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Deciphering mechanisms of pathogenicity and resistance induction in the interaction between *Phytophthora* spp. and European beech (*Fagus sylvatica* L.)

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Abstract

Phytophthora species belong to the most destructive plant pathogens in the world. They cause enormous economic losses in crops and massive damages in natural forest ecosystems. Unfortunately, currently there is no effective way available to control these pathogens. *Fagus sylvatica* is one of the most important tree species in European forests. The number of trees infected with *Phytophthora cambivora* and *P. plurivora* has increased since the last fifhteen years.

In this work, five parallel investigations are combined aiming to elucidate mechanisms of pathogenicity and resistance induction on the beech-*Phytophthora* interaction.

The molecular mechanism of elicitins, a conserved protein secreted by almost all *Phytophthora* species, was deciphered. It was demonstrated that blocking elicitins caused loss of pathogen virulence. As a consequence, elicitins could be a target in plant-*Phytophthora* interactions to prevent infection.

Phosphite, the potassium salt from phosphorous acid sprayed on leaves, was shown to be very effective in protecting beech seedlings against root infection by *P. plurivora*, in particular through activation of plant own defenses. It converted a high susceptible interaction into a resistant one. Since phosphite is environmental friendly, cheap and easy to apply in plants, it seems to be a powerful alternative to common pesticides.

An innovative, cheap and easy-to-built device, called zoospores trap, was developed to provide valuable information regarding the very early steps of plant-*Phytophthora* interaction in the rhizosphere. The activity of beech root exudate to attract zoospores of *P. plurivora* could be visualized and quantified. Another device was developed to control root temperatures and was constructed to investigate if differences in aggressiveness of two *Phytophthora* species were related only on their differential growth rates. The outcome of this analysis was that *P. plurivora* is still more aggressive than *P. pseudosyringae*, even when the growth rates of both pathogens are comparable.

Finally, carbon and nitrogen allocation of *F. sylvatica* saplings were investigated by use of stable isotope labeling techniques under infection with *P. plurivora* (susceptible interaction) and *P. pseudosyringae* (tolerant interaction). In general, Phytophthora infection had little impact on the plant internal resource allocation. The most important result of this analysis was that infected plants allocated less newly assimilated carbon in twigs possibly as an attempt to recover infection in the root system.

Altogether, the outcome of these five investigations provides an insight into the high virulence of *P. plurivora* towards its host and in general supplies possibilities to manage *Phytophthora* diseases; in particularly, by exploiting activation of plant own defenses.

Zusammenfassung

Phytophthora-Arten gehören mit zu den gefährlichsten Pflanzenpathogenen weltweit. Sie verursachen nicht nur hohe wirtschaftliche Einbußen durch Ernteverluste, sondern auch große Schäden in natürlichen Wald-Ökosystemen. In den letzten Jahren haben *Phytophthora*-Pathogene, wie *Phytophthora plurivora* und *P. cambivora* zu großen Ausfällen an unserer heimischen Rotbuche (*Fagus sylvatica*) geführt.

Diese Arbeit befasst sich mit einer Kombination unterschiedlicher Versuchsansätze, mit dem Ziel, zum einen Mechanismen für die hohe Anfälligkeit der Buche gegenüber *P. plurivora* aufzuklären und zum anderen Möglichkeiten der Resistenzinduktion bei *F. sylvatica* gegen das Wurzelpathogen aufzuzeigen.

Im ersten Versuch wurde die Bedeutung des Elicitins „Plurivorin" für die anfällige Interaktion zwischen *P. plurivora* und *F. sylvatica* entschlüsselt. Es zeigte sich, dass nach der Blockierung des Proteins durch spezifische Antikörper in der Rhizosphere von Buchensämlingen, *P. plurivora* nicht mehr in der Lage war, Wurzeln zu infizieren. Mehr als 90% aller Pflanzen überlebten. Laserscanning Mikroskopie bewies zudem, dass „Plurivorin", in Anwesenheit des Antikörpers, nicht mehr im Wurzelgewebe nachweisbar war. Des Weiteren zeigte sich, dass bei dieser Versuchsvariante wichtige Abwehrgene der Buchensämlinge, im Vergleich zu Kontrollen, signifikant exprimiert waren. Das „Plurivorin" scheint somit zum einen für das Eindringen von *P. plurivora* und zum anderen für die Down-Regulierung von Abwehrgenen in der Buche verantwortlich zu sein, was die hohe Virulenz des Wurzelpathogens gegenüber seiner Wirtspflanze *F. sylvatica* erklärt. Es bleibt abzuwarten, ob Elicitinen anderer Phytophthora-Pathogene eine ähnliche Bedeutung bei anfälligen Wirt-Phytophthora- Interaktionen zukommt.

