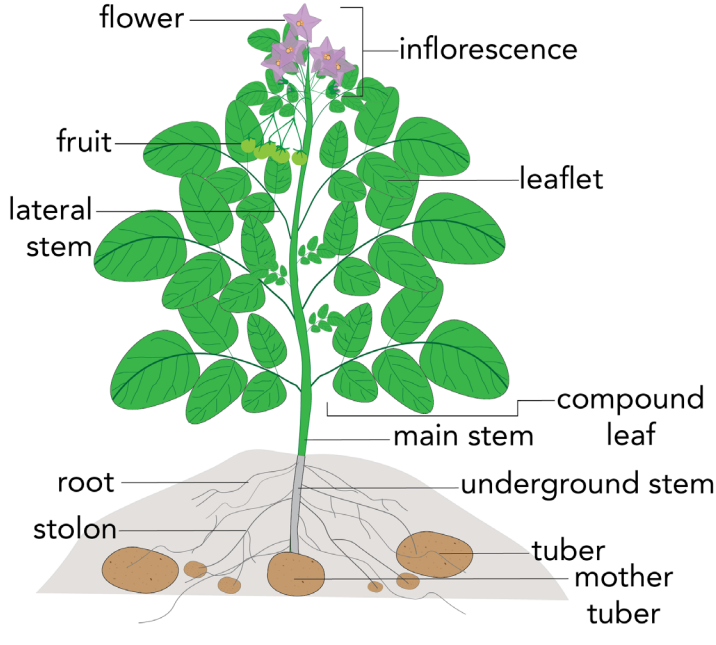

Figure 2 Annotated illustration of a potato plant

2.1.3 Plant diseases and immunity

Plant diseases are caused by either abiotic stressors, causing non-infectious disease, or biotic stressors, causing infectious disease. These stresses prohibit the normal function or adversely affect the plant, in severe cases this results in death of the plant. In this thesis, the focus lies on the infectious diseases caused by pathogenic organisms and 'disease' is, hereafter, used to refer to infectious disease. The major groups of plant pathogens are bacteria, fungi, oomycetes, nematodes, viruses, and parasitic plants (Strange and Scott, 2005). For disease to establish, three factors are required: 1) a virulent pathogen 2) a susceptible host 3) a favourable environment. The interaction of these three components is explained by the disease triangle (Figure 3) (Scholthof, 2007, Moore et al., 2011). Most plants are resistant to most pathogens, yet all forms of agriculture are vulnerable to infection by plant pathogens. This is due to the existence of highly adapted and quickly evolving pathogens. Additionally, the plants we use as crop plants often have a narrow genetic base and may not contain resistance against pathogens occurring in other areas than the crop's origin (Strange and Scott, 2005). Moreover, (large) monoculture fields present an ideal environment for rapid proliferation of pathogens and exert a large selection pressure for the continued evolution of pathogenicity against the narrow genetic base of the host (Oerke, 2006).

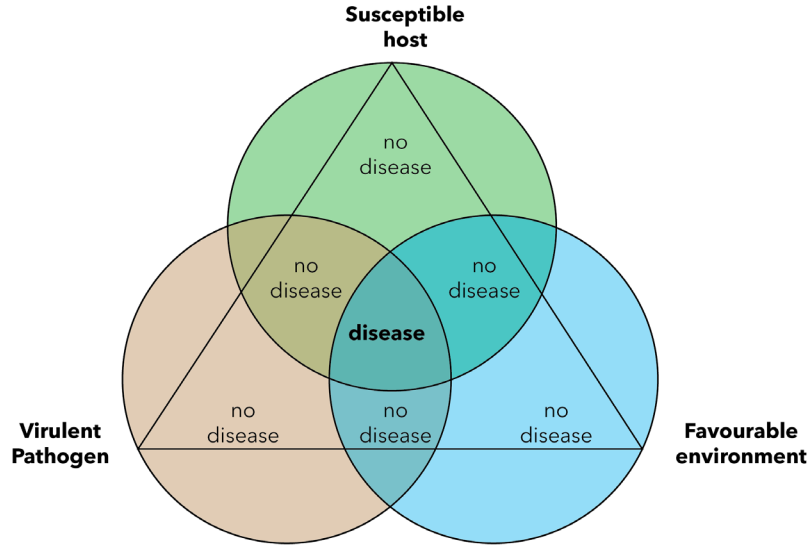

Figure 3 Disease triangle showing the three components required for the establishment of disease based on figure from Moore et al., 2011

For a plant pathogen to successfully infect, it first needs to be able to pass passive defences. The passive defences include physical barriers such as the cell wall, cuticle and anti-microbial compounds that are produced constitutively (Peyraud et al., 2017). Once a pathogen has managed to pass the passive defences this does not mean the fight is lost for the plant. Plants additionally possess an active immune system that can be activated after sensing the presence of a pathogen. Plants contain both cell-surface receptors present at the plasma membrane and intracellular cytoplasmic receptors, such as Nucleotide-binding Leucine-rich repeat Receptors (NLRs). Both the cell-surface and intracellular receptors can recognize danger signals and activate downstream defence pathways (Figure 4). The recognition receptors form complex networks to mediate immune responses (Wu et al., 2018). Danger signals can be Microbe/Pathogen Associated Molecular Patterns (MAMPs / PAMPs), plant originating Damage Associated Molecular Patterns (DAMPs), or pathogen derived effectors (van der Burgh and Joosten, 2019). Effectors are proteins secreted by pathogens that suppress plant immunity by manipulating or suppressing triggered immunity, or by

targeting susceptibility factors (Toruño et al., 2016, Engelhardt et al., 2018). Plant immunity has often been explained by the 'zigzag' model (Jones and Dangl, 2006) that separates a less forceful PAMP Triggered Immunity (PTI) in which the recognition of PAMPs by Pattern Recognition Receptors (PRRs) triggers immunity and the more specific and forceful Effector Triggered Immunity (ETI), in which the recognition of effectors by Resistance gene (R-gene) encoded NLRs triggers immunity. However, new insights showed that this strict danger signal-based separation does not hold, and van der Burgh and Joosten (2019) recently suggested the 'Spatial Immunity Model' (Figure 4). In this model, the focus lies on the place where a danger signal triggers a response and not on what type of danger signal, since both PAMPs and pathogen secreted effectors can be present in the apoplast and the cytoplasm. Extracellularly Triggered Immunity (ExTI) occurs upon the recognition of Extracellular danger signals or Immunogenic Patterns (ExIP) and Intracellular Triggered Immunity (InTI) occurs upon the recognition of Intracellular Immunogenic Patterns (InIP) (Figure 4).

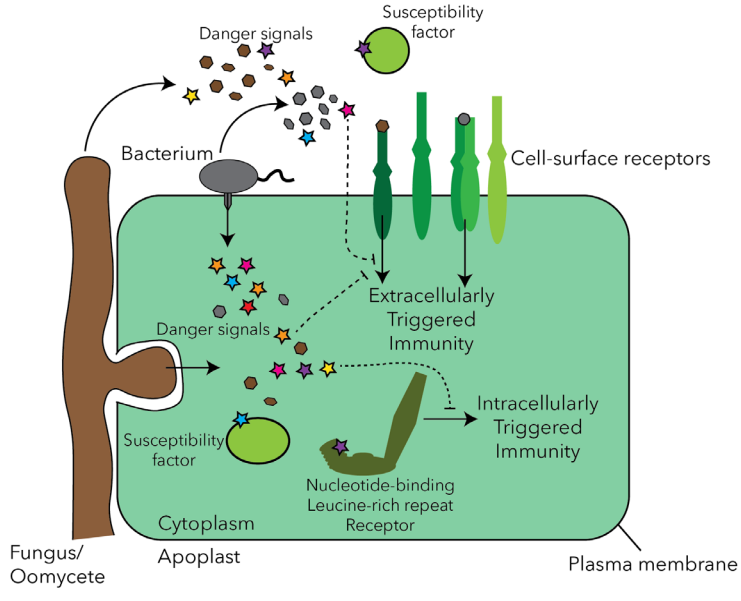

Figure 4 Schematic overview of the 'Spatial immunity Model' based on figure from van der Burgh and Joosten, 2019

2.1.4 Plant hormones

Plant hormones are organic signalling compounds within the plant that are required for the regulation of developmental stages and in response to specific stimuli, such as biotic and abiotic stresses (Bedini et al., 2018). The compounds that have been identified to function as plant hormones are: auxins, gibberellins, cytokinins, abscisic acid, ethylene, brassinosteroids, strigolactones, Jasmonic Acid (JA) and Salicylic Acid (SA) (Jiang and Asami, 2018). These hormones have specific functions in plant growth or stimuli responses; however, they also function in complex networks with each other. SA is a phenolic compound that plays an important role in the response to disease. Biosynthesis of SA is triggered after the recognition of PAMPs and/or effectors. The increase of SA due to pathogen recognition and the downstream responses are generally effective against biotrophic and hemibiotrophic pathogens; that is pathogens that utilize living plant cells for all or part of their disease cycle. JA is a lipid derived compound that also plays a role in the response to disease. The biosynthesis of JA rapidly increases after pathogen infection or insect attack. The increase of JA and downstream signalling often enhances resistance against necrotrophic pathogens; that is pathogens that kill host cells and feed from dead or dying tissue. The SA and JA pathways cross-talk and can have antagonistic but also synergistic effects (McDowell and Dangl, 2000, Pieterse et al., 2012, Mur et al., 2006). However, SA-JA interactions are part of a complex network including other hormones and signalling pathways that is fine-tuned differently in different plant species. Caution should be taken with generalizing findings from studies of one plant species or plant-pathogen interaction.

