Host-Pathogen Interactions in Root Infecting Oomycete Species

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Cover: Pea plant infected with *Phytophthora pisi* (left), control plant (right). Sporangia releasing zoospores (left) and oospores (right) in infected root tissue.
(Photo: Sara Hosseini)

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Abstract

The oomycetes include some of the most devastating pathogens on both cultivated crops and wild plants. In the genus *Phytophthora* some closely related species have a broad host range, while others are very host specific. The aim of this project was to gain an understanding of the mechanisms underlying the differentiation of a subgroup of root-infecting *Phytophthora* species and to gain knowledge about the plant immune responses triggered by distantly related oomycetes that adapted to the same legume host.

We investigated the zoospore chemotaxis of legume-root infecting *Phytophthora* species to different isoflavonoid compounds and explored a possible connection to host preference. Our results showed that specific chemotaxis towards host isoflavones is of limited importance in *Phytophthora sojae* and *Phytophthora vignae*, while, specific chemotaxis of *Phytophthora pisi* and *Phytophthora niederhauserii* indicated an adaptation to their pathogenicity on the host and lack of pathogenicity on non-host plants.

The comparative proteomic study of *P. pisi* and *P. sojae* in germinating cysts and hyphae, using tandem mass spectrometry, revealed specific and common pathogenicity factors involved in initiation of infection and host specificity such as serine proteases, membrane transporters and ricin-B lectin in these closely related species. Furthermore, the data suggested that germinating cysts catabolize lipid reserves through the β-oxidation pathway and the glyoxylate cycle to initiate infection.

The transcriptomic response of pea plants towards *Aphanomyces euteiches* and *P. pisi,* two distantly related oomycetes, was studied during early phase of infection, using a microarray approach. The results deciphered common and specific immune mechanisms towards these pathogens. Activation of cell wall modification, regulation of jasmonic acid biosynthesis and induction of the ethylene signaling pathway were among the common transcriptional responses to both pathogens. However, induction of chalcone synthesis and auxin signaling were specific transcriptional changes against *A. euteiches*.

Keywords: chemotaxis, effector, immune responses, isoflavonoid, pathogenicity factor, *Phytophthora*.

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Dedication

To my beloved parents Nasrin and Mostafa.

As you start to walk out on the way the way appears.
Rumi

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 **Hosseini, S.,** Heyman, F., Olsson, U., Broberg, A., Jensen Funck, D. and Karlsson, M., (2014). Zoospore chemotaxis of closely related legume-root infecting *Phytophthora* species towards host isoflavones. *Plant Pathology* 63(3), 708-7014.

II **Hosseini, S.,** Resjö, S., Liu, Y., Brandström-Durling, M., Heyman, F., Levander, F., Liu, Y., Elfstrand, M., Funck Jensen, D., Andreasson, E. and Karlsson, M., (2015). Comparative proteomic analysis of hyphae and germinating cysts of *Phytophthora pisi* and *Phytophthora sojae*. *Journal of Proteomics* 117, 24-40.

III **Hosseini, S**., Elfstrand, M., Heyman, F., Funck Jensen, D. and Karlsson, M., (2015). Deciphering common and specific transcriptional immune responses in pea towards the oomycete pathogens *Aphanomyces euteiches* and *Phytophthora pisi*. (*Submitted*).

The contribution of Sara Hosseini to the papers included in this thesis was as follows:

I Participated in the experimental design, set up the assay, performed the experiments. Wrote the paper assisted by co-authors.

II Participated in the experimental design, conducted the experiment, performed the bioinformatics analysis. Wrote the paper in cooperation with co-authors.

III Participated in the design of the project, performed the experiment and analysed the data. Wrote the paper in cooperation with co-authors.

Abbreviations

CHS	Chalcone synthases
CRNs	Crinklers
EIDs	Emerging infectious diseases
ET	Ethylene
ETI	Effector-triggered immunity
GO	Gene Ontology
GSL	Glucan synthase–like (Callose synthases)
JA	Jasmonic acid
KOG	Eukaryotic Orthologous Groups
LOX	Lipoxygenase
LRR	Leucine rich repeat
MS/MS	Tandem mass spectrometry
NB	Nucleotide binding domain
NIPs	Necrosis-inducing proteins
PAMPs	Pathogen-associated molecular patterns
PRRs	Pattern recognition receptors
PTI	Pattern-triggered immunity
RT-qPCR	Reverse transcription quantitative PCR
RXLR	Effectors containing Arginine-any amino acid-Leucine-Arginine motifs
SA	Salicylic acid

1 Introduction

Plant diseases are constant threats to crop production causing yield reduction and reduction of product quality in horticulture, agriculture and forestry. Emerging infectious diseases (EIDs) caused by fungi and oomycetes, the two most important groups of eukaryotic plant pathogens, are presenting a worldwide threat to food security (Fisher *et al.*, 2012). EIDs are those pathogens that are increasing in their incidence, geographic or host range, and virulence (Jones *et al.*, 2008). Among oomycetes, the genus *Phytophthora* (the "plant destroyers" in Greek) harbors some of the most destructive plant pathogens of dicotyledonous plants (Erwin & Ribeiro, 1996). Plant disease epidemics caused by these pathogens have affected the course of human history. Late blight caused by *Phytophthora infestans* is the most well-known plant disease epidemics that led to the Irish potato famine in the nineteenth century. Current EIDs involving *Phytophthora* species include the "Sudden Oak death", caused by *Phytophthora ramorum*, which causes devastating damage in ecosystems of Western America (Grunwald *et al.*, 2008) and the agent of "Eucalypt dieback" in Australia, *Phytophthora cinnamomi*, which extensively damages wild and cultured woody plants worldwide and is reported to have a host range in excess of 3000 species (Hardham, 2005). A striking feature of *Phytophthora* species is their ability to adapt to different environments. Despite their morphological similarities, closely related species can have different host range. Some species are able to infect a broad range of hosts, while others have a narrow host range.

Devastating *Phytophthora* species in agricultural production include *Phytophthora sojae*, the agent of stem and root rot of soybean, with an annual cost worldwide of $1-2 billion (Tyler, 2007). Root rot caused by *Phytophthora pisi* is an EID on pea and faba bean in Southern Sweden (Heyman *et al.*, 2013). Knowledge of the infection biology of root infecting oomycetes is limited compared the knowledge about the leaf infecting species, in spite of their

ecological and economic effects. As it is very difficult to target soil borne pathogens with traditional pesticide treatments, employment of resistant varieties is the best approach to control soil borne diseases. The crucial step towards management of root rot diseases caused by oomycete pathogens is to gain knowledge about the principles of their infection biology and host responses. Thus, this thesis contributes to the understanding of the mechanism underlining host specificity of legume-root infecting oomycetes, identification of pathogenicity factors at initiation of infection and the plant defence mechanisms that are affected early after infection.

1.1 Oomycetes as plant pathogens

Oomycetes are a diverse group of eukaryotic organisms that are classified to the kingdom *Stramenopila*. They cause destructive diseases on a vast variety of plant and animal hosts (Haas *et al.*, 2009) and have colonized many different niches, but more than 60 % of all known oomycete species are plant parasites (Thines & Kamoun, 2010). Based on the morphology and growth patterns oomycetes and fungi are similar (Latijnhouwers *et al.*, 2003), however they differ in many physiological traits. For example, the oomycete cell wall is mostly composed of cellulose, while chitin is absent. In contrast, in filamentous fungi chitin is one of the main structural components. Phylogenetic analysis revealed that fungi share a common ancestor with animals, whereas, the oomycetes closest relatives are the heterokont golden-brown algae (Baldauf *et al.*, 2000). Oomycetes are divided into the orders Saproleginales, which includes about 500 species, and Peronosporales, which includes 1300 species (Thines & Kamoun, 2010). Among Saproleginales, the genus *Aphanomyces* includes destructive pathogens on plants, crustaceans and fish (Gaulin *et al.*, 2008; Blazer *et al.*, 2002). The plant pathogenic species *Aphanomyces euteiches* (Jones & Drechsler, 1925) causes a disease with high yield reduction in pea production worldwide and also affects other legumes, such as alfalfa (Gaulin *et al.*, 2007; Levenfors *et al.*, 2003; Wicker & Rouxel, 2001). In contrast to the diversity of hosts among *Aphanomyces*, the genus *Phytophthora* that belongs to Peronosporales includes only plant pathogenic species. This diversity within the oomycetes could reflect different evolutionary histories and different mechanism of infection between Saproleginales and Peronosporales (Kamoun, 2001).

To date, the oomycete genus *Phytophthora* consists of over 100 described species (Kroon *et al.*, 2012), many of which are able to infect important crop species in agriculture, horticulture and forestry. Currently 10 clades are phylogenetically distinguished within the genus *Phytophthora* (Kroon *et al.*,

2012). Some species are able to infect a broad range of hosts and attack numerous plant species while others have a narrow host range, only capable to infect one or few plant species. *Phytophthora capsici* is an example of a broad host range pathogen that causes root, crown, foliar and fruit rot on multiple important plant families (Lamour *et al.*, 2012), whereas *P. sojae* is an example of narrow host range species (Kaufmann & Gerdemann, 1958) that causes root and stem rot in soybean.

1.1.1 *Aphanomyces euteiches* and *Phytophthora pisi,* a well-known and an emerging threat for pea cultivation

Legumes are important sources of proteins for human food and animal feed. In addition, legumes improve soil fertility and decrease the need for N fertilizers through symbiotic interaction with nitrogen fixing bacteria, and thus contribute to the sustainability of agriculture (Sugiyama & Yazaki, 2012). Field pea, *Pisum sativum*, is a legume crop that is grown on over 25 million acres worldwide as the forth most important legume crop. The major producing countries of field pea are China, India and USA. In Europe dry pea is the most produced legume and the major pea producing countries are France, Hungary, Turkey, Italy, Russia and Spain (http://www.fao.org/). Frozen green peas are a locally important cash crop in southern Sweden, and a large portion of the annual production is exported to the European market.

Aphanomyces euteiches (Jones & Drechsler, 1925), causing seedling damping off and root rot disease of many legumes, is considered as the most devastating pea pathogen, causing up to 80 % losses each year (Gaulin *et al.*, 2007). It is widespread in North America, Europe, Japan, Australia and New Zealand (Wicker *et al.*, 2003; Grau *et al.*, 1991). Almost a century after its description, Aphanomyces root rot is still considered the most important and destructive disease of pea. Plants at any age can be infected by this pathogen. The first symptoms on the roots appear as softened and water-soaked lesions that develop into the cortical cells affecting the entire root in a severe infection. The epicotyls then become dark and eventually collapse. The damage of the root system leads to wilting symptoms, stunted growth and yellowing leaves and in severe cases the plants die before forming any pod (Gaulin *et al.*, 2007).

Lately, a root rot disease of pea and faba bean (*Vicia faba*) caused by a *Phytophthora* species has been detected in southern Sweden. The agent of this EID is described as *Phytophthora pisi* (Heyman *et al.*, 2013), which is able to infect a group of closely related legumes such as common vetch (*Vicia sativa*), chickpea (*Cicer arietinum*), lentil (*Lens culinaris*) and sweet pea **(***Lathyrus odoratus*), in addition to pea and faba bean. Pea plants infected with this pathogen are stunted with soft rotted roots (Figure 1). In contrast to the

symptoms caused by *A. euteiches*, the rot symptoms caused by *P. pisi* in pea plants are limited to the roots, where a brown discoloration develops over time, and do not expand above the cotyledons (Heyman *et al.*, 2013).