Im zweiten Versuchsansatz wurde die Wirkung von K-Phosphit, das Kaliumsalz der Phosphorigensäure (H_3PO_3), auf die Interaktion von Buchen und *P. plurivora* getestet. Es zeigte sich, dass die Blattapplikation einer 0.5%-igen Lösung die hoch anfällige Wirt-Pathogen-Paarung in eine resistente Interaktion verwandelte. HPLC-Analysen wiesen K-Phosphit im Wurzelgewebe in Konzentrationen nach, die deutlich über dem ED50-Wert für *P. plurivora* lagen. Des Weiteren zeigten qPCR-Analysen, dass durch K-Phosphit wichtige Abwehrgene der Buche signifikant zu Kontrollen exprimiert wurden. Da K-Phosphit sowohl umweltverträglich, kostengünstig und zudem leicht von Pflanzen über die Blätter aufgenommen und in die Wurzeln transportiert wird, könnte es eine attraktive Alternative zu herkömmlichen Pestiziden für die Bekämpfung von *Phytophthora*-Pathogenen sein.

Im weiteren Verlauf der Dissertation wurde eine Zoosporen-Falle entwickelt. Dieses innovative und kostengünstige Gerät erlaubt es, die Attraktion von Zoosporen unterschiedlichster *Phytophthora*-Arten durch Wurzelexudate zu visualisieren und zu quantifizieren. Mit Hilfe dieser Zoosporen-Falle wurde gezeigt, dass Wurzelexudate von Buchensämlingen höchst attraktiv auf Zoosporen des Buchenwurzel-Pathogens *P. plurivora* wirken.

Im Rahmen einer weiteren Versuchsreihe wurde eine „Thermo-Box" entwickelt, die es erlaubt, den Infektionsverlauf zweier *Phytophthora*-Arten bei unterschiedlichen Wurzeltemperaturen vergleichend zu analysieren. Mit Hilfe der „Thermo-Box" wurde gezeigt, dass die tolerante Interaktion zwischen der Buche und *P. pseudosyringae* nicht auf ein langsameres Wachstum des Pathogens zurückzuführen ist. Bei Wurzeltemperaturen, die ein vergleichbares Wachstum für *P. plurivora* und *P. pseudosyringae* garantierten, war ersteres Pathogen höchst virulent gegenüber

seinem Wirt der Buche, wogegen *P. pseudosyringae* nicht in der Lage war *F. sylvatica* Sämlinge abzutöten. Die tolerante Interaktion muss folglich auf spezifische Abwehrmechanismen der Buche zurückgehen.

Zum Schluss wurden die Allokation von Kohlenstoff und Stickstoff mittels Markierung durch stabile Isotope in *F. sylvatica*-Setzlinge untersucht, die mit *P. plurivora* (anfällige Interaktion) und *P. pseudosyringae* (tolerante Interaktion) infiziert wurden. Insgesamt wurde die Allokation innerhalb der Pflanzen nur geringfügig durch die *Phytophtora* Infektion beeinflusst. Das Hauptergebnis dieser Analyse war, dass bei infizierten Pflanzen weniger neu assimilierter Kohlenstoff in den Zweigen nachweisbar war, möglicherweise als ein Versuch, der Infektion im Wurzelbereich entgegen zu wirken.

Zusammenfassend lässt sich feststellen, dass die Ergebnisse aller fünf Untersuchungen wertvolle Informationen zur hohen Virulenz von *P. plurivora* gegenüber seinem Wirt, der Buche, lieferten. Des Weiteren lassen sich Methoden zur Bekämpfung von Krankheiten durch *Phytophthora* ableiten, wie zum Beispiel die Induktion eigener Abwehrmechanismen.

List of abbreviations

A	Net rate of CO_2 uptake per leaf area [µmol/m^2s]
BSA	Bovine-serum albumin
C_a	Ambient CO_2 concentration [µmol/mol]
Cc	Central cylinder
cDNA	Copy DNA
C_i	Intercellular CO_2 concentration [µmol/mol]
Co	Cortex
Con	Control
CR	Coarse roots
DM	Dialyze membrane
dNTPs	Deoxynucleotide triphosphate (ATP, CTP, GTP, TTP)
Dpi	Days post inoculation
DW	Dry weight
EC_{50}	Half maximal effective concentration
ET	Ethylene
ETI	Effector-triggered-immunity
ETS	Effector-triggered immunity
F. sylvatica	*Fagus sylvatica*
FR	Fine roots
FW	Fresh weight
GC-MS	Gas chromatography – mass spectrometry
g_l	Leaf conductance [mmol/m^2s]
H2, H4	Harvests 2 and 4
HPLC	High-performance liquid chromatography
HR	Hypersensitive response
IEF	Iso-eletric focusing
JA	Jasmonic acid
J_{max}	Maximum rate of electron transport [µmol/m^2s]
LRR	Leucine-rich repeat
MAMP	Microbe-associated molecular pattern
MM	Molecular markers
NS	Statistically not-significant