2.2 *Phytophthora infestans*

2.2.1 Infestation and destruction- the arrival of the plant destroyer

Phytophthora infestans is, as its name, a combination of the Greek words φυτόν (plant), φθορά (destruction) and the Latin infestare (to infest), suggests, a devastating pathogen. Although initially considered to be a fungus and still often misclassified as one, *P. infestans* is an oomycete belonging to the kingdom of Chromista (Ho, 2018). *P. infestans* is the causal

agent of potato late blight disease that arrived in North America and Europe in the 1840s and played an important role in the occurrence of the Great Irish Famine (1845-1849) (Ribeiro, 2013). Its name was given by Anton de Bary, who showed that this fungus-like micro-organism was the cause of potato late blight disease and carefully described the different spore types of *P. infestans.* With this, he confirmed a theory posed a few decades earlier that plant diseases could be caused by micro-organism and hereby kick-started the field of plant pathology (de Bary, 1876, van West and Vleeshouwers, 2004, Judelson and Blanco, 2005).

Currently, *P. infestans* is still the number one pathogen leading to yield loss in potato production and even with extensive control it still causes an average global yield loss of 6% (Savary et al., 2019). The global cost of late blight control and losses was estimated to be €4.8 billion over a decade ago (Haverkort et al., 2008), and with increased global production, this figure is likely to be significantly higher nowadays.

Late blight disease is characterized by brown coloured lesions that can first appear water-soaked and chlorotic, but quickly result in large scale necrosis (Figure 5A). Due to the efficient spreading mechanism of the *P. infestans* spores, the disease quickly spreads from leaf to leaf and even to the tubers. When left uncontrolled the foliage of a plant turns completely necrotic and is destroyed in less than a week (Figure 5B) (Schumann and D'Arcy, 2000)

A

B

Figure 5 Potato leaflet infected with *P. infestans* in a potato field (A) and loss and browning of foliage due to *P. infestans* of a row of susceptible potato plants not sprayed with fungicides between rows of fungicide treated plants (B).

2.2.2 Lifestyle and cycle

P. infestans is a pathogen with a hemibiotrophic lifestyle, e.g. it starts its life on a host plant as a biotroph but at a certain point switches to necrotrophy. Reproduction can occur either asexually or sexually, when two strains with different mating types meet (Figure 6). Asexual reproduction occurs through the production of sporangiophores containing several sporangia. The asexual sporangia can either germinate directly or indirectly by the process of zoosporogenesis. During zoosporogenesis, multiple zoospores, cell wall-less spores that contain one tinselled and one whiplash flagellum, are released from the sporangium. The release of zoospores occurs at temperatures below 14°C (Schumann and D'Arcy, 2000, Judelson and Blanco, 2005).

The infection of a host plant starts with the landing of a zoospore or sporangium on a host leaf. A sporangium germinates either directly or indirectly, after a released zoospore encysts and starts growing a germ tube (Figure 6). The germ tube tip swells into an appressorium from which the plant is penetrated by the formation of a penetration peg. *P. infestans,* from there, continues its growth both inter- and intracellularly, on occasion forming an intracellular feeding structure, a haustorium. After 2-3 days the switch from biotrophy to necrotrophy is made and after complete colonization, sporulating hyphae surface through stomata and sporangiophores are produced, allowing the asexual life cycle to repeat over and over again over a growing season (van West and Vleeshouwers, 2004). Additionally to asexual reproduction, *P. infestans* is heterothallic and thus can also reproduce sexually when two strains with the different mating types, A1 and A2, are present. Until the 1980 only isolates with the A1 mating type occurred in Europe, but since the first report of A2 isolates in East Germany in 1980, A2 isolates have been reported all over Europe. The first A2 isolate in Sweden was reported in 1985 (Kadir and Umaerus, 1987, Drenth et al., 1993). The presence of both mating types and the occurrence of sexual reproduction results in increased genotypic variation and enhanced pathogen adaptability (Andersson et al., 2009).

The sexual spores called oospores are thick-walled and formed when hyphae of the two mating types meet and form the female and male gametangia, the oogonium and antheridium. The nucleus from the antheridium enters the oogonium, the nuclei from both gametangia fuse, and the diploid oospore is

formed (Schumann and D'Arcy, 2000). Oospores can survive harsh conditions such as soil temperatures up to 40°C and as low as -80°C (Drenth et al., 1995). Germinating oospores can either directly infect a host plant or form a sporangium that acts similarly to the asexually produced sporangia (Judelson and Blanco, 2005).

Towards the end of the growing season, and during harvest, tubers can become inoculated with *P. infestans*. The inoculum for the next year can come from infected tubers used as 'seed' tubers, from infected debris left in the field, oospores that can survive in the soil for many years, or from other perennial host plants (Zwankhuizen et al., 2000, Vetukuri et al., 2020).

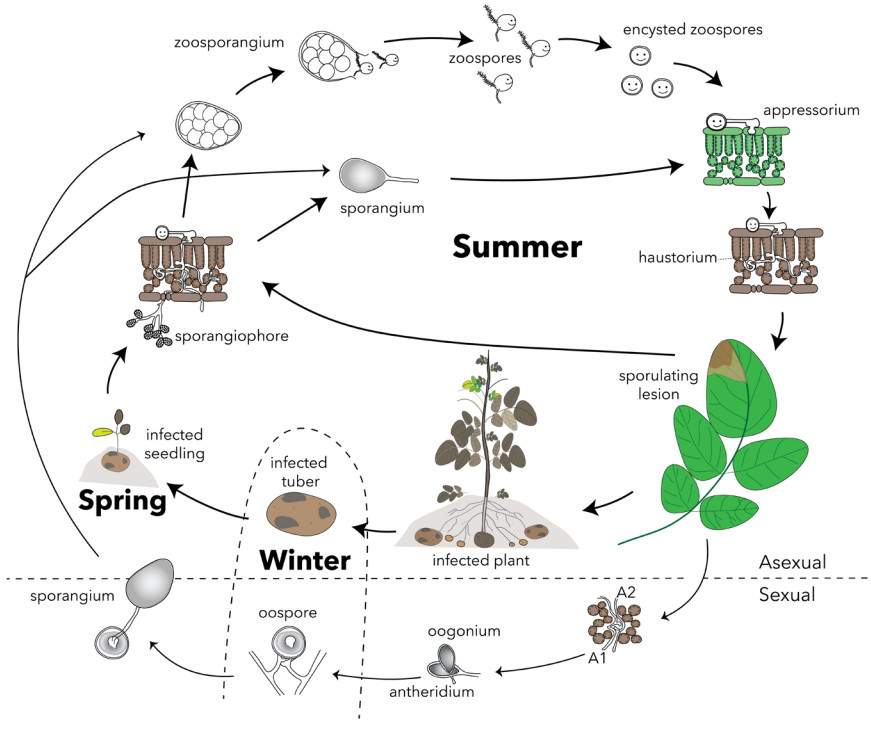

Figure 6 Life cycle of *Phytophthora infestans* based on figure from van West and Vleeshouwers (2004)

2.3 *Alternaria solani*

2.3.1 Disease and morphology

Alternaria solani is a fungal plant pathogen belonging to the *Alternaria* genus of ascomycete fungi. Many of the species within the *Alternaria* genus are plant pathogens, but the genus also contains allergenic species and species that can cause opportunistic infections in humans, especially in immunocompromised individuals (Pastor and Guarro, 2008). Several species of *Alternaria* have been indicated or shown to cause potato early blight disease such as *A. solani, A. alternata* and *A. grandis* (Landschoot et al., 2017). However, in Sweden *A. solani* is believed to be main agent causing potato early blight (Edin and Andersson, 2014).

Potato early blight, paradoxically to its name, is a disease that is usually observed late in the growing season and the susceptibility of potato plants has been shown to increase with maturity and the start of senescence. The name early blight was likely given to the disease since it was usually detected first or more severely in early maturing potato cultivars.

Early blight symptoms are characterized by dark brown round or irregularly shaped ring patterns, often described as “bull’s eye” like, lesions (Figure 7) The early blight lesions are necrotic and, in contrast with late blight lesions, very dry. Large lesions are sometimes observed to fall out of the potato leaves; it is unknown whether this is a strategy for pathogen spread, a protection mechanism of the plant, or both. Sporogenesis of conidia occurs from hyphae growing on top of the necrotic lesion (Figure 7). When left uncontrolled early blight results in large scale necrosis and defoliation (Figure 8).