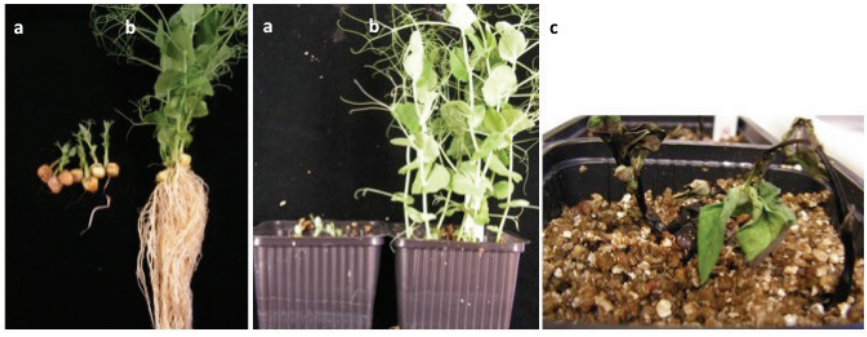

Figure 1. Symptoms caused by *Phytophthora pisi* in three weeks old pea (a) compared to the mock inoculated samples (b) and faba bean (c) in a pot experiment.

Once these root infecting oomycetes have become established at a location, they can be very difficult to control. The thick-walled oospores, which are the resting structures of these pathogens, can remain dormant in soil and organic debris for many years (Shang *et al.*, 2000). Effective chemical control for Aphanomyces root rot of legumes is not available. Using selection-based strategies, pea germplasms with partial resistance are obtained (Hamon *et al.*, 2011; Pilet-Nayel *et al.*, 2005), although no resistant or partially resistant pea varieties are yet commercially available. Currently, disease avoidance by applying crop rotation or bioassay methods to assess the inoculum potential in the soil appears to be the most effective practice to control root rot of pea (Gangneux *et al.*, 2014; Gaulin *et al.*, 2008).

1.1.2 Root infecting *Phytophthora* spp. in clade 7

Phylogenetic analysis shows that *P. pisi* belongs to clade 7b (Heyman *et al.*, 2013) (Figure 2). Clade 7 comprises species mostly pathogenic on roots and subclade b includes a number of important pathogens of the plant family Fabaceae (Heyman *et al.*, 2013). Among the species of this subclade is *P. sojae*, the causal agent of damping off and root rot of soybean (Hildebrand, 1959) that is a closely related species to *P. pisi.* Another species is *Phytophthora vignae*, which is host specific to cowpea (Purss, 1957). In contrast to these species that have a narrow host range, *Phytophthora niederhauserii* is a broad host range species and is capable of infecting plants from 25 different families (Abad *et al.*, 2014).

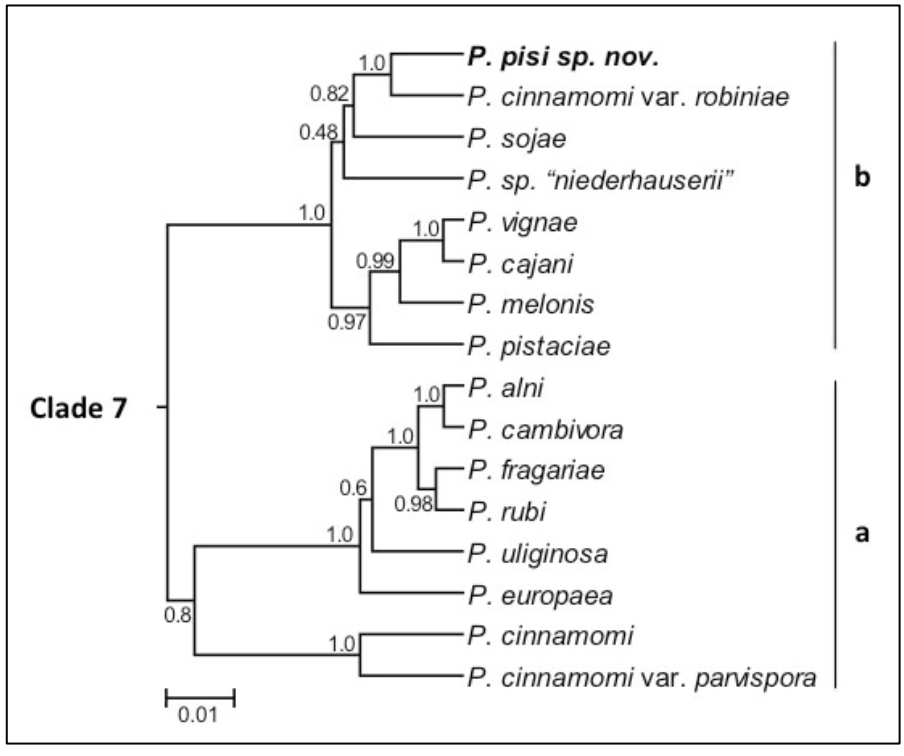

Figure 2. Phylogenetic analysis of *Phytophthora* species in clade 7 based on internal transcribed spacer and cytochrome oxidase 2 data. Numbers on nodes indicate Bayesian posterior probabilities. Reproduced from Heyman *et al*., 2013. *Plant Disease* 97:461-471, by permission from the publisher.

1.1.3 Zoospores, the key factors in initiating infection of oomycete

Oomycete species have asexual and sexual stages in their life cycle. In the sexual cycle, thick-walled oospores, which can survive in the soil up to 10 years, germinate during the growing season as a primary source of inoculum to infect the plants. In the asexual stage, in addition to hyphal growth, uninucleate bi-flagellated motile zoospores are formed by cytoplasmic cleavage within sporangia. Under flooding conditions zoospores are released and swim chemotactically towards plant roots. After attachment to the root surface they differentiate into cysts, which subsequently germinate and penetrate the roots (Figure 3). The speed of asexual formation of zoospores is thus an important factor for success of these root-infecting pathogens.

Most *Phytophthora* species have been described as hemibiotrophic pathogens. After penetration inside root tissues, the infection begins with a biotrophic phase, where the pathogen evades plant defence responses and retrieves nutrients from living host cells. Later, the infection turns to a

necrotrophic phase that leads to host cell death and a build up of pathogen biomass, typically including formation of survival propagules and sporangia, thereby making new infection cycles possible.

For host-specific pathogens and symbionts, the ability to recognize and move in the direction of the host plant signal may be crucial for survival (Morris *et al.*, 1998). Germination and chemotropism in response to a host-specific signal has been described in some plant-fungus interactions. For instance, host flavonoids and pterocarpan isoflavonoids stimulate spore germination of *Fusarium solani,* a pathogen in peas and beans (Ruan *et al.*, 1995), and the fatty alcohol fraction present in the surface wax of host avocado fruit induces spore germination and appressorium formation of *Colletotrichum gloeosporioide* (Podila *et al.*, 1993). Furthermore, in *Rhizobium* bacteria, expression of nodulation genes is induced by flavones or isoflavones (Hassan & Mathesius, 2012; Dharmatilake & Bauer, 1992). Zoospores of oomycetes, which play a key role in spreading the pathogen throughout the soil (Tyler *et al.*, 1996), also exhibit chemotactic responses to plant-derived compounds. For instance, zoospores of *A. euteiches* are highly sensitive to prunetin, the isoflavonoid secreted from the roots of its host plant pea (Sekizaki *et al.*, 1993). Zoospores of *P. sojae* are reported to be attracted specifically to the isoflavones daidzein and genistein, which are exuded by soybean roots. Specific attraction to host compounds is thus suggested as part of the mechanism that ascertains host range (Morris & Ward, 1992). Despite the importance of zoospores in the infection biology of root-infecting oomycetes, the knowledge about their potential role in host range determination is limited.

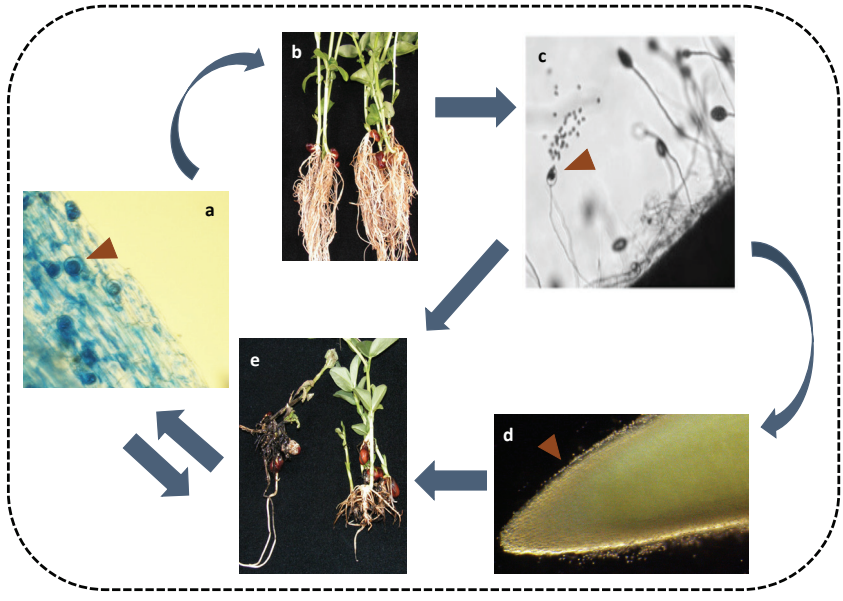

Figure 3. Life cycle of root infecting oomycetes. a) in the sexual life stage oospores are formed inside the root tissue and can survive in the soil for many years as the primary source of inoculum followed by germination during the growing season. b) Oospores germinate to produce hyphae, which may differentiate to sporangia and infect the healthy roots. c) Zoospores are released from sporangia and spread in the soil under flooding conditions. d) Zoospores get attracted to the roots and encyst. e) Cysts penetrate the root tissue and hyphae colonize the whole root tissue.

1.1.4 Oomycete genome structure and pathogenicity factors

The success of oomycetes as plant pathogens depend on their ability to suppress or evade host defence responses and to gain nutrition and proliferate. During infection, oomycete pathogens secrete a variety of extracellular proteins such as cellulose binding elicitor lectin (CBEL) (Gaulin *et al.*, 2006) and cell wall degrading enzymes that contribute to adhesion to the plant surface and plant cell wall degradation, respectively, and therefore to pathogenicity (Kamoun, 2006). In addition, *Phytophthora* species secrete effector proteins to modulate biochemical, morphological and physiological processes of their hosts. These proteins are divided into two broad categories, apoplastic and cytoplasmic effectors with different target sites in the plant. Apoplastic effectors accumulate in the plant interacellular space and include necrosis-inducing proteins (NIPs) (Qutob *et al.*, 2002), elicitins that are small cysteine-rich proteins (Kamoun, 2006) and different enzyme inhibitors such as serine protease inhibitor (EPI) (Tian *et al.*, 2005) and glucanase inhibitor (GIP)

(Denance *et al.*, 2013). Cytoplasmic effectors are translocated into the plant cytoplasm and include two expanded gene families in *Phytophthora*, known as RXLR effectors (Birch *et al.*, 2006) and Crinklers (CRNs) (Torto *et al.*, 2003). The RXLR effectors share the conserved RXLR amino acid motif (arginine, any amino acid, leucine, arginine), the domain required for delivery inside plant cells, followed by diverse, rapidly evolving carboxy-terminal domains that are responsible for the virulence-related function of the effectors (Birch *et al.*, 2008). CRNs are necrosis-inducing proteins that have a conserved FLAK motif for translocation, and are targeted to the host nucleus upon delivery (Schornack *et al.*, 2010). Differences in gene family expansion and diversity, in particular dynamic repertoires of effector genes, are probably responsible for different traits among *Phytophthora* species, such as altered host specificity. Unlike the RXLR effectors, CRNs are present in the genome and transcriptome of all examined plant pathogenic oomycete species including *Pythium ultimum*, *Albugo candida*, and *A. euteiches* indicating that the CRNs form an ancient effector family that arose early in oomycete evolution (Schornack *et al.*, 2010).