P. plurivora, Plu	*Phytophthora plurivora*
P. pseudosyringae, Pse	*Phytophthora pseudosyringae*
PAMP	Pathogen-associated molecular pattern
PBS	Phosphate buffered saline
PCD	Programmed cell-death
Phi	Potassium phosphite
Phosphite	Potassium phosphite
PPFD	Photosynthetic photon flux density
PRP	Proline-rich protein
PR-protein	Pathogenesis-related protein
PRR	Pattern-related receptor
PS II	Photosystem II
PTI	PAMP-triggered immunity
QPCR	Quantitative polymerase chain reaction
R_d	Mitochondrial respiration rate in the light [µmol/m^2s]
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
RSA	Relative specific allocation
RT	Room temperature
RuBisCO	Ribulose-1,5-bisphosphate-carboxylase
RuBP	Ribulosebisphosphate
SA	Salicylic acid
SD	Standard derivation
SE	Standard error
SOD	Sudden-Oak-death
V_{cmax}	Maximum RuBP saturated rate of carboxylation
WB	Western-blot

Chapter 1

General introduction

1.1 Phytophthora threatening agriculture and natural ecosystems

From 1845 to 1852, Ireland suffered a great famine in which approximately one million people died and other one million emigrated. The mass starvation was caused mainly due to a potato disease called potato blight. Later in 1876, Anton de Bary described *Phytophthora infestans* as the pathogen causing the disease. The genus *Phytophthora* was then created and with that, the discipline of plant pathology was founded (Hansen *et al*., 2012). The name *Phytophthora* comes from the greek Phyto – plants, Phthora – destroyer (the plant destroyer).

Over the years, the number of recognized species has steadily increased with a spike in the last decade (Levesque, 2011). To date the genus *Phytophthora* is found to have 114 species formally described, most of them associated with highly destructive plant-diseases (Hansen *et al.*, 2012). The host range of *Phytophthora* species is very broad, virtually all dicotyledons, as well as some certain cereals and monocotyledons are subjected to infection (Erwin and Ribeiro, 1996), resulting in economic losses in crops and damage in the environment that accounts over a total of $5 billion worldwide (Stokstad, 2004).

Evolution:

The genus *Phytophthora* is grouped in the Oomycetes class. The oomycetes are a group of organisms that includes pathogens of insects, crustaceans, fish, vertebrate animals, various microorganisms and plants (Margulis and Schwartz, 2000). Modern molecular phylogenies based on ribosomal RNA (rRNA) sequences, amino-acid data for mitochondrial proteins and four protein-encoding chromosomal genes have been used to finally set the position of the oomycetes as an unique lineage of stramenopile eukaryotes, unrelated to true fungi but closely related to heterokont photosynthetic algae (Fig. 1) (Sogin and Silberman, 1998; Baldauf *et al*., 2003; Adl *et al*., 2005). Analyses of the genome sequences of *P.*

infestans, *P. sojae* and *P. ramorum* suggested that a total of 855 genes shared similarity with photosynthetic organisms such as red algae and cyanobacterias (Tyler *et al*., 2006). According to Lamour (2007), highly destructive Phytophthora pathogens seem to have evolved from phototrophic ancestors. Beakes *et al*., (2012) concluded that oomycetes were originated in the sea and that hyphal-like pattern of growth and parasitism arose as these organisms moved to terrestrial environments. Kamoun (2001) stated that the modern phylogenetic analyses lead to a conclusion that oomycetes evolved the ability to infect plants independently of other eukaryotic plant pathogens, such as fungi and are likely to have distinctive mechanisms for doing so.