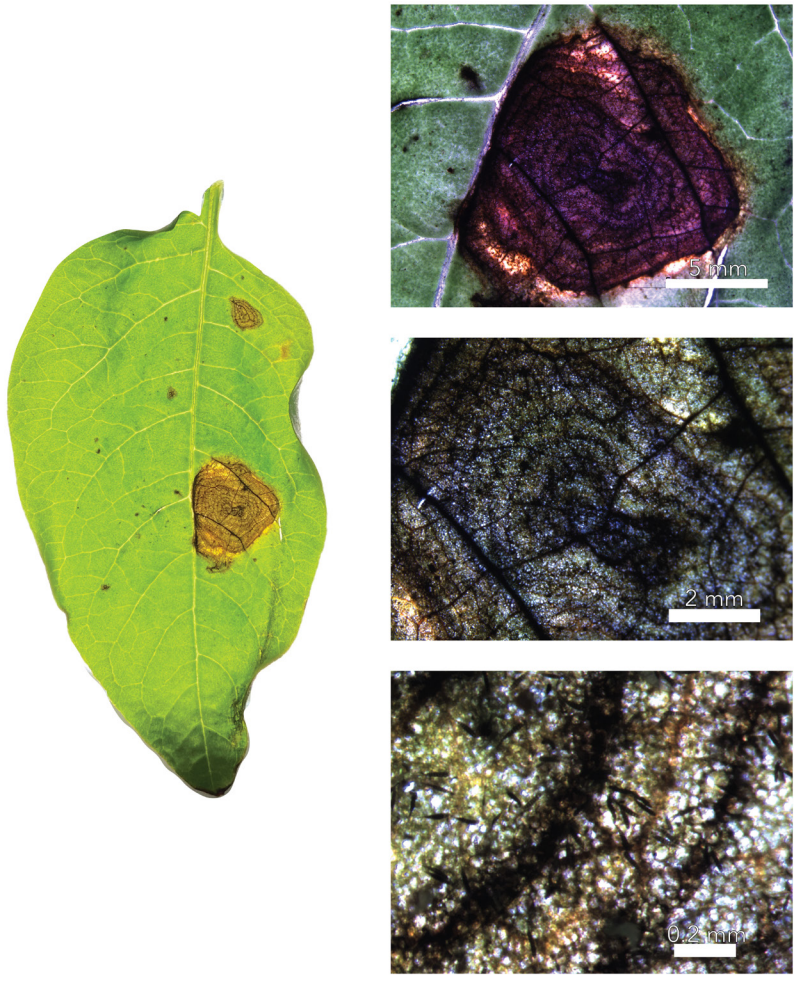

Figure 7 Potato leaflet displaying characteristic early blight lesions caused by *A.solani* and stereo micrographs of the largest lesion displaying the concentric ring pattern and conidia sporogenesis.

Figure 8 Necrosis and defoliation of potato plants due to early blight. Photo: E. Liljeroth

2.3.2 Life cycle

The *Alternaria solani* life cycle begins with the landing of a multicellular spore, a conidium, on a host plant leaf. The first leaves infected in natural infections are the leaves nearest the soil (Figure 9). A conidium can form one or multiple germ tubes and enter the plant leaf either through a natural opening, a pre-existing wound, or by forming an appressorium and penetrating the epidermis (Dita et al., 2007). *A. solani* is considered to be a necrotrophic pathogen and species belonging to the *Alternaria* genus are known to secrete multiple phytotoxins to kill host plant cells to obtain nutrients. However, there is limited knowledge on the specific toxins secreted by *A. solani* (Meena and Samal, 2019). Once the plant tissue is fully necrotic, sporogenesis of conidia from conidiophores occurs. These new spores are dispersed either by water or wind and multiple cycles of infection occur during the growing season (Kemmitt, 2002).

At the end of the growing season, tubers can become infected during the harvest and serve as an inoculum for the next year if used as seed tubers. However, in countries where certified disease-free ‘seed’ tubers are used, the

inoculum for new growing seasons is due to the sturdiness and resilience of the conidia that ensures they can survive in the soil and in infected debris. *A. solani* spores can survive in the soil for several years (Kemmitt, 2002) and thus, even after several years of crop rotations, new infections from soil-borne conidia can occur (Figure 9).

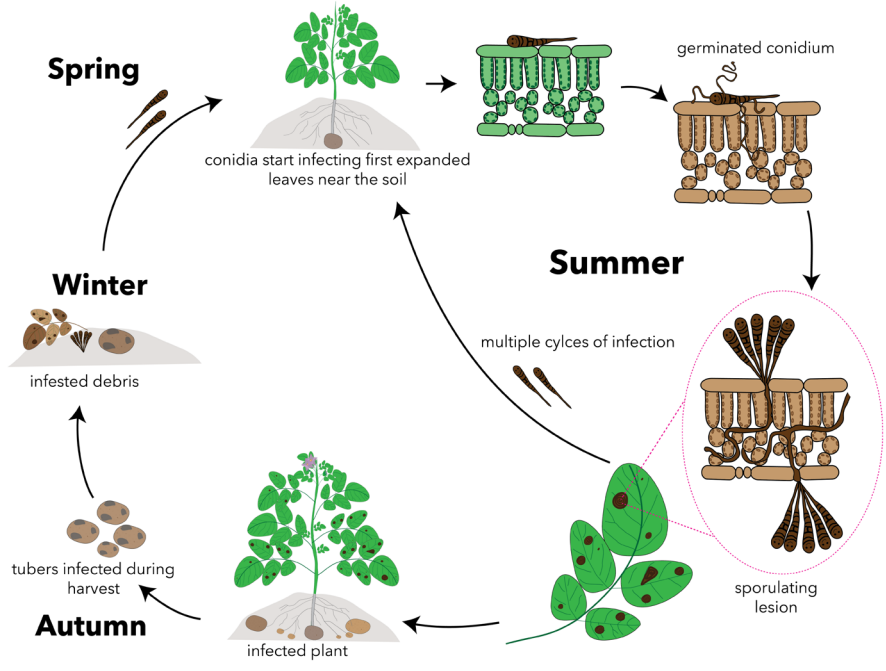

Figure 9 Life cycle of *Alternaria solani* based on figures from Kemmitt, 2002 and Adhikari et al., 2017

2.4 Management and control of early and late blight

2.4.1 General good practice for disease prevention

Since both *A. solani* and *P. infestans* can infect tubers and can overwinter in infected debris and volunteer potatoes, the first steps in preventing disease occurrence is to ensure removal of debris from the field and weed control

along with the use of disease-free 'seed' potatoes. Additionally, the use of cultivars with higher levels of resistance limits the occurrence of disease.

2.4.2 Plant resistance against early and late blight

Although potato cultivars with varying levels of *A. solani* resistance exist, the resistance is considered to be quantitative since no specific R-genes have been identified. Breeding for early blight resistance requires identification of suitable resistant sources and the development of genetic markers. However, a challenge when breeding for early blight resistance is that the resistance against early blight is related to the maturity of cultivars. Late-maturing cultivars generally show higher levels of early blight resistance (Boiteux et al., 1995). Yet, introducing late maturity in new cultivars is considered to be undesirable, since the larger number of days required for tuber production complicates the inclusion of potato in a cropping system and increases the chance of (a)biotic stresses affecting the production (Devaux et al., 2014, Kawar et al., 2018). A recent study employing QTL (Quantitative Trait Locus) mapping in a tetraploid crossing population, found two QTLs for foliar resistance to early blight independent of defoliation that could be used for the development of genetic markers to be used in breeding for early blight resistance in the future (Odilbekov et al., 2020).

Late blight resistance, in comparison, has been shown to be qualitative with multiple specific R-genes identified that can confer resistance to *P. infestans*. Many of these R-genes have been identified in wild *Solanum* species such as *S. demissum, S. bulbocastanum* and *S. venturii* (van der Vossen et al., 2003, Foster et al., 2009). Introduction for research purposes, either by traditional breeding or by genetic engineering, of these R-genes into potato cultivars has led to improved resistance to strains of *P. infestans* that secrete effectors that are recognized by these R-genes. However, when tested in the field the R-gene resistance has been continuously broken and *P. infestans* was nicknamed an 'R-gene destroyer' by Fry (2008). Stacking of multiple R-genes is generally considered to be a promising strategy for achieving more durable resistance. However, traditional breeding techniques are not capable of, within a relevant timeframe, introducing several R-genes into commercially interesting cultivars, since almost 50 years were required to introduce and bring cultivars to the market with just one R-gene (Zhu et al., 2012, Haverkort et al., 2016, Ghislain et al., 2019). However, efforts using

diploid potatoes, such as the recently sequenced diploid potato line Solyntus (van Lieshout et al., 2020) that can be propagated by true seeds, could in the future speed up this process (Jansky et al., 2016, Su et al., 2020).

2.4.3 Chemical control of early and late blight

Current field management, disease-free 'seed' potatoes and more resistant cultivars are, however, not enough to prevent the occurrence of these diseases and they are, therefore, mainly controlled by chemical pesticides.

Early blight is primarily controlled by the application of foliar fungicides. Both broad spectrum mode of action and single site-specific mode of action fungicides are used for the control (Yellareddygari et al., 2019). An example of a widely used specific mode group of fungicides are the Quinone outside Inhibitors (QoIs). Single site-specific mode of action fungicides are often highly effective but come with a high risk of resistance development (Odilbekov et al., 2019). For QoIs, mutations leading to a single amino acid substitution in the cytochrome b protein of *Alternaria* reduce the efficiency of these fungicides in the field (Pasche et al., 2004). Mutations rendering *A. solani* less sensitive to QoI treatment have been reported for populations in the USA, Canada, Europe and for *A. alternata* populations in South Africa (Pasche et al., 2004, Peters et al., 2008, Leiminger et al., 2014, Odilbekov et al., 2016, Dube et al., 2014). However, QoIs are not the only single-site mode of action fungicides for which resistance has been detected in *A. solani* populations, resistance to succinate dehydrogenase inhibiting (SDHI) fungicides has also been reported (Gudmestad et al., 2013, Bauske et al., 2017). It is therefore now recommended to alternate and combine fungicides to decrease the selection pressure on *A. solani* strains that are resistant (Odilbekov et al., 2019). For a third group of widely used fungicides, the triazoles, that interrupt the biosynthesis of the fungal cell plasma membrane (Ribas e Ribas et al., 2016), no observations of reduced efficiency have so far been reported in Europe. However, one study with isolates from Pakistan did report reduced efficiency of a triazole fungicide in inhibiting *A. solani* growth (Akram, 2018).