Whole-genome sequencing of oomycete species began with *P. sojae* (95 Mb) and *P. ramorum* (65 Mb) followed by *P. infestans* (240 Mb) (Haas *et al.*, 2009; Tyler *et al.*, 2006). Genome structure analysis of these three *Phytophthora* species revealed that the conserved genes are present in regions where gene density is high and repeat content is relatively low (the core genome), whereas non-conserved genes are located in regions with low gene density and high repeat content (the plastic genome). The core genome contains genes involved in cellular processes such as DNA replication, transcription and protein translation, whereas genes involved in plant infection, such as fast-evolving effectors, are predominantly located in the gene-sparse or plastic region, which is highly dynamic. This probably plays a crucial part in the rapid adaptability of these pathogens to host plants and derives their evolutionary potential (Haas *et al.*, 2009).

1.2 Plant defence mechanisms

1.2.1 Plant-microbe interactions

In nature, plants are under continuous biotic stress caused by different pathogens and pests, which exploits highly specialized features to establish a parasitic relationship with their hosts (Pieterse *et al.*, 2009). In addition, in the rhizosphere many plants have symbiotic interactions with mycorrhizal fungi or rhizobacteria, which may provide benefits to the plant. Therefore, plants have evolved processes to discriminate pathogenic and beneficial interactions in order to prevent the disease while supporting the advantageous interactions.

On the other hand, recent studies show that pathogenic and symbiotic filamentous fungi use common plant genetic elements for root colonization. For instance, in *Medicago truncatula* a cutin-derived signal is required for formation of both mycorrhizal hyphopodia and *Phytophthora palmivora* appressoria, which are the essential structures in symbiotic fungi and pathogenic oomycetes for initial invasion of plants (Wang *et al.*, 2012). Furthermore, the secreted isoflavonoids that have a role in initiation of Rhizobium-legume symbiosis interactions were also shown to be chemoattractants for pathogenic oomycetes (Morris & Ward, 1992). Taken together, the fact that symbiotic and pathogenic microorganism use the same plant signals or mechanisms for their interaction support the idea that plants evolved mechanisms for establishing symbiosis while pathogens have taken the advantage of these pathways (Rey *et al.*, 2014). Thus, plant-microbe interactions in the rhizosphere are a complex and dynamic process with cross interference of pathogenic and symbiotic relationships that also intersects with plant development (Evangelisti *et al.*, 2014).

The co-evolutionary arms race between the pathogen and the plant host has prompted varied pathogen virulence strategies and modified plant defence mechanisms. The plant host must protect itself against pathogens for survival, whereas the pathogen has to evade or suppress host immune response to proliferate (Lu & Desveaux, 2013). Therefore, plant-pathogen interaction is a dynamic interplay between host defence mechanisms and specialized pathogen factors.

In order to defend themselves against all different types of pathogens, plants have an array of mechanical and chemical barriers through the physical structures and production of antimicrobial metabolites and proteins. These types of defences are non-specific and called pre-invasive layer of defence (Pieterse *et al.*, 2009). Furthermore, plants have evolved sophisticated strategies to recruit a broad range of inducible defences upon an attack, which can be called post-invasive layer of defence (Jones & Dangl, 2006). In the primary layer of immunity, plants recognize conserved microbial features, known as microbe-associated molecular pattern (MAMPs) (Zipfel & Robatzek, 2010; Jones & Dangl, 2006). These molecules include bacterial flagellins, fungal chitin and oomycete glucans (Hein *et al.*, 2009; Ingle *et al.*, 2006) and are perceived by pattern recognition receptors (PRRs) that in turn initiate downstream signaling events that eventually result in activation of pattern-triggered immunity (PTI). This layer of immunity activates basal early defence responses such as an oxidative burst, and cell wall reinforcement to a broad range of pathogens. To counter this primary immune response, specialized pathogens evolved effectors that suppress PTI and result in effector-triggered

susceptibility (ETS), representing the first level at which molecular co-evolution between pathogen and plant occurs. In turn, plants have acquired resistance (R) proteins that recognize particular effectors or their activity leading to the next layer of defence called effector-triggered immunity (ETI) (Chisholm *et al.*, 2006; Jones & Dangl, 2006), which represents the second level of plant-pathogen co-evolution, as effectors evolve to evade detection and R proteins evolve to maintain detection. Ultimately, this "zig-zag" of firmly co-evolving molecular interactions regulates the final outcome of the infection process (Hein *et al.*, 2009; Chisholm *et al.*, 2006; Jones & Dangl, 2006) (Figure 4). The zig-zag model in oomycte-plant interactions is shown in figure 5.

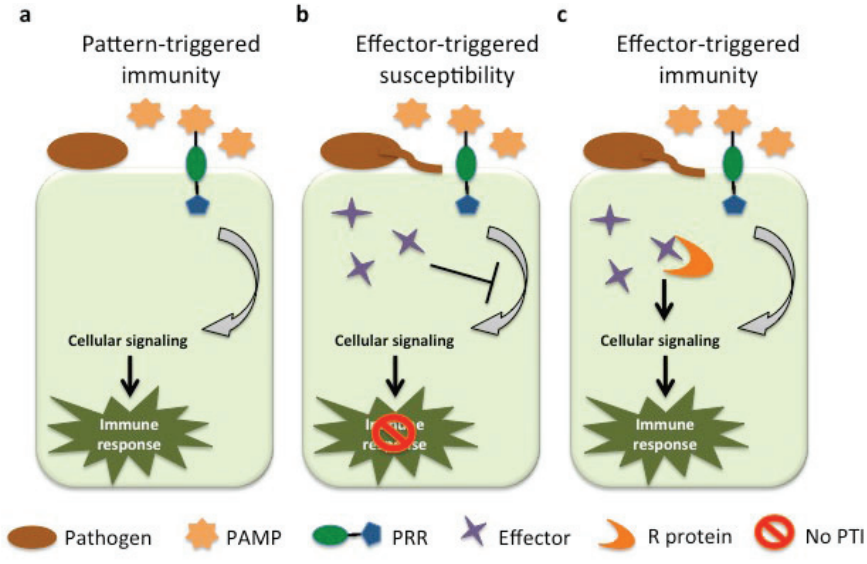

Figure 4. Simplified schematic representation of the plant immune system. (**a**) Upon pathogen attack, pathogen-associated molecular patterns (PAMPs) activate pattern-recognition receptors (PRRs) in the host, resulting in a downstream signaling cascade that leads to pattern-triggered immunity (PTI). (**b**) Virulent pathogens have acquired effectors that suppress PTI, resulting in effector-triggered susceptibility (ETS). (**c**) In turn, plants have acquired resistance (R) proteins that recognize these effectors, resulting in a secondary immune response called effector-triggered immunity (ETI). Adopted from Pieterse *et al*., 2009.

Immune responses are generally resource-expensive and thus plants need to prioritize towards either growth or defence, depending on the external and internal factors. Therefore, plants must avoid unnecessary responses to different types of microbes (Huot *et al.*, 2014). The specificity of pathogen recognition affects the balance between necessary and unnecessary immune responses. Therefore, the PTI responses, which are triggered by the non-

specific structural microbial molecules, start slowly at early phase and increase gradually in the case of continuous or increasing MAMP signalling in the late stage. In contrast to PTI, ETI responses that are activated upon recognition of the pathogen effectors by the corresponding R protein are strong and rapid at the early stage and remain robust at the late stage by network compensation (Katagiri & Tsuda, 2010). Although the pathogen is detected distinctly in PTI and ETI, these two modes of plant immunity are not separated but rather form an incorporated immune system that lead to activation of partly overlapping signalling sectors and responses (Cui *et al.*, 2014; Sato *et al.*, 2010; Pieterse *et al.*, 2009).

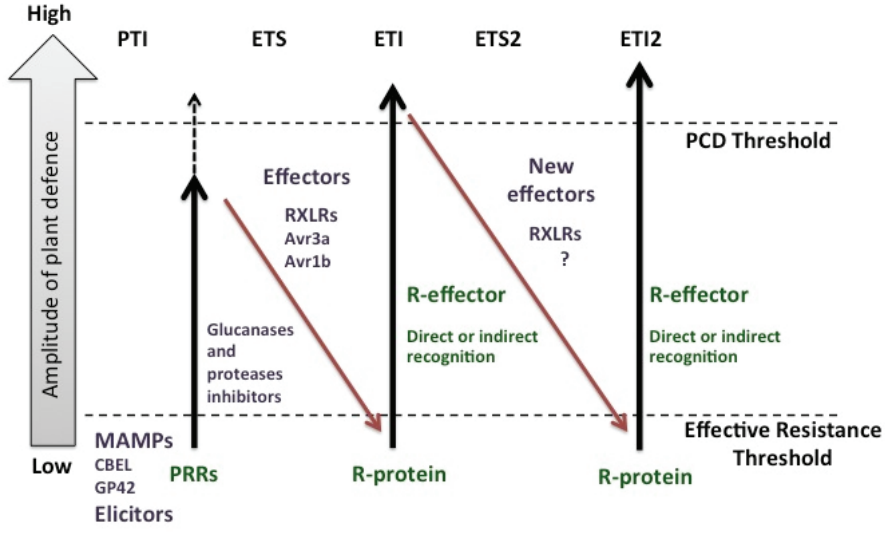

Figure 5. The zig-zag in oomycetes-plant interactions. Examples of oomycetes microbe-associated molecular patterns (MAMPs) that lead to pattern-triggered immunity (PTI): CBEL, cellulose-binding elicitor lectin; GP42, a member of transglutaminase. Elicitors can contribute to the host programmed cell death (PCD), which is represented by a dotted arrow extending PTI above the threshold. The amplitude of defence is shown on the *y* axis. Adopted from Hein *et al*., 2009.

1.2.2 Induced defence mechanisms with a focus on legume-oomycete interactions

Plant induced defence against oomycetes results in a range of responses such as ion effluxes, generation of reactive oxygen species (ROS), hypersensitive reaction (HR), cell wall reinforcement, synthesis of pathogenesis-related (PR) proteins and phytoalexin synthesis.

Typically the HR, a form of programmed cell death (PCD), is associated with ETI responses, while it can also be part of PTI responses (Thomma *et al.*,

2011). HR is highly effective against biotrophic pathogens, such as downy mildews and viruses. HR also can be effective against hemibiotrophic pathogens if it is triggered in the biotrophic phase of infection, while its delay can favour necrotrophic proliferation of the pathogen. Therefore, the ability of hemibiotrophic oomycetes to suppress or delay the HR of plant tissue is likely to be of major importance as a pathogenicity mechanism. The HR is reported to be suppressed by the *P. sojae* RXLR effector Avr1b in soybean (Dou *et al.*, 2008).

The PR proteins are plant-specific proteins currently classified into 17 functional families and possess antimicrobial activities through hydrolytic activities on cell walls, contact toxicity and possibly an involvement in defense signaling. PR proteins such as β-1,3-glucanases and chitinases are constitutively present in plants in several forms, with specific activities and thus can be only enhanced, rather than induced, by pathogen infection. The apparent association between PR-1 proteins, which are used as markers for pathogen-induced systemic acquired resistance (SAR), and enhanced resistance against oomycetes is noted (van Loon *et al.*, 2006). Legumes are a rich source of PR-10 genes. The PR-10 proteins are cytoplasmic with ribonuclease activity and are induced in roots and other organs in response to biotic and abiotic stresses (Samac & Graham, 2007). Silencing of a *Medicago* PR-10 resulted in reduced susceptibility to *A. euteiches* (Colditz *et al.*, 2007).