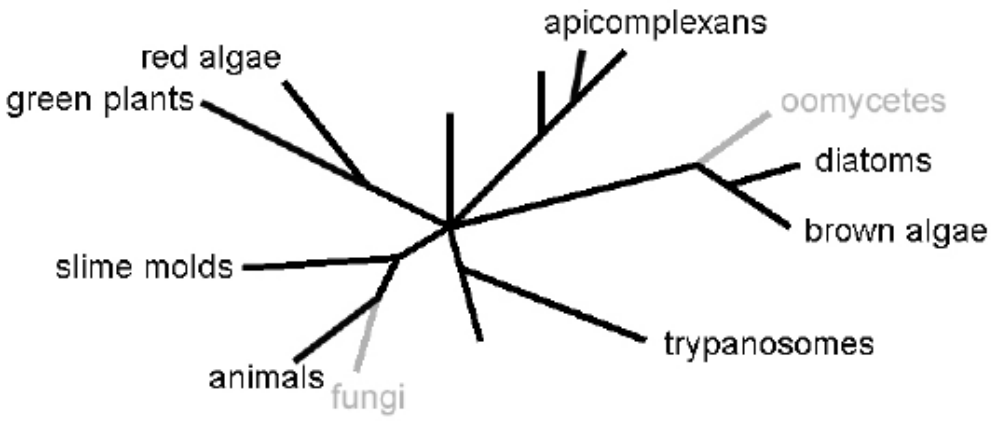

Figure 1: Schematic phylogenetic tree from eukaryotes (tree adapted from Baldauf, 2003).

For several years, when modern molecular techniques were still not available, Oomycetes were classified together with fungi species due to their morphological resemblance. Similarities in morphology, habitat, reproduction and some strategies of infection when comparing fungi and oomycetes are a good example of convergent evolution. Nonetheless, in contrast to fungi, several other differences lead to the conclusion that oomycetes are more related to plants than animals. A good example is the composition of the cell wall. Oomycetes cell walls mainly consist of cellulose, whereas cell walls of fungi

primarily contain chitin. Table 1 shows the main differences between oomycetes and true fungi.

Today the widely accepted classification of the Phytophthora genus goes as follows:

Domain: Eukariota; **Kingdom:** Chromalveolata; **Phylum:** Heterokontophyta; **Class:** Oomycetes; **Order:** Peronosporales; **Family:** Pythiaceae; **Genus:** Phytophthora.

Table 1: Major differences between oomycete and true fungi

Feature	Oomycete	True fungi
Neighboring taxonomic groups	Diatoms and golden-brown algae	Animals
Hyphal architecture	Aseptate and coenocytic tubular	Either single cells or septated hyphae
Ploidy of vegetative hyphae	Diploid, except for transient structures	Typically haploid or dikaryotic; often with a stable haploid nuclei in gametangia or semi-stable diploid stage following mating
Typical size of genome	50–250 Mb	10–40 Mb
Major glucans in cell walls	Cellulose	Usually chitin
Pigmentation	Usually un-pigmented	Very common for hyphae or spores, or secreted (for example, melanin, carotenoids and others)
Toxic secondary metabolites	None described	Common (typically aromatic, heterocyclic)
Mating hormones	Non-peptide, probably lipid-like	Usually small peptides or lipopeptides
Predominant asexual spore	Un-desiccated, unicellular	Desiccated single or multicellular conidia sporangia (multinucleate cells)
Motile asexual spores	Nearly universal, biflagellated	Uncommon, only in chytrids, which are zoospore monoflagellate
Sexual spores	Oospores, formed on the termini of specialized hyphae	Various types, often formed in large numbers, within complex enclosures (for example, each containing one viable perithecia, mushroom caps and others) zygotic nucleus
Mitochondria	With tubular cristae	With flattened cristae

Table modified from Rossman and Palm, 2006.

Biology:

Phytophthora exhibits filamentous mycelia growth during the vegetative stage (Erwin and Ribeiro, 1996). The mycel is diploid, coenocytic (few or no septa) with cellulose cell-walls, primarily formed with β-glucans. Reproduction can be sexual or asexual. The majority of the species are homothallic (self-fertile), some others are heterothallic, divided into A1 and A2 mating types, e.g. *P. ramorum*. Sexual spores, or oospores, are formed when the male structure, antheridium, associates with the oogonium (female). Assexual spores, the zoospores are differentiated inside the sporangia (zoosporangia). The sporangia release zoospores under optimal conditions, but are also employed as an infective propagule.

Zoospores:

The major infective agents that initiate plant infection for most *Phytophthora* species are the zoospores. The zoospores are wall-less motile cells surrounded only by a plasma membrane, which extends over the two flagella and probably contains the receptors that detect external stimuli. The flagella allow them to swim after detecting external stimuli (mainly coming from root exudates). Hosts are targeted through taxis responses such as chemotaxis, autotaxis, and electrotaxis (Walker and van West, 2007). Once the zoospores reach the host, they encyst and subsequently germinate, initiating the infection process (Hardham, 2005).