In Europe, 36 different pesticide products and or mixtures of pesticide products are registered for the control of late blight. These include pesticides with different modes of action, ranging from contact pesticides that are not

taken up in the plant and only protect the parts of the plant that have been sprayed to systemic pesticides that are distributed in the plant through the xylem and thus have the potential to be functional in the whole plant, not only the sprayed part (EuroBlight, 2020). However, *P. infestans* has developed resistance to several of these pesticides. For example, the systemic and contact pesticide metalaxyl was introduced in Western Europe in 1977, yet resistance was already recorded 3 years later in 1980 (Yun Lee et al., 1999). Another intensively used pesticide fluazinam introduced in 1992, has recently been shown to have reduced efficiency in the Netherlands (Schepers, 2018). The most recently registered pesticides are oxathiapiprolin based and are also highly active against plant pathogenic oomycetes. Yet the resistance development risk has been estimated as high, because resistance was shown to be achieved by three point mutations in *Phytophthora capsica* and *Phytophthora sojae* (Miao et al., 2016, Miao et al., 2018, Cohen and Rubin, 2020).

3. Thesis aims

The overall aim of this thesis was to gain an improved understanding of the interactions between potato, *Solanum tuberosum*, and the causal agents of two potato diseases, *Phytophthora infestans* and *Alternaria solani*. Both the binary interactions of potato with one pathogen, the interaction of both pathogens together, and, in addition, the tripartite interaction of all three organisms together were studied. An improved understanding of both the individual and combined interactions of these pathogens with potato is urgently required. This will allow the development of more sustainable control strategies since the current control of both diseases relies heavily on unsustainable practices. Furthermore, since most interaction studies focus only on binary interactions, whilst the actual situation in the agricultural field is unquestionably more complex, this data contributes to an understanding of some of that complexity.

The specific aims of the included research papers were:

1. To elucidate the importance of the phytohormones salicylic acid (SA) and jasmonic acid (JA) in potato defences against *A. solani* and to analyse transcriptional changes occurring upon infection with *A. solani* in hormone deficient and control potatoes (paper I).

2. To visualize and quantify the ionomic changes occurring upon inoculation with *P. infestans* in susceptible and resistant potato leaves (paper II).

3. To analyse the effect of *A. solani* and *P. infestans* on each other *in vitro* and to study the interaction of both pathogens together with potato in laboratory and field settings (paper III).

4. To analyse the transcriptional changes of potato and *A. solani* occurring after inoculation of potato with *A. solani*, including time points before penetration, during infection and after the occurrence of necrosis due to infection (paper IV).

4. Methods

4.1 Whole plant infections

In order to be able to study plant-pathogen interactions, a controlled laboratory set-up is often employed to ensure no interference from other stress factors and variations in the environmental conditions. Additionally, the laboratory set-up allows for experiments to be performed multiple times and at any time of the year, which is especially beneficial when working with a crop plant that has only one production cycle per year in the Northern hemisphere. To study interactions of *P. infestans* and potato, Detached Leaf/Leaflet Assays (DLAs) are often employed to achieve high replicate number infection assays (Malcomson, 1969). While DLAs generally work well for studying the compatibility of *P. infestans*-*S. tuberosum* interactions, results of DLAs with *A. solani* on potato leaves were previously shown to poorly correlate with results obtained from infection assays with intact plants (Vleeshouwers et al., 1999, Odilbekov et al., 2014).

In this thesis work all the infection assays of potato with *P. infestans* and *A. solani*, with the exception of the RNA sequencing sample preparation (paper IV), were performed in versatile environmental test chambers in which custom-made acrylic boxes were placed. The boxes were designed to contain a tray insert of 30 mm high to allow 1 litre of water to be placed in the box, without the plant pots standing in water (Figure 10). When the box is closed, the water in the bottom ensures a Relative Humidity (RH) of over 95%, required for successful infection, is reached. *In vitro* plantlets transferred to soil and subsequently grown in soil for 4 weeks, reaching a height of approximately 20 cm measured from the soil to the tip, were used for the infection assays. Three boxes, each containing 4 plants were placed

in versatile environmental test chambers (Panasonic model MLR-353H-PE, Panasonic Healthcare Co., Ltd., Japan). The chambers were programmed to have 16 hours of light and 8 hours of darkness with a stable temperature of 25°C during the light and 22°C during the dark hours. A maximum of 48 plants, placed in 12 boxes in four cabinets could be used for infection assays. This infection assay set-up allowed for a high infection efficiency of both *P. infestans* and *A. solani* in a very controlled environment.

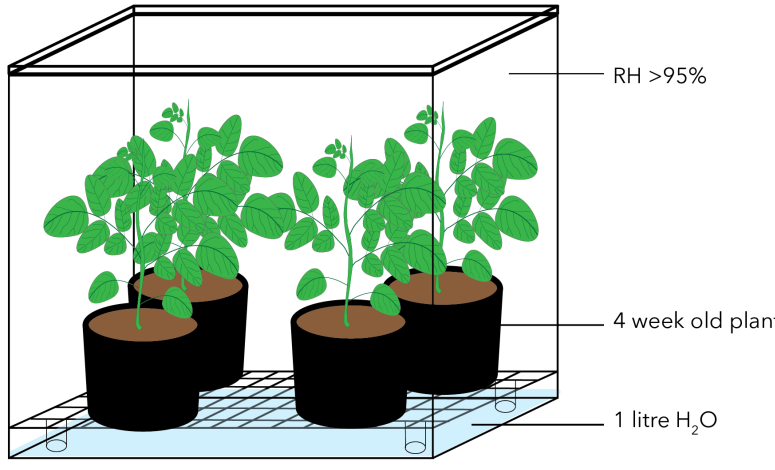

Figure 10 Schematic overview of an infection assay box

4.2 Field trial

While laboratory studies of plant-pathogen interactions supply valuable insight into the interactions without interference from unintended stressors but with the opportunity for increased replication of experiments, commercial potato production occurs in an open field. To translate basic research, obtained using laboratory experiments, successfully into field settings, it is important to confirm that the results obtained in the laboratory hold up in an agriculturally relevant setting.

In paper III a field trial was carried out to test the influence of pre-established *A. solani* infection on the potential for *P. infestans* to infect. The field contained 8 smaller plots that made up 4 replicate blocks. In each block one plot was inoculated with *A. solani* by spreading *A. solani* infected barley kernels in between the plants. All plots were later inoculated with *P. infestans* by brushing infected material collected from another field trial over the plants in the plots. The disease symptoms of both early blight and late blight were visually scored repeatedly for 5 weeks.

4.3 Transcriptomics

Transcriptome analyses, such as microarray (paper I) and RNA sequencing (paper IV), provide information on the amount and type of RNA produced in a sample. Transcriptome analysis can provide valuable information about the changes in gene expression occurring during infection of a plant by a pathogen. We employed microarray-based transcriptomics to analyse the differences in responses to *A. solani* infection in a potato cultivar and two hormone compromised plant lines with the same cultivar background (paper I). In paper IV we used Illumina based RNA sequencing of a potato cultivar infected with *A. solani* and subsequent mapping of the sequences to both a potato reference genome and an *A. solani* reference genome.

The microarray technique we used in paper I utilises an Agilent array with RNA probes with complementarity to a set of *S. tuberosum* predicted transcript sequences. The probes are spotted on to the array at a known place. The sample RNA is labelled with a fluorescent dye and subsequently added to the array where hybridization between the probes and RNA can occur. All unbound and non-specific bound sample is washed off and subsequently the intensity of fluorescence emission on the whole array is scanned after excitation with an appropriate wavelength for the fluorescent dye used. The acquired image is quantified, processed and the data analysed further to determine, for example, differential gene expression between different treatments (Page et al., 2007). While microarray is a powerful method to obtain transcriptome information from samples, this technique can only detect predefined transcripts or genes. Simply, if there is no probe on the array for a specific gene/ transcript, no information will be obtained about

this gene/transcript, regardless of the abundance of RNA for this gene/transcript in the sample.

RNA sequencing used in paper IV, in comparison, does not require prior knowledge of the transcripts in the sample. After RNA isolation a complementary DNA (cDNA) sequencing library should be prepared. Depending on the sequencing platform and type of RNA of interest the library preparation might vary. However, it generally involves a selection or depletion process to select the type of RNA of interest, reverse transcriptase of the RNA, fragmentation and size selection to acquire the optimum sequencing size, and adaptor ligation. The cDNA libraries are subsequently sequenced on a Next Generation Sequencing (NGS) platform generating nucleotide sequence read files. The read files require mapping to a reference genome and transcript assembly and quantification. Alternatively, *de novo* transcriptome assembly can be performed. After assembly and quantification, the data can be used, for example, to perform differential gene expression analysis (Kukurba and Montgomery, 2015).

4.4 Ionomics

The ionome represents the inorganic building blocks, the essential and nonessential element composition, of an organism (Figure 11). The study of ionomics focusses on measuring and quantifying the ionome of organisms and the changes that occur in the ionome due to changes in the physiological state e.g. (a)biotic stress, developmental changes, and/or genetic modifications (Salt et al., 2008). In paper II we have employed two different techniques, X-Ray Fluorescence (XRF) and Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES). These methods were used to obtain information about the element composition in potato leaflets either inoculated with *P. infestans* or mock-inoculated. The ionomes of *P. infestans* and mock-inoculated plants as well as compatible and incompatible *P. infestans*-potato interactions were analysed and compared.