Many of the secondary metabolites that are produced through the phenylpropanoid pathway can function as preformed or inducible physical and chemical antimicrobial barriers, as well as signal molecules involved in local and systemic signalling in plant immunity system (Naoumkina *et al.*, 2010; Dixon *et al.*, 2002). These compounds are natural products derived from the amino acid L-phenylalanine and regulate a wide range of physiological processes such as pigmentation of flowers and fruits and many of the plant responses to biotic and abiotic stimuli such as establishment of symbiotic interactions in legumes (Tanaka *et al.*, 2008; Dixon *et al.*, 2002). Phenylpropanoid-based polymers including lignin and suberin contribute substantially to the stability and robustness of plants against mechanical or environmental damage (Vogt, 2010). In addition, other phenolic compounds such as stilbenes and flavonoids are synthesized through the phenylpropanoid pathway. Flavonoids represent one of the largest classes of plant-specialized metabolites, which have about 10,000 structurally different members, and can be further subdivided into two main groups, namely flavonoids and isoflavonoids (Tahara, 2007). The structural diversity of flavonoids is derived by different modification of the carbon skeleton such as hydroxylation, glycosylation and methylation (Winkel-Shirley, 2001). Flavonoids can function

as antimicrobial agents, UV protectants, floral pigments, auxin transport regulators and inducers of the nodulation genes in symbiotic rhizobia. A subclass of flavonoids is composed of isoflavonoids, which are mostly limited to the plant family Leguminosae and are believed to represent the majority of phytoalexins produced by legume plants such as medicarpin from alfalfa, pisatin from pea and maackiain from chickpea (Hassan & Mathesius, 2012; Dixon *et al.*, 2002). Specifically local resistance of pea root tips against *A. euteiches* is shown to be associated with an increase in pisatin production in the border cells (Cannesan *et al.*, 2011). Furthermore, soybean partial resistance to *Fusarium solani* seems to be associated with the ability of soybean roots to produce the phytoalexin glyceollin (Lozovaya *et al.*, 2004). In addition to their role as phytoalexins, the isoflavonoids act as chemoattractants for oomycetes legume pathogens, thereby presenting the legume host with an evolutionary conflict.

1.2.3 Plant hormonal signalling in interaction with oomycetes

Plant immunity is activated by a complex signaling network, in which the network components and the network sectors interact with each other (Sato *et al.*, 2010). The regulation of pathogen-induced plant defence responses tightly depend on the phytohormones signaling, mainly mediated by the salicylic acid (SA) and jasmonic acid (JA)/ethylene (ET) pathways. Furthermore, other hormones such as auxin, brassinosteroids (BR), abscisic acid (ABA), cytokinins (CK) and gibberellins (GA) that were originally described to regulate plant development and growth, are recently reported as crucial regulators of defence responses (Robert-Seilaniantz *et al.*, 2011).

Classically, the SA-signalling is required for establishing the local and systemic acquired resistance (SAR) to different biotrophic and hemibiotrophic pathogens. A transcriptional factor, NPR1 (nonexpressor of PR genes 1), has been shown to have a crucial role in NPR1-dependent SA-mediated signalling pathway for several plant species such as *Arabidopsis*, tobacco and rice (Vlot *et al.*, 2009). In the absence of SA, NPR1 is localized in the cytoplasm as an oligomer. Accumulation of SA induces a redox change in the cell, leading to the dissociation of the NPR1 complex and migration of NPR1 monomers into the nucleus, where it regulates the expression of target genes (Figure 6) (Fu & Dong, 2013; Vlot *et al.*, 2009).

In contrast to SA, the JA/ET signalling is often found to be involved against necrotrophic pathogens (Loake & Grant, 2007; van West *et al.*, 2003). Jasmonates, which are lipid-derived molecules from α-linolenic acid, are involved in different processes such as development, light responses and

biotic/abiotic stress signaling. The JA responses are regulated through the F-box COI1 SCF (Skip/Cullin/F box) E3 ubiquitin ligase complex (UPP complex). In the presence of JA signals, the JAZ protein that negatively regulates a key transcriptional activator of jasmonate responses (MYC2) binds to the UPP complex SCF^{COL1} and is degraded by the proteasome, leading to activation of JA responses (Robert-Seilaniantz *et al.*, 2011). Ethylene is involved in many aspects of the plant life cycle such as seed germination, root hair development, root nodulation and fruit ripening and also have role in response to biotic and abiotic stresses. After perception of ethylene by the corresponding receptors that are located on the endoplasmic reticulum (ER), the transcription factor EIN3 accumulates in nuclei where it regulates the expression of many target genes, such as ERF1 (Figure 6) (Robert-Seilaniantz *et al.*, 2011).

The auxin phytohormone is involved in all aspects of plant developmental processes such as lateral root development, vascular differentiation, embryogenesis, flower development, cell division and elongation (Dharmasiri & Estelle, 2004). In addition, auxin plays an important role in plant-microbe interactions. Auxin signaling is mediated by the UPP complex SCF^{TIR1} (analogous to that involved in JA signaling) that accelerates degradation of auxin/indole-3-acetic acid (AUX/IAA), which are negative regulators of auxin responsive genes, in response to auxin. Degradation of these repressor proteins leads to activation of the transcription factors called auxin response factors (ARFs) that are positive regulators of auxin signaling (Figure 6) (Robert-Seilaniantz *et al.*, 2011; Dharmasiri & Estelle, 2004).

All these hormonal pathways interact to each other in a complex, huge network with highly interconnected components (Denance *et al.*, 2013; Robert-Seilaniantz *et al.*, 2007). The fact that plant growth regulators are tightly involved in the plant immune response suggests that developmental and defence signaling networks are interconnected (Pieterse *et al.*, 2009). The outcome of plant-pathogen interactions is strongly influenced by the balance of hormonal crosstalk. The primary mode of interaction between SA and JA appears to be mutual antagonism (Spoel *et al.*, 2003), however, synergistic interactions between SA and JA/ET are also reported in some pathosystems (Pieterse *et al.*, 2009). In many cases, JA and ET signalling pathways interact synergistically (Pre *et al.*, 2008; Lorenzo *et al.*, 2003). The CK, GA and BR are reported to positively interact with SA-mediated resistance to biotrophic pathogens. However, auxin and ABA are shown to modulate the SA sector negatively (Robert-Seilaniantz *et al.*, 2011). The current understanding of plant hormonal responses has been achieved through the study of a limited number of models, which may restrict our view on the true plant defence mechanisms

against pathogens. In general the output of the hormonal network depends on the specific plant-pathogen interactions (Robert-Seilaniantz *et al.*, 2011).

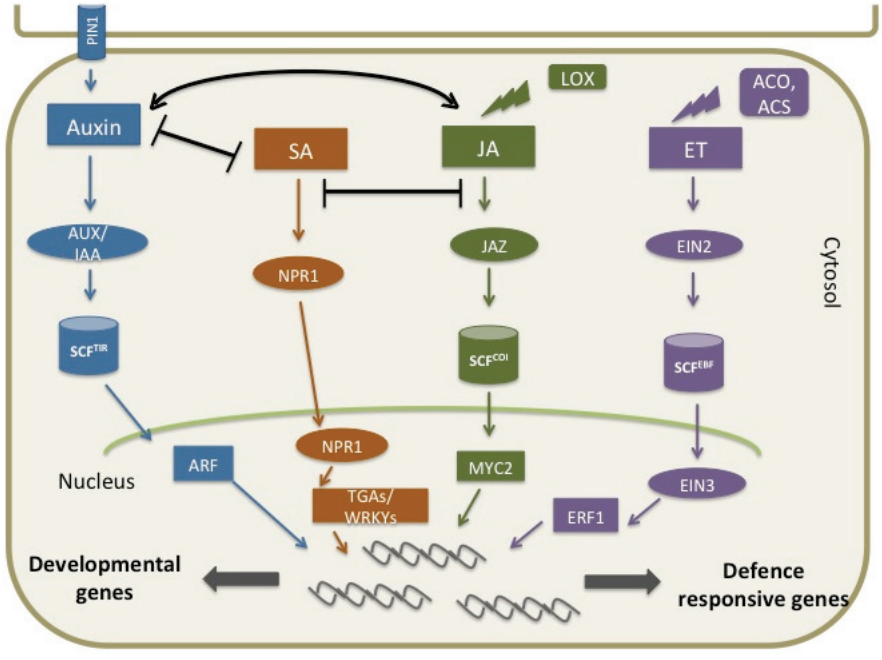

Figure 6. Schematic picture of hormonal signalling network in plant defence. Adopted from Pieterse *et al*., 2009 and Huot *et al*., 2014.

In soybean, application of 1-aminocyclopropane-1-carboxylic acid (ACC, an intermediate ET biosynthesis) was shown to increase resistance against *P. sojae* through induction of PR genes. Moreover, application of benzothiadiazole (BTH, an activator of SA signaling) was reported to increase soybean resistance to this pathogen. In contrast, GA and ABA were reported to increase the soybean seedlings susceptibility to *P. sojae* through antagonistic interaction on the SA and ET-signaling pathways (Sugano *et al.*, 2013). Furthermore, transcriptional activation of SA, ET and BR phytohormone signaling pathways has been reported in ten soybean near isogenic lines (NILs) in incompatible interaction with *P. sojae* (Lin *et al.*, 2014).

Recently suppression of the auxin pathway has been reported to increase susceptibility to *P. cinnamomi* in lupin (*Lupinus angustifolius*) and *A. thaliana*, indicating an important role of this pathway in defence against this oomycete (Eshraghi *et al.*, 2014b). However, application of auxin was reported to downregulate *A. thaliana* defence against the downy mildew oomycete

pathogen *Hyaloperonospora parasitica* trough inhibition of the SA-mediated defence response (Wang *et al.*, 2007).

The phytohormone ABA, which plays an important role in plant developmental processes and resistance to abiotic stress, is reported to increase susceptibility of plants to biotrophic and hemibiotrophic pathogens such as *P. infestans* (Henfling *et al.*, 1980) and *P. sojae* (McDonald & Cahill, 1999), whiles it enhances resistance to the necrotrophic pathogens such as *Alternaria brassicicola*, possibly through priming for callose accumulation (Ton & Mauch-Mani, 2004). It has been shown that in *A. thaliana*, ABA plays a role in the defence against *Pythium irregulare*, a necrotrophic oomycete. In this interaction, ABA enhances defences through callose priming and activation of JA biosynthesis, leading to regulation of defence genes (Adie *et al.*, 2007). Furthermore, recently ABA is linked to resistance against *P. cinnamomi* that has a predominantly necrotrophic lifestyle (Eshraghi *et al.*, 2014a).

1.3 The pea-*Phytophthora pisi* pathosystem

The *in planta* infection system of *P. pisi* on pea was established to enable laboratory studies of this plant-pathogen interaction system on a molecular level. In this infection system, which is described in details in the "Material and Methods" section, the pea roots were infected with *P. pisi* zoospores and the infection process was evaluated from 2 h post inoculation (hpi) to 72 hpi. By 6 hpi the pathogen colonises five cortical cell layers of pea root tissue using both inter- and intracellular growth, while by 27 hpi the deep cell layers are saturated by pathogen hyphae. By 48 hpi a mass of mature sporangia emerge from the root surface and numerous oospores are formed in the root tissue (Figure 7) (Hosseini *et al.*, 2012).