Water is essential and optimal conditions (e.g. pH higher 4) are required to release zoospores by sporangia. The dispersal capacity is limited, unless they are transported in moving water. The life-spam of *Phytophthora* zoospores in lab conditions is relatively short. Most of the zoospores encyst in the first 4 hours. However, a few can survive over a day, mainly at the expense of lipid reserves, since they are not able to uptake organic nutrients from the environment such as amino acids and sugars (Penington *et aI.,* 1989). Nevertheless, Vanini *et al*., (2012), found an unexpected resistance to environmental stresses, such as

drought for *P. cambivora* zoospores in substrate without any host, and pointed out the phytosanitary risks related to movement of not only plants but also associated substrates.

***Phytophthora* species:**

The genome sequence of *P. infestans*, *P. sojae*, *P. capsici* and *P. ramorum* was recently completed. These notorious plant pathogens contribute to major economic and environmental losses every year. Analyzes of their genome sequences will hopefully result in feasible management and control strategies of these pathogens and their diseases. Taking advantage of new technologies and decrease on sequencing costs, a consortium integrating several groups worldwide will sequence more than one hundred species in the next years, kicking forward the study of *Phytophthora* diseases.

The main species causing disease in crops worldwide are: *P. infestans*, mainly in potato and tomato (Hausbeck and Lamour, 2004); *P. sojae*, infecting soya beans (Kamoun and Smart, 2005; Tyler, 2007); *P. capsici*, with a wide host range including members of Solanaceae, Cucurbitaceae as well as Fabaceae family (Birch and Whisson, 2001) and *P. nicotianae* (syn. *P. parasitica*), infecting both herbaceous and woody hosts in a range of about 60 different plant families, including the Solanaceae and other cultivated crops of worldwide importance, such as citrus in USA, China and Brazil (Erwin and Ribeiro, 1996, Attard *et al*., 2007).

A large number of other *Phytophthora* species are associated with disease in woody plants, infecting trees and shrubs either as foliar pathogens, bark pathogens, or root pathogens (Hansen *et al*., 2012). Some few species are host-specific, such as *P. lateralis or P. alni* that infect cedar and alder trees respectively (Erwin and Ribeiro, 1996, Brasier and Kirk, 2004). However, the majority of the *Phytophthora* species can infect more than one host. One particular species, *P. ramorum* has been recently highlighted on the news regarding the

devastating effects on the oak populations in California and Oregon (USA). The disease was called Sudden Oak Death (SOD) and has now a status of epidemics in USA. According to Grünwald (2012), the recently emerged *P. ramorum* has originated from reproductively isolated populations and underwent at least four global migration events, pointing out the importance of strengthening the current bio-security protocols regarding trading of plants. *P. quercina* is also an important species responsible for oak decline in Europe (Jung *et al*., 1999; Cooke *et al*., 1999). *P. cinnamomi* is an aggressive soil-borne root pathogen with a broad host range and considered to be one of the world's most invasive species, present in more than 70 countries and able to infect more than 3000 plants (Hardham, 2005). In Australia it is responsible for infecting several hundreds of native plants, damaging forests and removing habitats for small mammals. *P. cinnamomi* dieback of Eucalyptus has been also associated with the increased number of fires in damaged forests, reaching large areas including urban zones. *P. pseudosyringae*, although not as aggressive as the above mentioned species, is also associated with disease of several forest trees, mainly beech and oaks from European forests (Jung *et al.*, 2003).

The number of *Phytophthora* species associated with disease in forest trees has increased over the last decade. The main reasons are: intensive sampling campaigns, particularly in forest soils and streams; new technologies facilitating detection of invasive species in new hosts and detailed genetic analyses in some morphologically defined *Phytophthora* varieties that were later found to actually consist of several cryptic species (Hansen *et al.*, 2012). For example, the species formerly known as *P. citricola* has recently been divided into four distinct species, *P. plurivora*, *P. multivora*, *P. pini*, and *P. citricola* s.s., all of which are pathogenic to woody plants (Jung and Burgess, 2009).

Phytophthora plurivora is a hemibiotrophic root pathogen with worldwide distribution, attacking mainly woody plant species, in particular *Fagus sylvatica* (European beech) (Werres, 1995), (Fleischmann *et al*., 2005), (Jung *et al*., 2005). *P. citricola* and *P. plurivora* differ in length and breadth ratio of sporangia, colony growth patterns and optimal growth temperature. *P. plurivora* shows homothallic, paragynous, antheridia and semipapillate sporangia resembling lemons (Fig. 2). It is assumed that *P. plurivora* was imported from overseas on living plant stock and could spread in Europe because of perfect climate conditions and ubiquitous presence of host plants. *P. plurivora* has been reported to cause several severe plant diseases; collar rots, bark cankers, extensive fine root losses or dieback of crowns on young and mature of hundreds of tree and shrub species (Jung *et al*., 2005; Orlikowski *et al*., 2011).