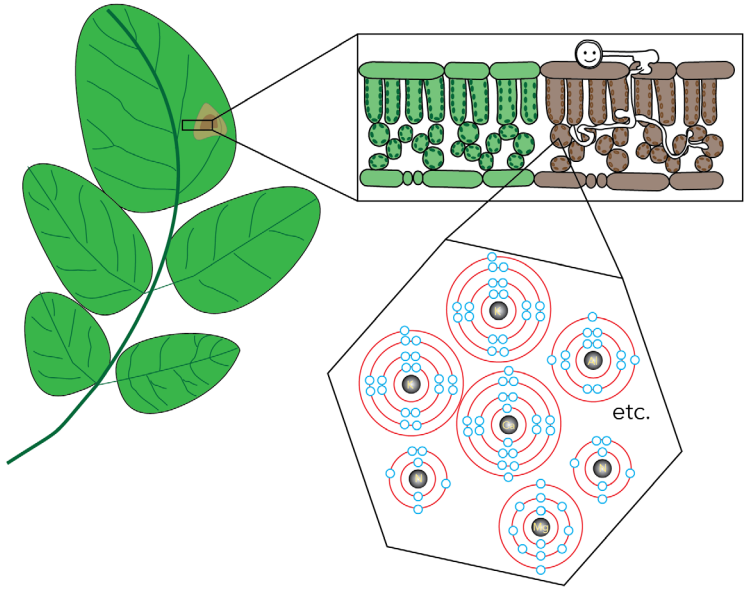

Figure 11 Schematic illustration of the ionome

XRF is the process whereby a photon is emitted from an atom after excitation with high energy X-rays. During excitation with high energy X-rays, atoms absorb the X-ray energy, and this results in the expulsion of an electron from an inner orbital of the atom. The atom relaxes by bringing an electron from a higher orbital to the 'free' spot in the lower orbital. The energy difference between the two states is released from the atom, and this energy is referred to as the fluorescence. Since each chemical element has electronic orbitals at characteristic energy levels, the released fluorescence can be used to determine which chemical elements are present in an excited sample by collecting and measuring the energies of fluorescence photons (Figure 12). The high energy X-rays required for XRF methods can be supplied by conventional high-voltage X-ray tubes, sealed radioactive gamma ray sources, or synchrotron radiation. (Salt et al., 2008, Shackley, 2018).

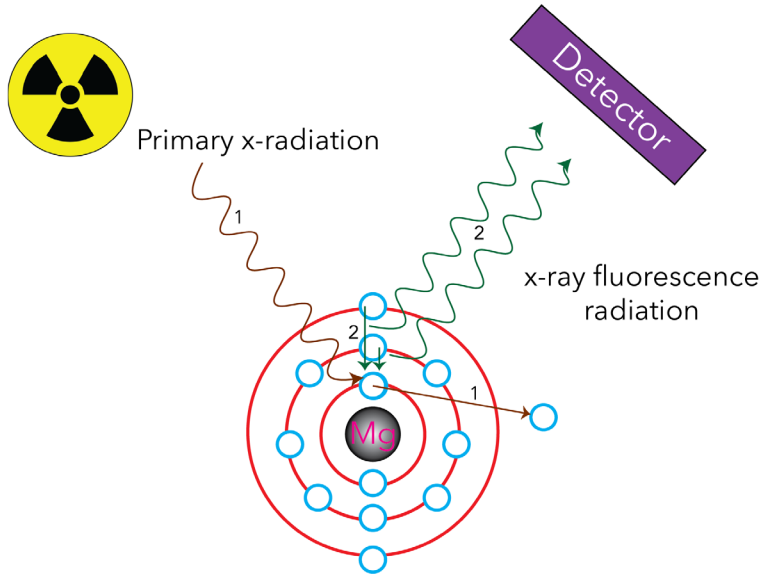

Figure 12 Schematic overview of the X-ray fluorescence process

In paper II we employed, both a commercially available benchtop micro (μ) XRF machine equipped with a rhodium target X-ray tube, and synchrotron based μXRF to obtain element maps showing the distribution of both macronutrient and micronutrient elements in control and *P. infestans* inoculated potato leaves.

ICP-OES combines ionization of atoms through inductively coupled plasma with detection through optical emission spectroscopy. ICP is a process in which a plasma is generated, and atoms are brought into the ionized state. Inductive heating is required to maintain the plasma. The sample of interest, after appropriate preparation including digestion, is introduced into the plasma in a stream of argon gas after aerosol creation by a nebulizer. The atoms from the sample are ionized, generally into a singly charged positive ion. OES is subsequently used to detect the atoms in the sample. Once the atoms fall back to the ground state, in a manner similar to the XRF process, photons of wavelengths characteristic to each chemical element are emitted.

The emitted light from the plasma is focused and passed through optical slits into the optical emission spectrometer. In the spectrometer, photons are separated by wavelength, achieved through optical filtering, and the individual intensities are subsequently detected by a charge injection device detector (Figure 13). Quantification of elements in a sample is achieved by comparing the photon intensities of the sample with those measured for a reference material with known concentrations (Salt et al., 2008, Caruso et al., 2017).

In paper II we employed ICP-OES for absolute quantification of elements in bulk samples of control and *P. infestans* infected leaflets.

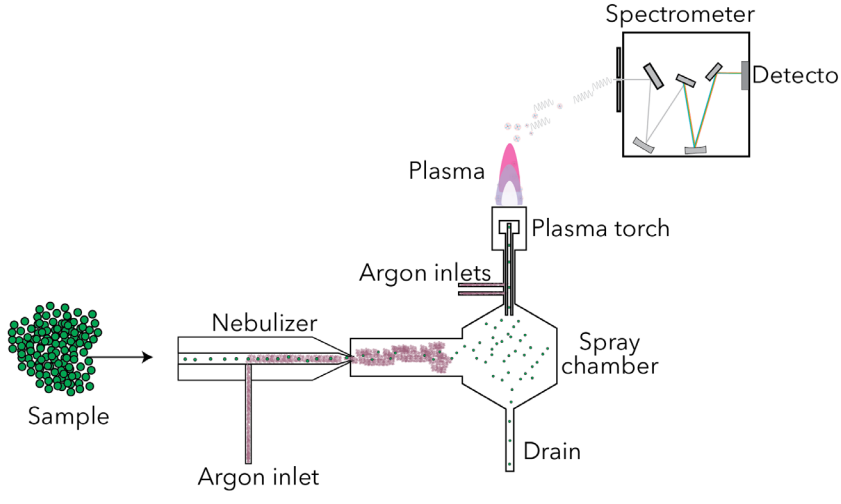

Figure 13 Schematic overview of ICP-OES based on figure from Caruso et al., 2017

4.5 Microscopy and tissue staining

In order to follow the progression of infection of potato leaves when inoculated with either *A. solani*, *P. infestans,* or both in more detail, we employed both conventional stereo and compound Bright Field (BF) microscopy of stained and unstained samples. Additionally, we have performed live cell imaging of potato leaves infected with fluorescent strains

of *A. solani* and *P. infestans* using Confocal Laser Scanning Microscopy (CLSM).

In paper IV *A. solani* infected leaves were stained with trypan blue to visualize the appressoria formed by *A. solani* to penetrate the potato epidermis. Trypan blue is a dye that has been used extensively in biosciences as a cell viability dye due to its high binding capacity to membranes of dead cells. Whilst this generally holds true for plant cells, trypan blue can bind to the cell wall of fungi and oomycetes regardless of the viability (van Wees, 2008, Fernández-Bautista et al., 2016, Liesche et al., 2015). Trypan blue was chosen for this experiment for both its ability to stain fungal growth and as a viability stain for plant cells. The stained samples were visualized using an inverted bright field microscope.

In paper I, 3,3'-diaminobenzidine (DAB) was used to stain the hydrogen peroxide (H_2O_2) present in potato leaves infected with *A. solani*. In the presence of haem containing proteins, for example peroxidases, DAB is oxidized by hydrogen peroxide and a brown precipitate is generated. The more H_2O_2 present the more brown precipitate. The samples were visualized using a stereo bright field microscope.

In paper III the infection processes of *A. solani*, *P. infestans,* and both pathogens together were followed and visualized using CLSM. We used a *P. infestans* strain constitutively expressing tdTomato, a very bright red fluorescent protein, in the 88069 background (McLellan et al., 2013) and an *A. solani* strain constitutively expressing enhanced Green Fluorescent Protein (eGFP) in the NL03003 background (created by P.J. Wolters, Wageningen, NL: see paper III).

CLSM has an advantage over conventional fluorescence microscopy due to the ability to focus light at one narrow depth level, the focal plane. CLSM utilizes lasers as a light source, since coherent laser light is ideal for precise focusing on a small part of a sample, the focal volume. Due to this precise illumination, the fluorophores predominantly in the focal volume are excited and emit fluorescence. The emitted light is subsequently passed through a dichroic mirror and a pinhole, filtering out non-fluorescent and out-of-focus light, to the detector. The detector usually contains Photo Multiplier Tubes

(PMTs) and a Charged Coupled Device (CCD) that respectively amplify and transform the low signal light into an electrical signal (Figure 14). In order to be able to form an image, scanning is used to collect signals from several focal volumes. Scanning can be performed in the *x, y,* and *z* directions, allowing for collection of 2D and 3D images (Hardham, 2012, St. Croix et al., 2005). The ability to collect images in the z direction and create 3D images makes CLSM an ideal tool to study the infection progression of plant pathogens that during infection grow deeper into the plant tissue. CLSM allows for determination of infection structures in different cell layers of the plant. Additionally, the use of fluorescent pathogens allows for live-cell imaging without the need for fixation and toxic stains that can alter the morphology of the plant and pathogen cells (Hardham, 2012).