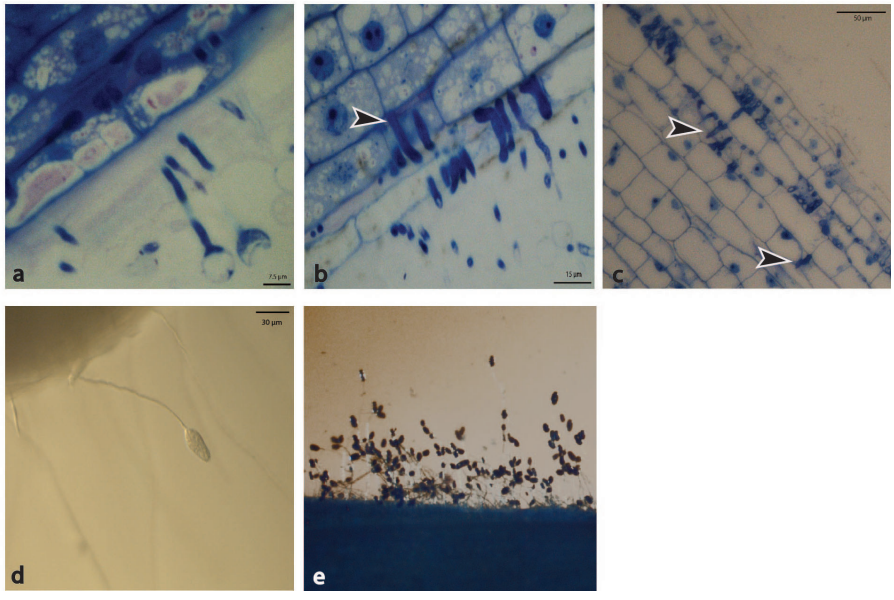

Figure 7. Microscopy and stereomicroscopy observation of *Phytophthora pisi* during *in planta* infection of pea roots. a) Germinated cysts at 2 hpi. b) Colonization of epidermal cells and one cortical cell layer by hypha at 2 hpi. c) Colonization of epidermal cells and five cell layers within the cortex by hypha at 6 hpi. d) Emergence of sporangia (about 40 µm in length) from the root surface at 20 hpi. e) A mass of mature sporangia emerging from the root surface by 48 hpi. Arrows indicate *P. pisi* hyphae (Hosseini *et al*., 2012. *European Journal of Plant Pathology*). The figure is reproduced by permission of the publisher.

2 Objectives

The overall objective of this thesis was to increase the knowledge of the mechanisms underlying host specificity of legume root-infecting oomycetes. More specifically, the objectives were to

- Determine the chemotactic behaviour of zoospores from closely related legume-root infecting *Phytophthora* species and explore a possible connection to host preference (Paper I).

- Test for differential protein abundance in infective and vegetative life stages of the closely related root-infecting *P. pisi* and *P. sojae* and identification of putative specific pathogenicity factors (Paper II).

- Test for differential trancriptomic responses of pea during interaction with two distantly related oomycete species, *A. euteiches* and *P. pisi* (Paper III).

3 Material and Methods

3.1 Biological material

Different strains of *Phytophthora* spp. and *A. euteiches* were maintained on dilute Granini Juice agar (4% filtered multi-vegetable juice and 2% Bacto Agar). For zoospore production, flasks containing 25 ml dilute lima bean broth (Schmitthenner & Bhat, 1994) were inoculated with mycelium and incubated in darkness at 25°C for 72 h. The mycelia were then washed in two or three steps with autoclaved river water, followed by incubation in darkness at 25°C for approximately 20 h (Hosseini *et al.*, 2012). The zoospore concentration in all experiments was adjusted to 10^5 (zoospore/ml). In paper II, for preparation of germinating cysts, zoospores were stimulated to encyst by vigorous shaking, incubated for 2-3 h and harvested by centrifugation. Hyphal samples were grown in clarified lima bean broth for 48 h at 25°C followed by harvest while zoospores were collected by centrifugation. Seeds of different legumes were surface sterilized in sodium hypochlorite (10 % v/v) for 5 min and were germinated in moist autoclaved paper towels by incubation in darkness at 25°C for four days. Healthy germinated seedlings with a root length between 4 and 5 cm were selected. All the material for transcriptomic and proteomics studies were immediately frozen in liquid nitrogen and stored at -80°C.

3.2 Inoculation methods and zoospore assays

3.2.1 Pathogenicity test, zoospore-root attraction and chemotaxis

The pathogenicity of *Phytophthora* species to legume plants was tested using a modification of the inoculum layer method (Walker & Schmitthenner, 1984). An agar culture of each species was placed between 2 layers of vermiculite in a plastic pot, followed by planting the seeds. Pots were incubated at 22°C and 16 h light for 3 weeks. Root symptoms and weight of the dried shoots were scored

as criteria for disease assessment. Root symptoms was inspected visually and expressed as the percentage of the root system with short length, discoloration (necrosis) and soft rotted lesions. Furthermore, the percentage of shoot weight reduction of treated plants compared with control plants was measured. For the zoospore-root attraction test, the distal ends of the roots were cut 3 cm from the tips and attraction of zoospores to the detached roots was studied. The chemotaxis assay was performed on a chamber cavity microscopy slide (Assistent) with capillary tubes as described by Tyler et al. (1996). The degree of chemotactic attraction of zoospores to the capillary tube (Drummond) filled with three isoflavone compounds prunetin, genistein and daidzein (Sigma-Aldrich) at four concentrations (1 mM, 100 µM, 10 µM and 1 µM) was assayed in comparison with the attraction to the tube filled with water as control by counting the number of cysts under a microscope.

Analysis of variance (ANOVA) was conducted on the pathogenicity test data using a Factorial General Linear Model in SAS (2008) package version 9 (SAS Institute). For the zoospore-root attraction data, a Generalized Linear Model with a negative binomial distribution was applied in the Glimmix procedure of the SAS (2008) package. Student's *t*-test was performed on the zoospore attraction data toward each isoflavone compound in Statistica version 9.1 (StatSoft).

3.2.2 *In planta* infection system of pea with *Phytophthora pisi* and *Aphanomyces euteiches* zoospores

Germinated pea seedlings with approximately 4 cm roots were mounted on supporting racks made from the top of 96-tip pipette boxes (Figure 8). Racks carrying the peas were then placed in zoospore suspension and the roots were incubated for 30 min, followed by incubation in autoclaved river water in a climate chamber (16 h light, 27°C). Roots were harvested at 30 min, 1, 2, 6, 20 and 48 hpi in quadruplicates, each consisting of 10 distal ends of the roots.

Figure 8. In planta infection system of pea roots with zoospores of *Phytophthora pisi* and *Aphanomyces euteiches*.

3.3 Analysis of root exudates

Seeds were surface sterilized and germinated as described and the end of the roots of five-day-old legume plants was placed in autoclaved water for 12 h. LC-MS/MS was performed on an HP1100 LC system (Hewlett-Packard) using a Reprosil-Pur ODS-3 column connected to a Bruker maXis Impact mass spectrometer with an ESI-QTOF. The isoflavones daidzein, genistein and prunetin were analysed by isolating and fragmenting the corresponding [M+H]+ ions and subsequently, extracted ion chromatograms were constructed for diagnostic fragment ions (m/z 199**.**075, 215**.**069 and 229**.**085, for daidzein, genistein and prunetin, respectively), and comparisons were made with data from analysis of authentic reference compounds.

3.4 Nucleic acid manipulations and gene expression studies

RNA was extracted from frozen material using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). For the microarray experiment, a phenol-chloroform extraction step was used before the mentioned kit. Traces of DNA were removed by DNase I treatment (Fermentas, St-Leon-Rot, Germany). DNase treated RNA was further diluted to 1-2 µg/µl for the microarray experiment. For cDNA synthesis, 1 µg total DNase treated RNA was reverse transcribed with the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Transcript levels were assessed by reverse transcriptase quantitative PCR (RT-qPCR) in an iQ5 qPCR System (Bio-Rad, Hercules, CA) using SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA). Data normalization was conducted with the expression levels of different genes (*H3* and *β-tubulin* for pea, *actin* and *S3a* for *P. pisi*), and relative quantification was carried out using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001). Analysis of variance (ANOVA) was conducted using a General Linear Model implemented in SPSS ver. 21 (IBM, Armonk, NY). Pairwise comparisons were made using the Fisher's test at the 95% significance level.

3.5 Protein extraction and MS/MS-analysis

To produce the protein lysates, samples were suspended in an extraction buffer consisting of 50 mM Tris-HCL, pH 7,4, 2 mM EDTA, 0.25 % SDS, 50 mM sodium pyrophosphate, 1 mM sodium fluoride and 50 µM sodium orthovanadate followed by sonication and centrifugation. Thirty micrograms of

the protein extracts were separated in an 1-D SDS-PAGE gel. After staining the gel for visualization, the lanes were cut into small slices, washed with NH_4HCO_3 and EtOH, treated with DDT to reduce and alkylate cysteine residues and digested using trypsin (Promega Trypsin Gold, Mass Spectrometry Grade). Samples were desalted using Nest columns (Nest Group, Inc., Southborough, MA) and eluted in formic acid.

The samples were analyzed using a LTQ-Orbitrap Velos (Thermo Fisher Scientific, Inc., Waltham, MA, USA) mass spectrometer interfaced with an Easy-nLC nanoLC system (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The raw data from the Orbitrap was converted to Mascot generic file format (MGF), using ProteoWizard (Kessner *et al.*, 2008). The *P. sojae* data was searched against *P. sojae* V3-GeneCatalog-protein (release date 2011, 04, 01) and the *P. pisi* data was searched against the *P. pisi* protein catalog version 1. For quantitative analysis, a label free approach based on precursor intensities was used (Sandin *et al.*, 2011). The resulting quantitative peptide data was analysed using the DanteR software (Taverner *et al.*, 2012). The "Model Based Filter/Impute/Anova" feature of DanteR was then used to identify proteins with increased or decreased abundance in the different life stages. *P*-values were adjusted using the Benjamini-Hochberg procedure for calculating q (Reiner *et al.*, 2003) and proteins with $q < 0.05$ were considered significant.

3.6 Microarray experiment

The microarray analysis was performed at Affymetrix center at Swegene center for integrative biology at Lund University, Sweden. *Medicago* MedGene-1-0-st array, which is designed based on *M. truncatula* A17 genome version 2 (Mt2.0), was used. cDNA synthesis and labeling was carried out on 200 ng of DNAse treated samples. The experiment included three biological and two technical replicates. Basic Affymetrix chip and Experimental Quality Analyses were performed and data normalization was done as described by Irizarry et al. (2003). This analysis was performed using the Expression Console Software V1.1.2. Signals were $\log_2$ transformed.

3.7 Data analysis

Gene Ontology (GO) annotations were obtained by BlastP or BlastN (Altschul *et al.*, 1997) in Blast2GO (Conesa *et al.*, 2005). Enrichment analysis of GO was conducted in Blast2GO and significance was tested using Fisher's exact test at $P \leq 0.05$ after correction for false discovery rate. Conserved protein

domains were identified using the SMART (Letunic *et al.*, 2012) and InterPro (Hunter *et al.*, 2009) analysis tools.

In paper II, eukaryotic Orthologous Groups (KOG) annotations were obtained by BlastP in webMGA server (Wu *et al.*, 2011) and enrichment analysis was conducted with the hypergeometric distribution test by using the phyper function in the R software (Ver. 3.0.1). Signal peptide assignment was done using a local copy of SignalP v4.1 (Petersen *et al.*, 2011). Orthology between individual proteins of *P. sojae* and *P. pisi* was determined by reciprocal BlastP of their predicted proteins. A pair of proteins was assigned orthology if both proteins were each other's best reciprocal Blast hit with an E-value $\leq 10^{-50}$. Effectors were identified using BlastP against the non-redundant database at NCBI (cut off $\geq$ 40% identity and E-value $\leq 10^{-6}$) and secondly by manual search for the RXLR and CRN motifs within the first 30-60 residues from the N terminus of the hypothetical proteins.