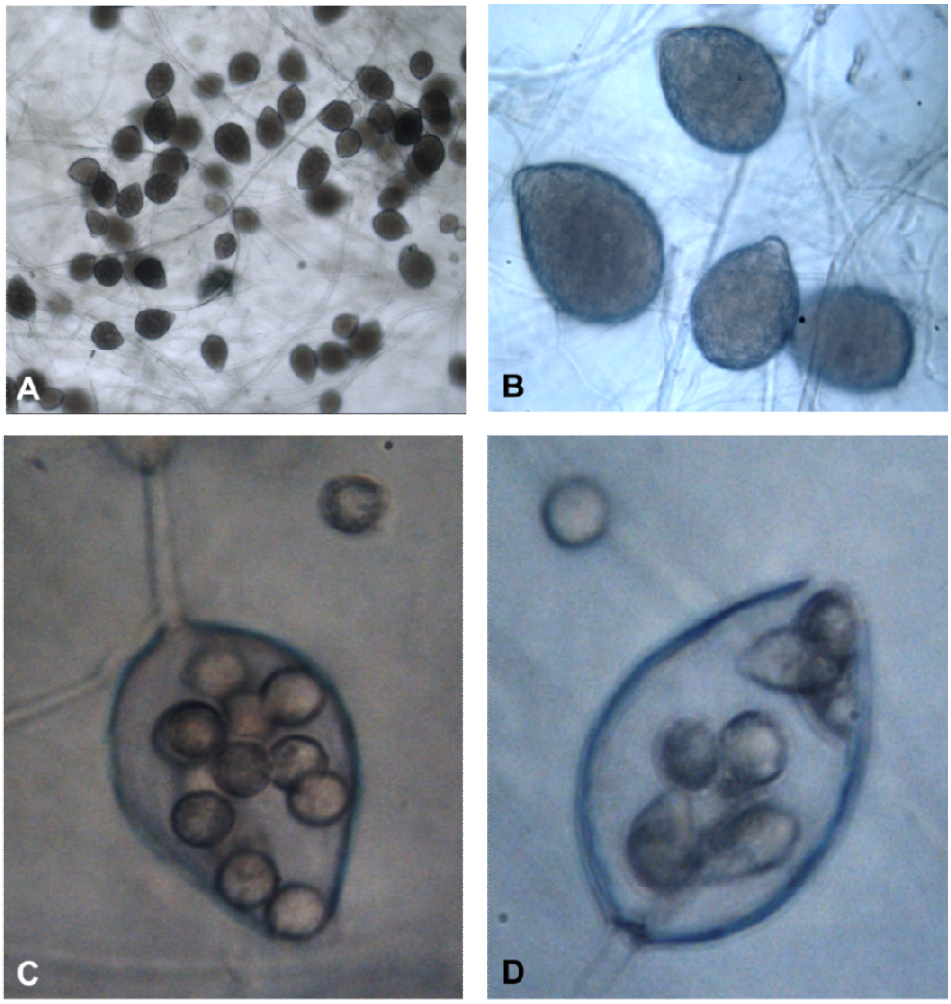

Figure 2: (A) shows zoosporangia of *P. plurivora* grown on V8-agar. (B) shows the typical limonene form of a semipapillate sporangia of *P. plurivora*. (C and D) Sporangia releasing zoospores. (copyright M. Humez).

The life cycle of *P. plurivora* is similar to most of the soil-borne species (Fig. 3). It starts from dormant spores (oospores) in unsuitable environmental conditions. Their life-span in the soil can last over several years. After the environmental conditions become suitable (high soil moisture, soil temperature > c. 10 °C, pH higher than 4), the resting spores germinate forming sporangia which release motile, biflagellate zoospores into the soil water (Fig. 2) (Jung *et al.*, 2009). The zoospores are chemotactically attracted to the surface of potential hosts. After penetration in plant roots, the typical non-septate hyphae grows inter- and intracellular inside fine root tissue. After damaging plants, the pathogen starts its necrotrophic phase and resting spores are set free into the soil environment; the cycle starts then all over again (Tsao, 1990).