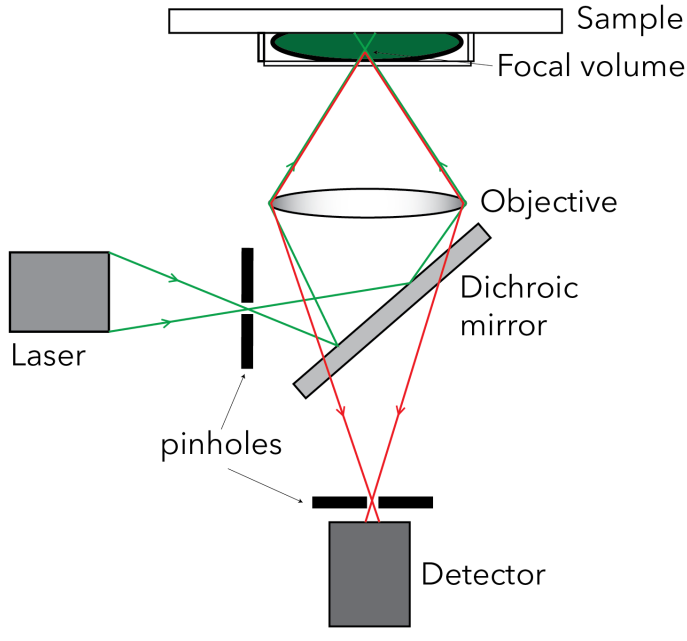

Figure 14 Schematic overview of the concept of confocal laser scanning microscopy (CLSM). The excitation light path is shown in green and the in-focus emission light path in red.

5. Results and Discussion

5.1 'It is all hormones and genes' - The potato - *A. solani* interaction and the role of phytohormones in potato defences

In order to improve control of early blight, a deeper understanding of resistance mechanisms and the interaction between potato and *A. solani* is required. In papers I and IV, we performed infection studies and transcriptomics to study the effect of *A. solani* infection on potato, the role of the signalling pathways of two plant hormones involved in disease responses and immunity, and transcriptional changes occurring in host and pathogen during the early stages of *A. solani* infection.

In paper I, the importance of salicylic acid (SA) and jasmonic acid (JA) in the potato immune responses to the necrotrophic pathogen *A. solani* was investigated. Two plant lines expressing *NahG,* a bacterial gene encoding a salicylate hydroxylase, rendering the plants SA deficient, and two JA insensitive plant lines in which *coi1* was silenced by RNA interference (RNAi) were used together with the background cultivar (cv.) Désirée to study the role of SA and JA. Bioassays of all plant lines infected with *A. solani* revealed that the SA deficient lines showed increased early blight lesion development compared to the JA insensitive lines and the untransformed cultivar. Additionally, fungal biomass determination by quantitative Polymerase Chain Reaction (qPCR) showed that the SA deficient lines had increased fungal biomass compared to all the other plant lines (paper I: Fig.1). These results indicate that the SA deficient lines facilitate faster growth of the pathogen and the observed larger visible lesions follow this faster growth.

The transcriptional changes due to infection with *A. solani* in a SA deficient line, JA insensitive line and the cultivar were analysed using custom Agilent microarrays with probes based on predicted transcripts from the *Solanum tuberosum* Group Phureja DM genome (v. 3.4) (Hancock et al., 2014). RNA was collected from the whole infected leaflets, to allow for detection of transcriptional changes in the whole leaf, and not only close to the spot of inoculation. Infected and mock inoculated leaflets for all three plant lines were sampled at 3 time points: 24, 72, and 120 hours post inoculation (hpi). Differential gene expression analyses were performed for *A. solani* versus mock inoculated samples at the three different time points for all three plant lines. In general, more differentially expressed genes (DEGs) were found for the SA deficient line (paper I: Table 1).

At the earliest time point, 24 hpi, DEGs were found only in the SA deficient lines and upregulated genes were associated with secondary metabolism and cell death. At 72 hpi, the first DEGs were found in the untransformed cultivar. Six genes were downregulated, the only annotated gene of these encodes polyubiquitin. A homolog of this gene in *Arabidopsis thaliana* was shown to be affected by pathogen inoculation too, with downregulation in response to the biotrophic oomycete *Hyaloperonospora arabidopsidis* and upregulation after inoculation with the necrotrophic fungus *Botrytis cinerea* (Wang et al., 2011, Data from Ausubel Lab and Scheel Lan deposited in the *Arabisopsis* eFP browser; Winter et al., 2007). At 120 hpi most DEGs are found for all plant lines (paper I: Table 1). At this time point *Pathogenesis Related* (PR) genes such as chitinases and PR proteins appear to be upregulated in the cultivar and the SA deficient plant line. Additionally, the cultivar showed upregulation of multiple genes encoding ion transporters (paper I: Table 3). In the JA insensitive line stress related genes such as small cytoplasmic heat shock proteins were found to be upregulated (paper I: Table 2).

In paper IV we performed RNA sequencing of cv. Désirée inoculated with *A. solani*, to study the transcriptional changes occurring upon inoculation and subsequent infection with *A. solani* in more detail. Samples for sequencing were collected from *A. solani* and mock-inoculated leaflets as early as one hour after inoculation when the *A. solani* spores had just started germinating and through a time course up to 48 hpi when necrosis of multiple plant cells

due to infection had occurred. To allow for the detection of local differences in gene expression due to inoculation, samples consisted of 8mm leaf discs containing the inoculation droplet. Differential plant gene expression analyses were performed for *A. solani* versus mock-inoculated samples at the different time points. Additionally, differential *A. solani* gene expression analyses were performed by comparing the *A. solani* inoculated samples at 6, 12, 24, and 48 hpi to the *A. solani* inoculated samples harvested at 1 hpi.

Differentially expressed potato transcripts (DETs) were found for all time points, the number of DETs increased with time, yet more were found at 24 hpi than at 48 hpi (paper IV: Table 2). At 1 hpi we found differential expression of 25 transcripts. One of the most highly upregulated transcripts encodes a Mutt domain protein (paper IV: Table 3). The Mutt domain is also called the Nucleoside diphosphates linked to some moiety X (Nudix) box. Genes containing a Nudix box such as several *A. thaliana* Nudix hydrolases (AtNUDX) have been shown to play important regulatory roles in biotic stress responses (Dong and Wang, 2016).

Gene ontology enrichment analysis (paper IV: Figure 4) revealed enrichment of catabolic processes, localization and transport at 6 hpi before penetration occurred. At 12 hpi, when the first appressoria are formed we found enrichment in drug metabolic processes, with upregulation of a pectinestarase transcript. This transcript could be an interesting target for further validation, since a pectinestarase gene in *A. thaliana*, was shown to be a susceptibility factor for infection with the necrotrophic fungus *B. cinerea* (Raiola et al., 2011). Uncharacteristically for a necrotrophic pathogen, we detected downregulation of several JA and ethylene biosynthesis related transcripts during the first 24 hours. At 24 and 48 hpi, similar to the last time point in paper I, we find upregulation of ion transporter related transcripts.

At the last time point, photosynthesis related genes showed downregulation, indicating either an energy preservation strategy or a reaction to the increasing *A. solani* induced necrosis. A similar downregulation of photosynthesis has also been observed in other studies of fungus-plant interactions including the interactions of the necrotrophic fungi *B. cinerea* and *Alternaria brassicicola* with *A. thaliana* (Bilgin et al., 2010).

Differential transcript expression for *A. solani* was analysed by comparing all the later time points individually to the first time point. DETs were found for all time points and similarly to the potato transcripts, the number of DETs increased with time, with the exception of 48 hpi for which the lowest number of DETs were found (paper IV: Table 4). Four transcripts were differentially expressed in all time points (paper IV: Table 5), of which two have functions in pectate hydrolyses and mannitol biosynthesis that have previously been shown to be important for the virulence of *Alternaria brassicicola* (Cho et al., 2015, Calmes et al., 2013). Additionally, we found differential expression of several transcripts encoding proteins with functions that have been shown to play a role in pathogenicity of other fungal pathogens (paper IV: Table 6).

Overall, the studies presented in paper I and IV found that intact SA signalling is required for potato defences against *A. solani.* Moreover, we identified several interesting target genes for future validation studies of potato defences against early blight, the way intact SA signalling influences limitation of early blight lesion development, and potential virulence factors of *A. solani.*

5.2 'Elementary, my dear Watson' - Ionomic responses of potato to inoculation with *P. infestans*

The ionome, the chemical element composition, of crop plants is determined by the nutrients available in the soil in which the plant is grown. Modern agriculture requires fertilization, often in the form of inorganic fertilizers, to provide enough nutrients for intensive production. The best fertilizer treatments and formulations for optimal crop production have thus been widely studied both by academic scientists and by agrochemical companies. Optimized fertilization ensures sturdiness and minimizes the occurrence of nutrient deficiency diseases with symptoms such as chlorosis, tissue death and stunted growth that can lead to yield losses (Oldroyd and Leyser, 2020, Hosier and Bradley, 1999). Multiple studies have shown that fertilization with specific nutrients can also influence resistance to pathogens, mostly in a positive way, but sometimes negative effects on resistance have been observed (Dordas, 2008, Elmer and Datnoff, 2014, Gupta et al., 2017).

Additionally, ion channels facilitate Ca^{2+} fluxes during e.g. pathogen recognition and studies on mutants of several ion channels in *A. thaliana* indicate more complex involvement of ion channels in plant defences (Moeder et al., 2011). As previously mentioned, we detected upregulation of several ion transporter genes, during potato infection with *A. solani* in paper I and IV. However, limited knowledge exists on the changes occurring in the ionome in plants with sufficient nutrient levels with varying levels of resistance in response to pathogen infection.

In paper II we have studied the changes occurring in the potato leaf ionome after inoculation with the hemibiotroph, *P. infestans*. The ionomes of cv. Désirée, a transgenic line expressing a single R- gene, and a transgenic line expressing three R-genes were visualized using benchtop µXRF and synchrotron µXRF and the cv. Désirée ionome was additionally quantified by ICP-OES (paper II: Figure 1). The µXRF obtained element distribution maps reveal that several of the analysed elements show redistribution patterns within and outside of the lesion in the susceptible cultivar. In the resistant R-gene(s) expressing lines we observed specific redistribution patterns for calcium (Ca), magnesium (Mg), manganese (Mn) and silicon (Si) that differed from the patterns observed in the susceptible cultivar (paper II: Figures 3, 4 and 5). Bulk analysis of *P. infestans* infected leaflets versus control leaflets by ICP-OES showed a significant increase in the concentrations of phosphorus (P), sulphur (S), molybdenum (Mo) and nickel (Ni) in the *P. infestans* infected leaves compared to the mock inoculated leaves (paper II: Table 3).