In paper III, the differentially expressed genes were identified using the Limma model in R software. The genes that were statistically differently regulated at each time point compared to the corresponding control samples ($P \leq 0.05$), were considered as responsive genes at that time point. Among these genes, the ones with $\log_2$ expression ratio treatment/control ≥ 0.584 (> 1.5 fold induction or ≤ 0.67 repression) were regarded as differentially expressed genes. The Venny online tool (Oliveros, 2007) was used to generate the venn diagram. Hierarchical clustering of the genes was performed using the HCE3.5 software (Seo *et al.*, 2006) with the complete linkage method and the Manhattan measure. Functional category assignment for differentially expressed genes was conducted using the WEGO online server (Ye *et al.*, 2006). KEGG orthology (KO) and enzyme commission (EC) numbers were obtained in KAAS (Ver. 1.69x) online tool (Moriya *et al.*, 2007). Enzymes were mapped to the KEGG (Kyoto Encyclopedia of Genes and Genomes) database using KEGG mapper-reconstruct pathways tool (Kanehisa & Goto, 2000).

Results and Discussion

3.8 Paper I: Zoospore chemotaxis of closely related legume-root infecting *Phytophthora* species towards host isoflavones

Isoflavonoids such as genistein and daidzein isoflavones, which are rapidly released from the legume roots and seeds in response to biotic and abiotic signals, play a crucial role in establishment of the *Rhizobium*-legume symbiosis by acting as chemo attractants and inducers of nodulation genes (Hassan & Mathesius, 2012). Therefore, chemotactic oomycete legume pathogens have developed mechanisms for recognition of the same chemical signals as *Rhizobium* for their pathogenesis (Morris & Ward, 1992). Zoospores with high chemotactic affinities for isoflavones that are specifically secreted from their host plants could hypothetically be expected to accumulate at higher densities as cysts on these plants compared to non-hosts.

To investigate and compare the relationship between zoospore chemotactic behavior to isoflavones and pathogenicity, the attraction pattern of zoospores of *P. pisi, P. sojae, P. vignae, P. niederhauserii* and *A. euteiches* towards three isoflavones; prunetin, genistein and daidzein, was investigated at four concentrations of those compounds from 1mM to 1 µM. Furthermore, the attraction of zoospores from these *Phytophthora* species to the detached roots of pea, soybean, faba bean, cowpea, lentil and clover as well as their pathogenicity to these legume species was analyzed. Moreover, the presence of these three isoflavones in the root exudate of legume plants was analysed.

Zoospores of *P. sojae* and *A. euteiches* were attracted to all isoflavones ($P \leq 0.040$) (Table 1), which is in agreement with the previous studies (Tyler *et al.*, 1996; Sekizaki *et al.*, 1993; Sekizaki & Yokosawa, 1988). Zoospores of *P. sojae* encysted on all plant roots but caused significant root symptoms ($P < 0.001$) only on soybean (60%) and lentils (49%). The attraction of *P. sojae*

zoospores to the isoflavones genistein and daidzein, is consistent with its attraction to the soybean and cowpea roots. Our analysis showed the presence of these compounds in the root exudate of these plants. The detection of these two isoflavones in soybean root exudate as well as daidzein in cowpea root exudate confirms the results from previous studies (Dakora, 2000; Graham, 1991). However, no isoflavones were detected in any of the other tested plant root exudates. Similarly to zoospores of *P. sojae*, zoospores of *P. vignae* strains showed attraction to all tested isoflavones ($P \leq 0.031$) and all plant roots, however, it showed high specificity to cowpea, causing significant ($P < 0.001$) root symptoms (65%) (Table 2), thus confirming the results from the pathogenicity test reported by Purss (1957).

Interestingly, zoospores of different *P. pisi* and *P. niederhauserii* strains were attracted to prunetin ($P \leq 0.040$), but were not attracted to genistein or daidzein at any of the tested concentrations (Table 1). However, they were to some degree attracted to all tested plant roots. In the pathogenicity test, *P. pisi* showed severe symptoms ($P < 0.001$) on pea (77.5%) and faba bean (65%) and caused 67.5% and 70.5% reduction in dried shoot weight of these plants, respectively ($P \leq 0.002$). Furthermore, it showed pathogenicity on lentil ($P < 0.001$) with 40% root symptoms, but not on soybean, cowpea and alsike clover (Table 2). Thus, the lack of affinity for soybean isoflavones in *P. pisi* is consistent with its lack of pathogenicity on soybean and cowpea. *Phytophthora niederhauserii* was pathogenic to all tested legume plants except soybean and clover (Table 2). It was highly virulent on faba bean, with a severity of 78%, and less virulent on lentil, pea and cowpea (47, 32 and 26.5%, respectively) ($P < 0.001$). This result confirms the view that this species has a broad host range (Abad *et al.*, 2014).

Table 1. *The oomycete zoospore chemotaxis to three isoflavones*

Oomycete species	**Prunetin**	**Genistein**	**Daidzein**
A. euteiches	+	+	+
P. sojae	+	+	+
P. vignae**,** 2 strains	+	+	+
P. pisi, 4 strains	+	-	-
P. niederhauserii*, 3 strains*	+	-	-

Attracted (+), non-attracted (-).

Table 2. *The* Phytophthora *species pathogenicity test on legume plants.*

***Phytophthora* sp.**	**Pea**	**Soybean**	**Cowpea**	**Faba bean**	**Lentil**	**Clover**
P. pisi	++++	-	-	++++	+++	-
P. niederhauserii	++	-	++	++++	+++	-
P. sojae	-	++++	-	-	+++	-
P. vignae	-	-	++++	-	-	-

The number of + refers to the severity of virulence while – refers to non-pathogenicity.

The attraction of zoospores to all tested plant roots, regardless of their secreted isoflavones pattern, might indicate that other factors also affect the zoospore behavior. For instance, different sugars and amino acids are among the attractants for zoospores of most *Phytophthora* species (Khew & Zentmyer, 1973) and even electrical gradients are shown to influence zoospore behaviour and attraction in the rhizosphere (van West *et al.*, 2003). The results of this study suggest that specific chemotaxis toward isoflavones is not the only factor governing zoospore attraction to plant roots and support the studies where zoospore attractions to the roots reported as a non-host-specific behavior (Raftoyannis & Dick, 2006; van West *et al.*, 2003; Deacon, 1988). However, several studies have reported a correlation between zoospore encystment density and disease severity of certain *Phytophthora* species (Erb *et al.*, 1986; Chi & Sabo, 1978), suggesting that the preferential attraction to the host plants root forms the first layer of host specificity determination.

To conclude, attraction of *A. euteiches*, *P. sojae* and *P. vignae* to multiple isoflavones is in contrast with their host specificity on pea, soybean and cowpea, respectively. On the other hand, specific chemotaxis of *P. pisi* to prunetin but not to non-host isoflavones, may indicate a recent adaptation to its host specialization towards pea, as this species is closely related to *P. sojae* (Heyman *et al.*, 2013). The specific affinity to prunetin for *P. niederhauserii*, which is a multihost pathogen, shows that such affinity is not only found in legume-specialized species.

3.9 Paper II: Comparative proteomic analysis of hyphae and germinating cysts of *Phytophthora pisi* and *Phytophthora sojae*

We investigated the proteomic differences between the germinating cysts and hyphae life stages from *P. pisi* and *P. sojae* by MS/MS analysis to identify candidate proteins involved in early infection, vegetative growth and host specificity. Germinating cysts were exposed to pea seeds exudate in order to synchronize cyst germination and to induce pathogenicity factors (Nelson, 1990). Altogether, 2755 *P. pisi* proteins and 2891 *P. sojae* proteins were identified, corresponding to on average 12% of the total predicted proteomes of these two species, which is comparable with other proteomic studies on *P. sojae* (15%) (Savidor *et al.*, 2008). For relative protein expression analysis, protein abundance was determined. In total, quantitative data were obtained for 1613 proteins in *P. pisi* and for 1419 proteins in *P. sojae*. From these, 205 proteins in *P. pisi* and 276 proteins in *P. sojae* showed different abundance between germinating cysts and hyphae.

In *P. pisi*, 215 proteins and in *P. sojae*, 191 proteins were predicted to be secreted proteins, which is about 7% of all identified proteins in each of these species and is consistent with the proportion of secreted proteins estimated from the genome of *P. sojae* (Tyler *et al.*, 2006). Among the putatively secreted proteins, 10 and 29 putative RXLR proteins and 24 and 70 putative CRN proteins were identified in *P. pisi* and *P. sojae*, respectively.

Identified proteins were associated with GO and KOG annotations, and proteins were clustered into functional groups based on their KOG annotation. Among the differentially abundant proteins, proteins assigned to the KOG categories Lipid transport and metabolism, and Energy production and conversion were more abundant ($P \leq 0.05$) in germinating cysts compared to hyphae in both species (Figure 9), suggesting that both pathogens utilize stored lipid reserves to produce energy needed for cyst germination. Among the proteins in this category were those involved in the mitochondrial β-oxidation pathway for fatty acid degradation (Shen & Burger, 2009; Poirier *et al.*, 2006), including acetyl-CoA dehydrogenase and enoyl-CoA hydratase. The analyses further revealed that the KOG categories Carbohydrate transport and metabolism, and Coenzyme transport and metabolism were enriched ($P \leq 0.05$) in the hyphal proteome compared to germinating cysts in *P. pisi* (Figure 1A). In *P. sojae*, the KOG category Secondary metabolites biosynthesis, transport and catabolism was enriched ($P \leq 0.05$) in hyphae compared to geminating cysts (Figure 9).

Figure 9. Distribution of protein functional categories (based on KOG annotation) in the differential abundance proteomes of germinating cysts vs hyphae of *Phytophthora pisi* and *Phytophthora sojae*. Only the classes with more than 3% abundance are described.

Orthologous proteins between *P. pisi* and *P. sojae* were identified through reciprocal BlastP searches in order to identify candidate proteins involved in early infection, vegetative growth and species specificity (Figure 8). In total, 58 orthologous protein pairs were more abundant in germinating cysts of both organisms and therefore were identified as candidates for involvement in early infection. Among these shared orthologs, enzymes associated with energy production such as one malate synthase and one isocitrate lyase (ICL), which

are signature enzymes of the glyoxylate cycle (Dubey *et al.*, 2013; Dunn *et al.*, 2009), were identified, suggesting the involvement of this cycle as an additional mechanism of fatty acid catabolism.

Proteins uniquely present in germinating cysts of either *P. pisi* or *P. sojae*, without interspecific orthologs, were considered as candidates for species-specific pathogenicity factors that may be involved in host specificity. Thus, in total, 37 and 19 proteins were recognized as candidates for host specificity in *P. pisi* and *P. sojae*, respectively (Figure 10). Among the specific *P. pisi* candidate proteins for germinating cysts was a serine protease that may be involved in degradation of host proteins during infection. Other candidates were ABC and MFS transporters that may be associated with detoxification, drug resistance and metabolic transport (Torto-Alalibo *et al.*, 2007) and may have role in protection of *P. pisi* against exogenous toxic compounds from the host, or against endogenous compounds (Connolly *et al.*, 2005). Of the specific candidates for *P. sojae* germinating cysts were a serine protease and a ricin b lectin protein.