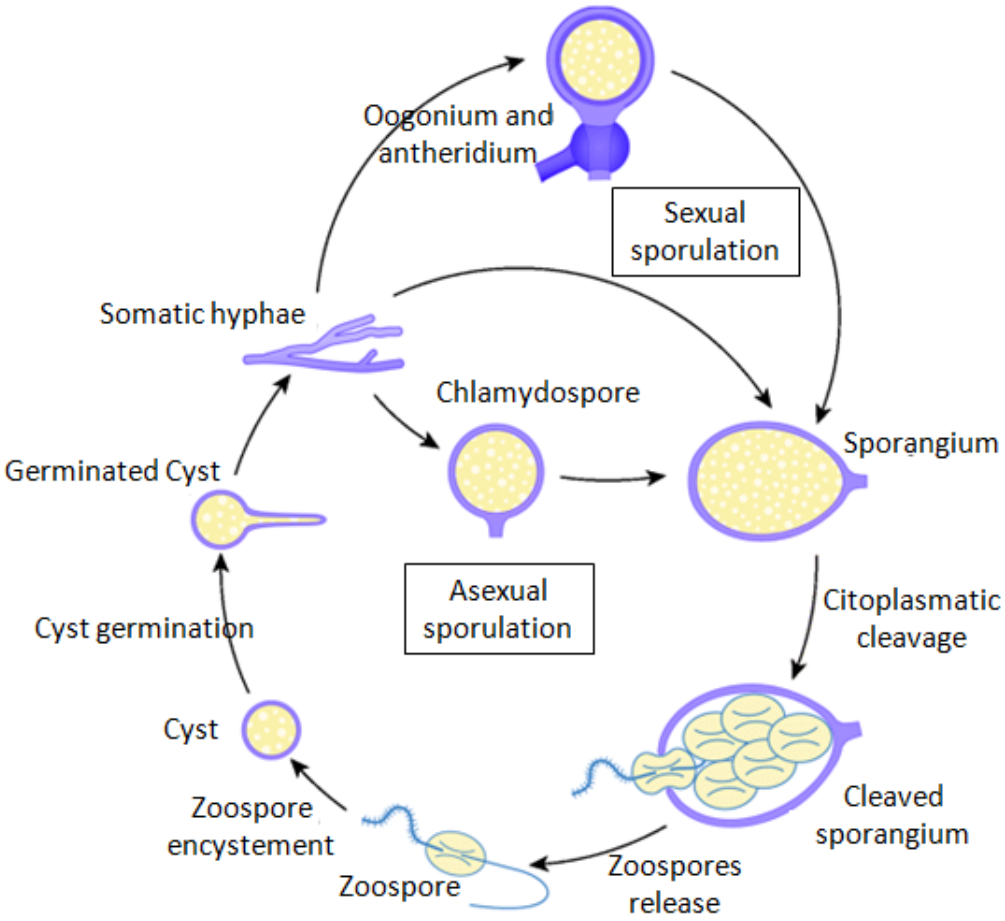

Figure 3: Schematic life cycle of a typical soil-borne Phytophthora (adapted from Hardham, 2005).

Management:

Even after almost 150 years since the genus was described, controlling diseases caused by *Phytophthora* species is still a challenge for plant-pathologists worldwide. Attard *et al*., (2008) stated in their publication that "to date, pesticides that are adapted to prevent or cure oomycete diseases do not exist". The main reason for that lies on the biochemical differences between oomycetes and true fungi already mentioned above. Most of the fungicides target chitin or ergosterol biosynthesis. Unfortunately oomycetes do not synthetize sterols and their cell walls mainly consist of cellulose and β-glucans, thus fungicides used in agricultute to control fungal diseases are totally inefficient. Metalaxyl a pesticide developed to combat several pathogens was widely used against *Phytophthora*- disease right after its homologation

in 1977. It is know to target RNA-polimerase-1 and thus inhibiting protein biosynthesis (Sukul and Spiteller, 2000). Unfortunately, only four years after the metalaxyl employment in agriculture, the first cases of resistance were already reported. Nowadays, resistance against metalaxyl is a general feature of *P. infestans* and *P. capsici* (Lee *et al*., 1999; Parra and Ristaino, 2001).

Several new compounds have been developed and tested in the last years but in most of the cases conventional breeding strategies are employed. Nevertheless, in recent years phosphite and phosphite-based compounds have arisen as an alternative to common pesticides. Figure 4 shows structure of K-phosphite and Fosetyl-Al.