The results presented in paper II show that ionomic changes occur during the infection of potato with *P. infestans*. Additionally, differences in redistribution patterns of elements were found in the compatible interaction of a susceptible host plant and the pathogen compared to the incompatible interaction of a resistant host plant and the pathogen.

5.3 'Double trouble?' - Moving away from the study of binary plant-pathogen interactions

Plant-pathogen interactions are often studied in a laboratory setting and as a binary interaction between the host plant and one pathogenic organism.

However, for crops such as potato that are commercially produced in an open field, that single plant-pathogen interaction is only a fraction of the network of interactions occurring. Rarely is there only one pathogenic organism present in a field, not to mention the plethora of other organisms in the plant microbiome that are absent in most sterile laboratory plant-pathogen interaction studies. In field studies conducted by our group, *A. solani* and *P. infestans* were several times observed in the same fields and an observation of reduced *P. infestans* infection after established *A. solani* infection triggered an intrigue in the tripartite interaction of the host plant with both of these pathogens (paper III: sFigure 1)

In paper III the binary interaction between *A. solani* and *P. infestans* as well as the tripartite interaction including *S. tuberosum,* was investigated from *in vitro* to agriculturally relevant field setting, *in agro* (paper III: Figure 1). *In vitro* solid medium experiments showed that the growth of *P. infestans* was reduced when plated together with *A. solani*, compared to when it was plated with itself (paper III: Figure 2 and sFigure 2). Additionally, *in vitro* experiments of consecutive growth of the pathogens in liquid medium, showed that the growth of *P. infestans* was inhibited in medium that previously harboured *A. solani* growth (paper III: Figure 3). The *P. infestans* sporangia that were used as inoculum often displayed cytoplasmic leakage after several hours in liquid medium that previously harboured *A. solani* (paper III: Figure 4A). Furthermore, hyphal tip swelling and subsequent bursting was occasionally observed for growth from sporangia that did manage to germinate (paper III: Figure 4B). Similar hyphal bursting was observed when *P. infestans* was grown in the presence of the oomycete pesticide flumorph (Hua et al., 2015).

In planta infection bioassays showed that the simultaneous co-inoculation of both pathogens resulted in larger necrotic lesions than single inoculations, however, when consecutively inoculating both pathogens on the same spot with a 24 hour interval, larger lesion development was only observed when *A. solani* was inoculated on top of *P. infestans* (paper III: Figure 5). CLSM imaging of the infection of both pathogens co-inoculated revealed that simultaneous inoculation rarely resulted in plant tissue colonization by *P. infestans.* In the consecutively inoculated samples plant tissue colonization of *P. infestans* was similarly rare when *A. solani* preceded

P. infestans inoculation. However, the ability for *A. solani* to colonize the plant tissue was not prohibited by already established *P. infestans* colonization in the samples where *P. infestans* preceded *A. solani* inoculation (paper III: Figure 7). Similar results were obtained when the effect of pre-established *A. solani* infection on the infection of *P. infestans* was tested *in agro*. Over the whole assessment period, the severity of late blight was significantly negatively correlated with the presence of early blight (paper III: Figure 8).

Overall, the results of paper III show that *P. infestans*, the pathogen most damaging to potato production worldwide, is negatively influenced by *A. solani*. The *in vitro* liquid medium experiments indicate a direct effect of *A. solani* on *P. infestans* by means of one or several unidentified *A. solani* secreted molecule(s). Additionally, the *in planta* experiments both in the laboratory and in the field show that the disease symptoms and occurrence in the host plant are altered depending on the order of inoculation with both pathogens. Since the inoculation with both pathogens in the field in comparison to the laboratory experiments did not result in inoculation of both pathogens on the same spot of the leaf, the plant responses induced by *A. solani* infection likely play a role in the reduced occurrence of late blight when the plants were previously inoculated with *A. solani*.

6. Conclusions

The development of disease is the result of a complex interplay of a pathogen, host, and all the environmental conditions in which the host-pathogen system is situated, thus including the presence of other pests, pathogens and (micro-) organisms. However, in phytopathological research, we often minimize or exclude the other component and focus on simple binary interactions between one host and one pathogen. In order to extend this binary view of disease interactions, the work in this thesis involved the study of not only the binary but also the tripartite interactions between *Solanum tuberosum*, *Phytophthora infestans* and *Alternaria solani* from *in vitro* to *in planta* and *in agro.*

For the interactions between potato and *A. solani* we focused on the role of phytohormones in potato defences against *A. solani*, the transcriptional changes in both potato and *A. solani* during infection and the influence of adding *P. infestans* into the mix on the *A. solani* infection process.

In *Arabidopsis thaliana* jasmonic acid was previously shown to be the main defence hormone against necrotrophic pathogens. However, we found that intact signalling of the salicylic acid is required for potato defences against *A. solani*. This study exemplifies the need to study plant defences against pathogens in the appropriate system, since assumptions based on model organisms, e.g. *A. thaliana,* do not always hold for other plant species.

Additionally, we determined differential expression of several potato and *A. solani* genes that present a group of valuable candidates for validation studies into their role in disease development and could reveal new targets for disease control strategies.

Moreover, during the early infection of *A. solani*, we found downregulation of biosynthesis genes for the hormones jasmonic acid and ethylene that are generally considered to be involved in defences against necrotrophs. We also identified putative secreted effectors and other pathogenicity factors expressed by *A. solani* during infection of potato. Furthermore, co-inoculation of *A. solani* with *P. infestans* appeared to aid the development of early blight characteristic lesions. Altogether, our data indicate that *A. solani* triggers plant responses that are different to those usually seen against necrotrophs, and benefits from the presence of a hemi-biotroph that during the first part of infection and colonization secretes effectors to manipulate the plant to prevent cell death.

The interaction of potato and *P. infestans* was further elucidated by visualizing and quantifying changes in the ionome of potato after inoculation with *P. infestans* on both susceptible and resistant plant lines. This is the first time that ionome changes have ever been studied during potato disease. We identified that the ionomes of plants with different levels of susceptibility show distinct changes. In summary, we identified that ion transport and changes in the ionome, an understudied field of research in phytopathology, may be important factors in both plant defence and diseases. This conclusion is based on the results from our ionomic study together with previous studies on effects of fertilizer treatments on disease tolerance, along with the differential expression of ion transporters found in our transcriptomic studies of the potato- *A. solani* interaction.

Lastly, the influence of the presence of *A. solani* on *P. infestans*' infection potential was analysed. We found that *A. solani* negatively influences *P. infestans*. The addition of *A. solani* into the mix led to reduced *P. infestans* growth *in vitro,* reduced *P. infestans* colonization of plant tissue *in planta*, and reduced the severity of late blight *in agro.* The *in vitro* experiments suggest the existence of *A. solani* secreted molecule(s) that directly negatively influence *P. infestans.* However, it cannot be excluded that the presence of, and the plant responses to, *A. solani in planta* ensure that the required conditions for *P. infestans* infection are not met. Overall this study points out that plant-pathogen interactions can be influenced by other pathogens, highlighting the fact that the situation found in an agriculturally

relevant setting is generally more complex and effects of, e.g. treatments, can differ from experiments in controlled environments.

In conclusion, this tale of two blights broadens our understanding of the interaction between potato and the two studied individual pathogens on new levels such as the transcriptome for *A. solani* and the ionome for *P. infestans* but also emphasizes the importance of combining the studies of multiple plant diseases and moving away from a separative view of diseases.

7. Future perspectives

In order to fulfil future food production demands in an environmentally and economically sustainable way, alternatives to disease control are required. To aid the development of alternative and more sustainable control methods, a detailed understanding of the interactions that lead to disease is required. However, the generation of new knowledge alone is never enough to change a system. In order to change a system, translation of knowledge into practices is required. To ensure integration of the acquired knowledge from the studies in this thesis, additional research is suggested in the hereafter discussed directions.

The resistance and susceptibility differences of potato cultivars to early blight is still poorly understood. In paper I, we demonstrated the importance of intact salicylic acid signalling for potato defences against *A. solani*. Yet, the exact mechanisms by which SA signalling contributes to these defences remains to be elucidated. Additionally, it is not known what the influence of varying levels of salicylic acid on the potato responses to early blight is. Studies utilizing, for example, targeted gene or regulatory gene element editing techniques to increase or decrease the biosynthesis of SA and/or downstream signalling in several different cultivars followed by disease bioassays and field trials would allow for a more precise understanding of the roles of SA signalling in potato defences against early blight.

In both paper I and IV several potato and *A. solani* genes were detected that would be of interest for validation studies of their roles in resistance or susceptibility of potato and virulence of *A. solani.* Bioassays and infection studies using e.g. CRISPR-Cas9 mutant plants and genetically modified *A. solani* could help elucidate the roles of these genes and their role in the

establishment of disease. Additionally, since the findings of our studies on potato-*A. solani* interactions revealed different plant responses than those usually seen in response to necrotrophy, further elucidation of the nutrient acquisition of *A. solani* as well as characterization of the potential secreted effectors and mycotoxins is advised. For example, by combining laser dissection microscopy of infection structures and specific tissues with transcriptomics and metabolomics.