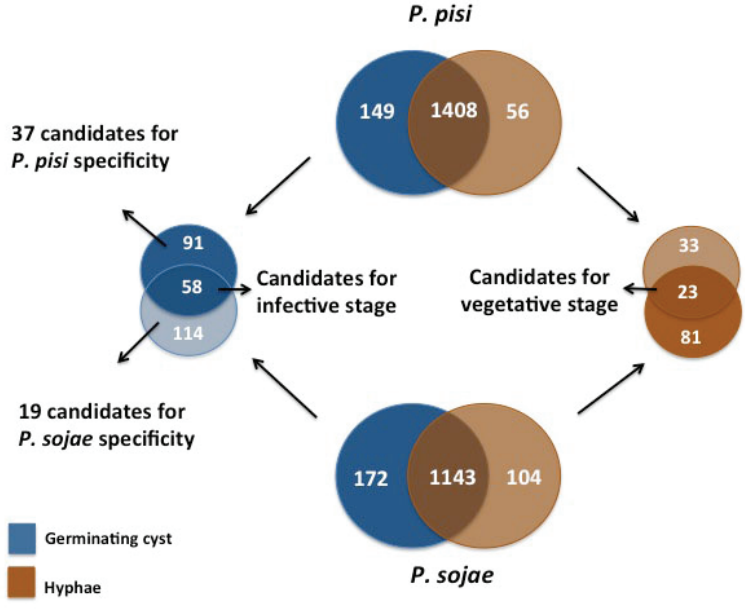

Figure 10. Identification of candidate proteins for involvement in early infection and vegetative growth. The non-overlapping regions represent the differentially abundant proteins in each life stage, whereas the overlapping regions represent the remaining identified proteins. In total 37 abundant proteins in germinating cysts of *P. pisi* and 19 abundant proteins in germinating cysts of *P. sojae* without interspecific orthologs, are candidates for host specificity.

In total, 23 orthologous protein pairs that were abundant in hyphae compared with germinating cysts of both organisms were identified as candidates for involvement in vegetative growth (Figure 10). Among these were one annexin-like protein that is a cell wall associated proteins (Grenville-Briggs *et al.*, 2010; Meijer *et al.*, 2006), and one phospholipase d-domain (PLD)-containing protein, which is a conserved enzyme between *P. pisi*, *P. sojae* and *P. ramorum* (Savidor *et al.*, 2008), involved in various cellular processes including phospholipid metabolism, signal transduction and vesicle trafficking (Meijer *et al.*, 2005; Hube *et al.*, 2001).

To conclude, the results suggest that both pathogens utilize stored lipid compounds for energy production during cyst germination. Furthermore, proteins specifically abundant in germinating cysts of either pathogen are identified and defined as candidate pathogenicity factors for each. The results from this study expand our knowledge on processes and proteins that are common for cyst germination of *P. pisi* and *P. sojae*, but also on factors that may be involved in host specificity.

3.10 Paper III: Deciphering common and specific transcriptional immune responses in pea towards the oomycete pathogens *Aphanomyces euteiches* and *Phytophthora pisi*.

The transcriptome response of pea to *P. pisi* and *A. euteiches*, which are distantly related oomycetes with different pathogenicity factor repertories, was investigated at two time points (6 and 20 hpi) during early phase of infection, using a *M. truncatula* microarray. Limited information is available about the pea defence mechanisms against oomycete infections. Thus, this study provides a global view of genes and possible defence pathways differentially regulated in the susceptible interaction between pea plants with these two pathogens, giving some insight into the transcriptional immune responses induced early after infection.

Genes that were statistically differently regulated at each time point compared to the corresponding control samples ($P \leq 0.05$) were considered as responsive genes at that time point. Among these genes, those with $\log_2$ expression ratio treatment/control ≥ 0.584 (> 1.5 fold induction or ≤ 0.67 repression) were regarded as differentially expressed genes.

Of 37,976 sequences analyzed, 574 and 817 genes were differentially expressed in response to *A. euteiches* at 6 hpi and 20 hpi, respectively. In response to *P. pisi*, 544 and 611 sequences were differentially expressed at 6 hpi and 20 hpi, respectively (Table 3).

Table 3. *Number of differentially regulated genes in pea in response to* Aphanomyces euteiches *and* Phytophthora pisi *at 6 and 20 hours post infection.*

Treatments	Induced genes	Suppressed genes
***A. euteiches*, 6 hpi**	254	320
***A. euteiches*, 20 hpi**	466	351
***P. pisi*, 6 hpi**	310	234
***P. pisi*, 20 hpi**	324	287

Hierarchical clustering of the differentially regulated genes revealed four distinguished clusters. Clusters 1 and 2 represented genes mainly induced at 6 hpi in response to *P. pisi* and *A. euteiches*, respectively. Clusters 3 and 4 included genes mainly induced at 20 hpi in response to *A. euteiches* and *P. pisi*, respectively (Figure 11A). Interestingly, only a limited number of differentially regulated genes were common in response to the two pathogens at 6 and 20 hpi, while large sets of genes were uniquely regulated in response to each

pathogen and at each time point (Figure 11B and 11C). Among all differentially regulated genes, 89 (4%) were associated with the signal transduction process (GO: 0007165) and therefore possibly involved in pathogen perception and signaling. Of those, 41 genes were identified as putative resistance genes (*R*-genes) and receptor-like kinases (*RLK*-genes). Hierarchical clustering of this group of genes showed four distinguished clusters associated with each time points and species, similar to those of all differentially expressed genes. Furthermore, a majority of these genes were specifically upregulated or downregulated at each time point in response to either pathogen. Induction and suppression of genes associated with signal transduction pathway at 6 hpi might suggest that immunity responses (associated with PTI or ETI) occurs early in infection.

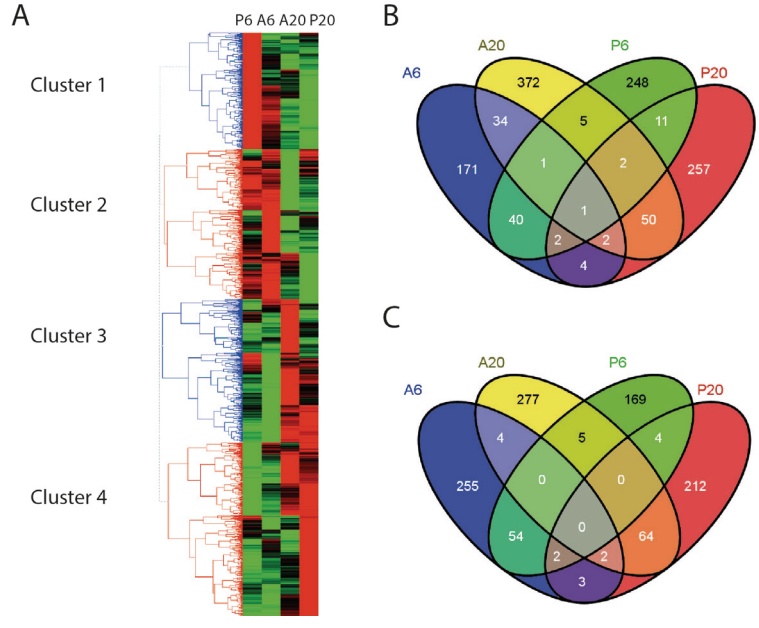

Figure 11. Differentially expressed pea genes in response to *A. euteiches* and *P. pisi.* (A) Hierarchical clustering of all differentially expressed genes ($P \leq 0.05$, ≥ 1.5 fold induction or ≤ 0.67 fold repression) at 6 hpi and 20 hpi compared to the mock-inoculated control samples generated by HCE3.5 software with the complete linkage method and the Manhattan distance measure. Red and green represent up regulated and down regulated genes, respectively. The overlap between (B) up regulated and (C) down regulated gene sets is shown in the Venn diagrams.

Comparisons between time points and pathogen species revealed that some transcriptional changes are common in response to both pathogens. For instance, genes involved in cell wall reinforcement such as two callose

synthases (1,3-β-glucan synthase; *GSL*) and a cinnamyl-alcohol dehydrogenase (*CAD*) that is responsible for the last enzymatic step in the monolignol biosynthesis, were induced in response to both pathogens (Figure 12A), suggesting that activation of cell wall modification in pea is a common immune response against both these oomycetes. Moreover, among the genes associated with hormonal signaling a putative lipoxygenase (*LOX*) gene was suppressed at 6 hpi in response to both pathogens (Figure 12B). Previously, a LOX was shown to be involved in the establishment of incompatibility in tobacco-*P. parasitica* interaction (Rance *et al.*, 1998). Furthermore, JA-deficient mutant tomato plants were shown to be more susceptible to *P. infestans* (Thaler *et al.*, 2004), suggesting the role of JA as an important mediator in plant defence signalling against *Phytophthora* species. Induction of a putative aminocyclopropane-carboxylate oxidase (*ACO*) gene (Figure 12B), which is catalyzing a step in ethylene production (Rudus *et al.*, 2013), suggests that the ET pathway is activated at 6 and 20 hpi in response to both pathogens.

Recently, Lin et al., (2014) showed that in soybean lines resistant to *P. sojae* SA, ET and BR pathways are transcriptionally activated at 24 hpi, while JA pathway is suppressed. In contrast, they reported that in a susceptible interaction JA pathway is activated; ET is suppressed while no changes were reported in SA and BR pathways. The results reported by Lin et al., (2014) is in agreement with our data where JA appears to be activated in pea in a susceptible interaction with *P. pisi* and *A. euteiches* at 20 hpi. Taken together, one speculation from our transcriptional data is that the oomycetes *P. pisi* and *A. euteiches* suppress JA biosynthesis in pea during the early phases of susceptible interactions. Another possible conclusion is that hormonal-based defence responses are delayed due to lack of pathogen perception and signaling.

In contrast to the genes and pathways that are regulated similarly in response to both pathogens, some transcriptional changes appear to be specific to infections by *A. euteiches* or *P. pisi*. For instance, three genes encoding putative auxin-induced SAUR family proteins, which are rapidly and transiently induced in response to auxin (Markakis *et al.*, 2013; McClure & Guilfoyle, 1987), were induced specifically against *A. euteiches* at 20 hpi (Figure 12B). Consequently, the induction of these proteins may indicate an accumulation of auxin in roots during *A. euteiches* infection. The importance of auxin signaling in resistance against different oomycetes has been previously reported (Eshraghi *et al.*, 2014b; Wang *et al.*, 2007; Tiryaki & Staswick, 2002). Recently, an effector of *P. parasitica* has been reported to modulate, possibly to decrease, the *A. thaliana* auxin content locally at the root apex to favour infection (Evangelisti *et al.*, 2013). Thus, a possible interpretation of our data is

that effectors from *A. euteiches* actively target the auxin homeostasis in pea roots to evade defence reaction.

Furthermore, in the phenylpropanoid biosynthesis pathway, five genes putatively encoding chalcone synthases (CHS) that are involved in the early steps of flavonoid biosynthesis were up regulated at 20 hpi specifically in response to *A. euteiches*. Naringenin chalcone, the product of the CHS reaction, is a substrate for the production of a wide range of secondary metabolites such as flavones, isoflavonoid and anthocyanins that are utilized in defence against pathogens and as signaling molecules by legume plants (Dixon *et al.*, 2002). Induction of genes in the phenylpropanoid pathway has been reported in legume plants against fungi and oomycetes (Samac *et al.*, 2011; Foster-Hartnett *et al.*, 2007; Moy *et al.*, 2004; Torregrosa *et al.*, 2004). Thus, the induction of CHS genes during infection by *A. euteiches* might suggest that biosynthesis of secondary metabolites e.g. isoflavonoid phytoalexins, is a part of plant immunity response to this pathogen.

A

Gene	P6h	A6h	P20h	A20h
GSL				
AC137603_39.4	1.0	**1.6***	1.2	0.8
AC155803_42.5	1.0	**1.4**	0.8	1.0
AC155803_41.5	**1.6***	0.9	0.5	**0.5***
AC155803_43.5	1.2	0.9	1.0	0.9
AC122723_35.5	1.1	1.1	1.1	0.9
CU012050_22.4	1.0	0.9	1.0	1.2
AC202574_37.3	1.1	1.1	1.0	**0.8**
PE				
AC148775_45.5	0.9	**0.8**	1.0	1.0
AC150204_16.5	**0.6***	0.8	0.9	1.0
AC153005_36.5	1.0	**1.2**	0.9	1.1
AC153005_9.5	0.9	0.7	**0.3***	0.7
AC174141_27.4	1.3	**1.5***	1.1	1.1
AC202348_3.4	1.0	1.1	1.0	**1.3**
CT009653_39.4	1.0	1.0	1.3	**1.7***
AC152919_7.5	1.0	0.8	**0.6***	**0.7**
PEI				
AC122165_33.4	1.2	1.0	**1.6**	1.4
AC160097_13.5	0.9	0.9	1.0	1.0
AC160097_28.5	0.9	0.9	1.0	**1.4**
AC165218_17.4	**1.3**	1.3	1.0	1.1
AC173289_18.5	1.1	1.3	**1.7**	**1.6***

B

Gene	P6h	A6h	P20h	A20h
LOX				
AY515253_32.4	1.0	0.8	1.2	**1.5***
AC146571_7.4	0.7	**0.7**	**2.3***	1.3
AC140032_2.4	0.8	0.8	**1.5***	1.3
AC149580_13.5	**0.6***	**0.6***	1.0	0.7
AC149638_35.4	0.9	1.0	0.8	**0.7**
AC149580_9.5	0.9	0.9	1.0	**0.8**
AC149580_19.5	0.9	1.0	**0.8**	**0.9**
AC140032_6.4	0.9	1.0	0.9	**0.8**
AC140032_7.4	1.0	1.1	1.0	1.0
ACO				
AC146817_44.4	1.1	**1.4**	1.2	1.2
AC174337_3.4	1.0	**1.3**	0.8	1.3
AC169513_37.4	**2.0***	**1.8***	0.6	0.9
AC125389_65.5	**1.6***	1.3	1.1	0.9
AC119419_24.4	**1.4**	1.0	0.8	0.8
AC158372_38.4	1.5	1.4	**0.6***	0.7
AC146817_41.4	0.8	0.5	**2.9***	2.1
AC174337_15.4	1.2	0.9	**2.0***	1.4
AC202309_24.3	1.3	1.1	**1.5***	**0.7**
AC124966_44.4	1.3	1.2	0.9	**1.8***
CT025839_48.5	1.0	**0.5***	1.2	1.4
CU013517_26.4	1.0	0.9	**1.3**	1.0
AC197464_13.4	1.0	1.0	**0.9**	**0.8**
AC158372_65.4	1.0	1.1	0.7	**0.6***
AC169513_36.4	0.9	1.0	0.8	**0.7**
AC158372_67.4	0.7	0.8	0.7	**0.7**
AC158372_42.4	1.0	1.0	0.9	**0.7**
AC169513_23.4	0.8	**0.7**	0.9	0.1
AC169513_26.4	0.9	**0.8**	1.0	1.1
SAUR				
CU326390_14.3	1.1	1.1	1.3	**1.9***
AC149578_14.4	1.0	1.0	**1.3**	**1.3**
AC148242_22.4	1.1	1.0	**1.4**	1.2
AC148242_37.4	1.0	1.0	1.2	**1.3**
AC148242_50.4	1.1	1.3	1.2	**1.7***
AC148242_51.4	1.0	1.0	1.1	**1.4**
AC146705_44.5	1.0	1.1	**1.2**	**1.2**
AC146705_10.5	1.1	1.0	1.2	**1.3**
AC146705_14.5	1.1	1.1	**1.2**	**1.1**
AC146705_13.5	0.9	1.0	1.2	**1.7***
AC146705_14.5	1.1	1.1	**1.2**	**1.1**
CU024876_8.4	0.9	1.1	**1.3**	1.1
CU024876_38.4	1.1	1.1	**1.3**	**1.2**
AC146705_4.5	**1.4**	1.2	0.8	0.8

Figure 12. Expression of pea genes involved in cell wall modification (A) and hormonal signalling (B) pathways. The values indicate ratios of fold change (FC) expression levels of genes at 6 hpi and 20 hpi in response to *A. euteiches* and *P. pisi* compared to mock inoculation. Bold values correspond to the treatments ($P \leq 0.05$). Asterisks indicate if the FC expression levels are differentially expressed (≥ 1.5 fold induction or ≤ 0.67 fold repression). The heat map goes from blue to brown with increasing expression values. Abbreviations (A): callose synthases (GSL), pectin esterease (PE), pectin esterease inhibitor (PEI). Abbreviations (B): lipoxygenase (LOX), 1-aminocyclopropane-1-carboxylate oxidase (ACO), auxin-induced SAUR family (SAUR).

Taken together, these results suggest that different pathogenicity mechanisms of *A. euteiches* and *P. pisi* trigger distinct pea genes associated with signaling and pathogen perception, leading to common and distinct transcriptional responses. Cell wall reinforcement and modulation of JA and ET pathways are similar in response to both pathogens, while induction of the auxin pathway and chalcone synthesis is a specific response to *A. euteiches* (Figure 13).

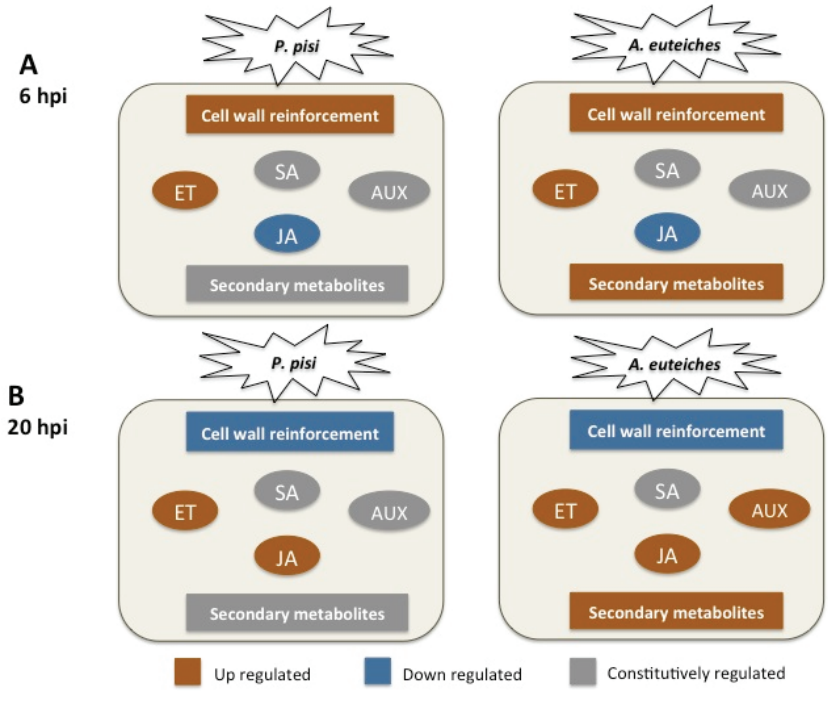

Figure 13. Scheme of differentially regulated defence sectors in pea in susceptible interactions with *Aphanomyces euteiches* and *Phytophthora pisi* at 6 hpi (A) and 20 hpi (B). Abbreviations; Ethylene pathway (ET), Salicylic pathway (SA), Auxin (AUX), Jasmonic acid pathway (JA).

4 Conclusions

Project I: The results suggest that the closely related root-infecting *Phytophthora* species in clade 7 have adopted different chemotactic behaviour that in some taxa might contribute to their host specificity.

- *P. sojae* and *P. vignae* were attracted to a pea isoflavone as well as soybean isoflavones, which is in contrast with their lack of pathogenicity on pea, suggesting that specific chemotaxis towards host isoflavones is of limited importance in these taxa.

- *P. pisi* and *P. niederhauserii* were specifically attracted to a pea isoflavone but not to the non-host soybean isoflavones, indicating an adaptation to their specific host plant.

- The affinity of *P. niederhauserii* to a pea isoflavone shows that this trait can also be present in taxa that are not specialized only to legumes.

Project II: The results suggest that *P. pisi* and *P. sojae*, which are closely related species, have specific proteins in their pathogenicity factor repertories that may play a role in the mechanism of their specificity towards pea and soybean, respectively.

- Proteins such as cysteine proteinases, isocitrate lyase and EGF-type proteins are involved in infection initiation while proteins such as annexin-like, coproporphyrinogen-III oxidase and proteins containing PLD-domain are associated with vegetative growth in both species.

- In germinating cysts lipid reserves are catabolized through the β-oxidation pathway and the glyoxylate cycle to drive the protein synthesis necessary to initiate the infection.

- Unique candidate pathogenicity factors in either *P. pisi* or *P. sojae* such as serine proteases, membrane transporters and a berberine-like protein, may be involved in their host specificity.

Project III: The results suggest that *P. pisi* and *A. euteiches*, two distantly related oomycetes, have evolved pathogenicity to the same host plant by modifying different signaling pathways, leading to some common and some specific transcriptional immune responses.

- Activation of cell wall modification, regulation of JA biosynthesis and induction of ET signaling pathway are among the common pea transcriptional responses to *A. euteiches* and *P. pisi.*

- Induction of chalcone synthesis and the auxin signaling are specific pea transcriptional changes against *A. euteiches*.

- Differential regulation of genes associated with signal transduction pathways, such as *NB-LRR* and *LRR-RLK*, at 6 hpi in response to both pathogens suggest that immunity responses associated with PTI or ETI occur early during infection.

5 Future prospects

Plant-pathogenic oomycetes remain a major threat for agriculture. Today the number of described *Phytophthora* species is over 100, many of them causing emerging infectious diseases. So far the most efficient control strategy is the use of plant resistant varieties. However, today no pea resistant variety is available against root-infecting oomycetes. As the first step, understanding the mechanisms behind initiation of the infection and molecular infection biology of these pathogens plays an important role in developing new, effective and long-term control strategies.

In this work we gained knowledge about the different chemotactic behaviour of some closely related species. For the taxa that have specific attraction to their host isoflavone, such as *P. pisi* and *P. nierdehauserii*, the biosynthesis of the attractant compound can be silenced and the pathogenicity could be tested against isoflavonoid-silenced host line. Furthermore, as the *P. sojae* receptors involved in chemotaxis were identified (Yang et al., 2013), the candidate G-protein-coupled receptors (GPCRs), which are key cellular components that mediate extracellular signals into intracellular responses, can be silenced in the other closely related species to study their function in pathogenicity and to test if they are conserved among these species.

Furthermore, in this thesis common and specific proteins involved in vegetative and infective stages of *P. pisi* and *P. sojae* are identified. The functional role of the candidate proteins associated with initiation of infection could be analysed applying gene knock-out techniques or RNA silencing methods to test their role in pathogenicity or virulence during interaction with the host plant. Moreover, candidate proteins can be expressed in the legume roots, applying Agrobacterium transformation method, to study their functions during plant interactions.

In the last project of the thesis we focused on the plant side during pea-oomycete interactions. This study shed light on the pathways that are

commonly or specifically targeted by these pathogens. The role of different hormonal pathways can be studied more in detail by inhibiting or inducing the hormones and studying the differences in susceptibility. Additionally, the interaction between relevant pathogen effector proteins and host target proteins can be investigated using in planta co-immunoprecipitation (co-IP) assays (Zhang *et al.*, 2015; Bozkurt *et al.*, 2011).

Furthermore, the on-going comparative genomic project of *Phytophthora* spp. in clade 7 will facilitate many aspects of molecular research about this group of pathogens such as questions on factors involved in host range, pathogenicity and evolution.

In a long-term perspective the finding of this study may assist breeding strategies, classical or molecular-based, by modifying the candidate pathways or genes involved in resistance.

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