The ionomic distribution differences to inoculation with *P. infestans* in susceptible and resistant plant lines observed in paper II, present excellent targets for further elucidation. The combination of ionomics and other 'omics' techniques such as transcriptomics, metabolomics and proteomics is highly suggested. By combining these techniques, genes or proteins involved in immunity and susceptibility related redistribution of chemical elements in the plant tissue could be identified as new targets for optimized resistance breeding strategies. Additionally, the specific element redistribution patterns associated with resistance and susceptibility could be considered as biomarkers and utilized for phenotyping resistance breeding material. Lastly, the results from ionomic studies such as in paper II can be a base for the development of crop optimized and disease limiting fertilizer regimes in future precision agriculture.

The results from paper III indicate the existence of *A. solani* secreted molecule(s) that directly negatively influence the germination and growth of *P. infestans*. Identification through metabolomics and proteomics of such molecules and additional identification of the *P. infestans* target site of these molecules, could be used for the development of new control agents. Transcriptomics of the tripartite interaction of *S. tuberosum, P. infestans*, and *A. solani* could help discover new, or core/common plant responses to disease by analysing the differences in plant gene expression between single and double infections and identifying commonalities that could be exploited in future breeding efforts. Additionally, metabolomics could clarify whether *P. infestans* inhibiting molecule(s) are present in the plant and/or the tripartite interaction. By combining metabolomics and transcriptomics, the gene(s) involved in the molecule(s) synthesis and the expression levels during infection could be elucidated.

Finally, in my opinion, it is of utmost importance to validate results obtained in controlled environments, such as laboratory experiments, in agriculturally important field settings in representative climates for the potato growing regions and by using multiple cultivars used in potato production. In science there is often an air of prestige and superiority associated with basic research exemplified by higher impact factors and whatnots. Whilst extremely valuable, only when combining basic and applied knowledge can we, as scientists, truly offer solutions to pressing world problems. I, therefore, hope, in the future, we will see more papers comparing *in vitro, in planta* and *in agro* data.

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Popular science summary

Whether boiled, fried as chips or fries, used in curries, or as a binding agent in the form of potato starch, it is impossible to imagine modern world cuisine without potatoes. The humble spud has become the third most important crop consumed by humans. In order to fulfil the global demand for potatoes, subsistence and commercial farmers grow them all over the world. However, the production of potatoes, like all other crops, is not without problems. Several potato diseases have the potential to reduce yield dramatically. The two most destructive diseases are potato early blight and late blight, caused by the fungal pathogen *Alternaria solani* and the fungus-like pathogen *Phytophthora infestans* respectively. Both diseases, when no control measures are taken, lead to death of the leaves and thus prevent the plants from generating the energy required to produce the desired tubers. Additionally, if the plant has already produced tubers, the diseases can make the tubers inedible. Farmers, therefore, employ several methods to prevent and control disease. The most used and efficient way to control both blights is the application of fungicides. However, due to fungicide resistance in the pathogens, the changing climate, and adverse effects of some fungicides on the environment, improved fungicides or other control strategies are required. In order to contribute to the development of more sustainable control of both blights in the future, the research presented in this thesis focused on improving our understanding of how the pathogens influence and interact with the plant to cause disease. The gene expression of plants during infection with the early blight pathogen and the role of plant hormones in the severity of the early blight symptoms were studied. We found several genes that were differentially expressed during disease and that the plant hormone salicylic acid is important for potato plants to limit the early blight symptoms. Additionally, the chemical elements inside the plant and changes in the distribution patterns of these elements were studied for plants with varying levels of resistance to late blight. Plants with high levels of late blight resistance were found

to have very different element distribution patterns. Lastly, the combined effect of both diseases in the same plant was analysed both in the laboratory and in the field, and we found that late blight occurs less if more early blight is present in a field. Altogether, we have identified several targets for further study and development of future proof disease control methods.

Populärvetenskaplig sammanfattning

Oavsett om den är kokt, friterad som chips eller pommes frites, använd i curryrätter eller som ett bindemedel i form av potatisstärkelse, är det omöjligt att föreställa sig det moderna världsköket utan potatis. Potatisen har blivit den tredje viktigaste konsumerade grödan av världens befolkning. För att uppfylla den globala efterfrågan på potatis odlas den både i hemträdgårdar och av kommersiella jordbrukare över hela världen. Däremot är produktionen av potatis i likhet med de flesta andra grödor inte utan problem. Flera potatissjukdomar har potential att minska avkastningen dramatiskt. De två mest destruktiva sjukdomarna är torrfläcksjuka och potatisbladmögel, orsakade av svamppatogenen *Alternaria solani* och den svampliknande patogenen *Phytophthora infestans*. Båda sjukdomarna leder till att bladen dör och vissnar ner om inga bekämpningsåtgärder vidtas. Detta hindrar växterna från att generera den energi som krävs för att producera de önskade knölarna. Dessutom, om växten redan har producerat knölar, kan sjukdomarna göra knölarna oätliga. Jordbrukare använder därför flera metoder för att förebygga och bekämpa sjukdomar. Det mest använda och effektiva sättet att bekämpa båda sjukdomarna är genom applicering av fungicider (svampgifter). På grund av att patogenerna blir mindre känsliga mot fungiciderna, det förändrade klimatet som kan gynna sjukdomarna och negativa effekter av vissa fungicider på miljön, behövs ständigt nya och förbättrade fungicider eller utveckling av bättre bekämpningsstrategier. För att bidra till utvecklingen av mer hållbara växtskyddsmetoder mot båda sjukdomarna fokuserades forskningen i denna avhandling på att förbättra vår förståelse för hur patogenerna påverkar och samverkar med växten. Jag studerade bl.a. genuttrycket av växter vid infektion med patogenen för torrfläcksjuka och växthormonernas roll i utvecklingen av sjukdomssymptomen. Jag hittade flera gener som uttrycktes olika under sjukdomsförloppet och fann att växthormonet salicylsyra är viktigt för potatisplantornas förmåga att begränsa utvecklingen av symtomen. Dessutom

studerades förändringar i fördelningsmönstret av kemiska grundämnen i potatisplantor med olika grad av motståndskraft mot potatisbladmögel. Plantor med hög grad av motståndskraft mot potatisbladmögel visade sig ha mycket olika fördelningsmönster av grundämnen. Slutligen analyserades i laboratoriestudier och i fält den kombinerade effekten av infektion med båda sjukdomarna. Jag fann att potatisbladmögel utvecklas i mindre omfattning om torrfläcksjuka förekommer tidigare i ett fält. Sammantaget har vi identifierat flera områden för vidare studier som skulle kunna leda till utveckling av nya sjukdomsbekämpningsmetoder.

Populair wetenschappelijke samenvatting

Of het nu gekookt, gebakken, gebruikt in curry's of als bindmiddel in de vorm van aardappelzetmeel is, aardappelen zijn niet weg te denken uit de moderne wereldkeuken. De aardappel is het door mensen op twee na meest geconsumeerde groentegewas. Om aan de enorme wereldwijde vraag te voldoen, worden ze over de hele wereld zowel voor eigen gebruik als commercieel verbouwd. De aardappelteelt is echter niet zonder problemen en verschillende ziektes kunnen de opbrengst drastisch verminderen. De twee meest destructieve aardappelziektes zijn alternaria en phytophthora, respectievelijk veroorzaakt door de schimmelpathogeen *Alternaria solani* en de schimmelachtige ziekteverwekker *Phytophthora infestans*. Beide ziektes leiden zonder bestrijdingsmaatregelen tot het afsterven van de bladeren en de planten kunnen hierdoor niet genoeg energie opwekken voor de knollenproductie. Bovendien, als de plant al knollen geproduceerd heeft, kunnen de ziektes de knollen oneetbaar maken. Boeren gebruiken daarom verschillende methodes om ziekte te voorkomen en te bestrijden. De meest gebruikte en efficiënte manier om beide pathogenen te bestrijden is de toepassing van fungiciden (schimmelbestrijdingsmiddelen). Verbeterde fungiciden of andere bestrijdingsstrategieën zijn echter nodig, voornamelijk vanwege groeiende fungicideresistentie, een veranderend klimaat en nadelige effecten van sommige fungiciden op het milieu. Om bij te dragen aan de ontwikkeling van een duurzamere bestrijding van beide aardappelziektes in de toekomst, richtte het in dit proefschrift gepresenteerde onderzoek zich op het verbeteren van ons begrip over hoe de pathogenen en de plant elkaar beïnvloeden en tot ziekte leiden. De genexpressie van planten tijdens *A. solani* infectie en de rol van plantenhormonen in de ontwikkkeling van ziekte symptomen werden bestudeerd. We vonden verschillende genen die differentieel tot expressie kwamen tijdens ziekte en dat het plantenhormoon salicylzuur belangrijk is voor aardappelplanten om ziekte te beperken. Bovendien werden de chemische elementen in de plant en veranderingen in de distributie van

deze elementen bestudeerd voor planten met verschillende niveaus van resistentie tegen phytophthora. Planten met een hoge mate van resistentie bleken zeer verschillende verspreidingspatronen van elementen te hebben ten opzichte van de controle planten met matige resistentie. Tenslotte werd het gecombineerde effect van beide ziektes in dezelfde plant en het aardappelveld geanalyseerd. We ontdekten dat phytophthora minder voorkomt als er meer alternaria in een veld aanwezig is. Samenvattend hebben we verschillende doelwitten geïdentificeerd voor verdere studie en draagt dit onderzoek bij aan de ontwikkeling van toekomstbestendige ziektebestrijdingsmethodes.

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'It was the best of times, it was the worst of times, it was the age of wisdom, it was the age of foolishness'

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The production of potato is plagued by several yield reducing diseases. The major causal agents of disease are *Phytophthora infestans* and *Alternaria solani*, causing late blight and early blight respectively. This tale of two blights broadens our understanding of potato–pathogen interactions on new levels, such as the transcriptome for *A. solani* and the ionome for *P. infestans* but also emphasizes the importance of studying the impact of multiple diseases together.

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