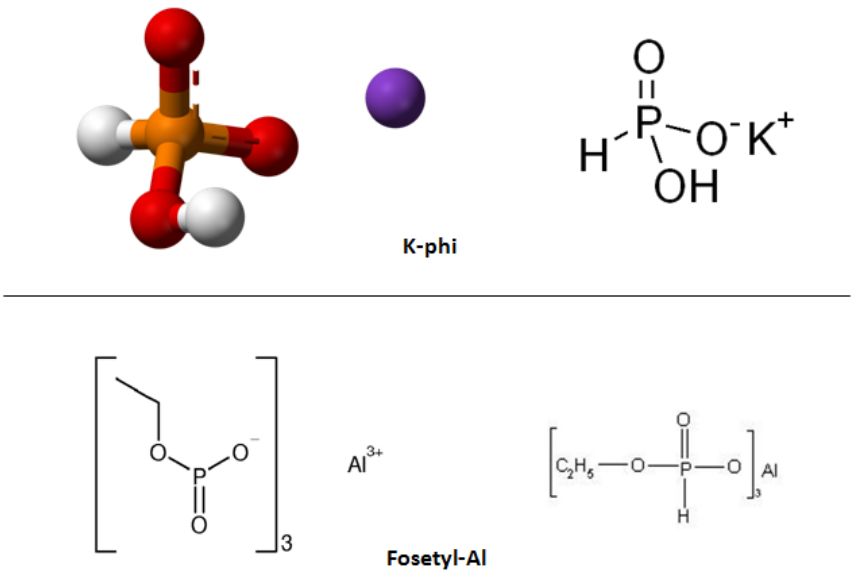

Figure 4: Structure of Potassium-phosphite (K-phi) and Fosetyl-aluminium (Fosetyl-Al), also commercialized as Aliette. Both are phi-based compounds and are largely used as an alternative to common fungicides in controlling diseases caused by oomicetes.

Phi-based compounds have been effectively used worldwide, especially in Australia, combating *P. cinnamomi* diseases in forest trees after trunk injections (Scott *et al*., 2012; Gentile *et al*., 2009; Garbelotto, 2007). However, there are already reports of resistance acquirement and its mode of action is largely unknown. Table 3 summarizes currently used pesticides and their efficacy towards *Phytophthora* pathogens.

Table 3: Commonly used fungicide classes and their activity on oomycetes

Class	Fungicide (example)	Inhibitor of	Activity on oomycetes	Risks to the environment
Triazole	Flutriafol	Sterol synthesis	Not active	Moderate
Polyoxin	Polyoxorim (PolyoxinD)	Chitin synthesis;	Not active	Moderate
Phenylamide	Metalaxyl	RNA polymerase-1	Active, but resistance evolves rapidly	High
Phosphite	K-phi	Not clear	Active, but resistance was already reported	Virtually no risk

Table modified from Attard *et al*., (2007).

1.2 *Fagus sylvatica* L.

Fagus sylvatica Linné, 1753, European beech, is one of the most important trees of natural and managed forests from the north of Europe. Due to its high shade tolerance it dominates the natural forests in temperate and warm-temperate climates of Europe (Packham, 2012). Climate changes associated with spread of *Phytophthora* diseases have substantially increased the number of declined trees in the past years. In this section we will give an overview of *F. sylvatica*, its biology, distribution and threat.

Biology:

European beech is a large broadleaved tree reaching about 30 to 40 meters height, with a stem diameter of up to 2m (Degen, 2001). Normally, individuals show a single stem, but massive branches can be often observed. Buds are fusiform, 1-2 cm, and grow in two phases, the first one in the early May after eruption, and the second phase in July (Mitchell, 1996). The leaves are 4 to 10 cm long, alternate, ovate to elliptic, with five to nine veins (Packham, 2012). Individuals are monoecious (male and female flowers on same branches). Flowering is developed in individuals of approximately 40 years old, pollination occurs mainly by wind and the reproduction is mostly sexual. Seeds are largely produced in mast years. They are 12

to 18 mm long, dispersed by birds and mammals, but most of them accumulate around the parental tree (Packham, 2012). Figure 5 illustrates some of those structures mentioned above.

In the environment, seed germination occurs in spring and requires moisture and low temperatures. In the lab, dormancy is frequently broken after incubating seeds in wet medium at 4°C. Seedlings show epigeal germination, and cotyledons are usually storage organs at the beginning and subsequently they are also involved in photosynthesis (Packham, 2012) (Fig. 6). The first leaves are completely expanded within 30 days after germination. Roots are pivotal; with several branches developed relatively fast after germination (Fig. 5). In adults the root system is considered to be shallow and intensive in comparison with other competitive tree species (Bakker *et al*., 2008).

Figure 5: Fagus sylvatica. a) leafy branch, b) inflorescences, c) cupula, d) seed, e) seedling.

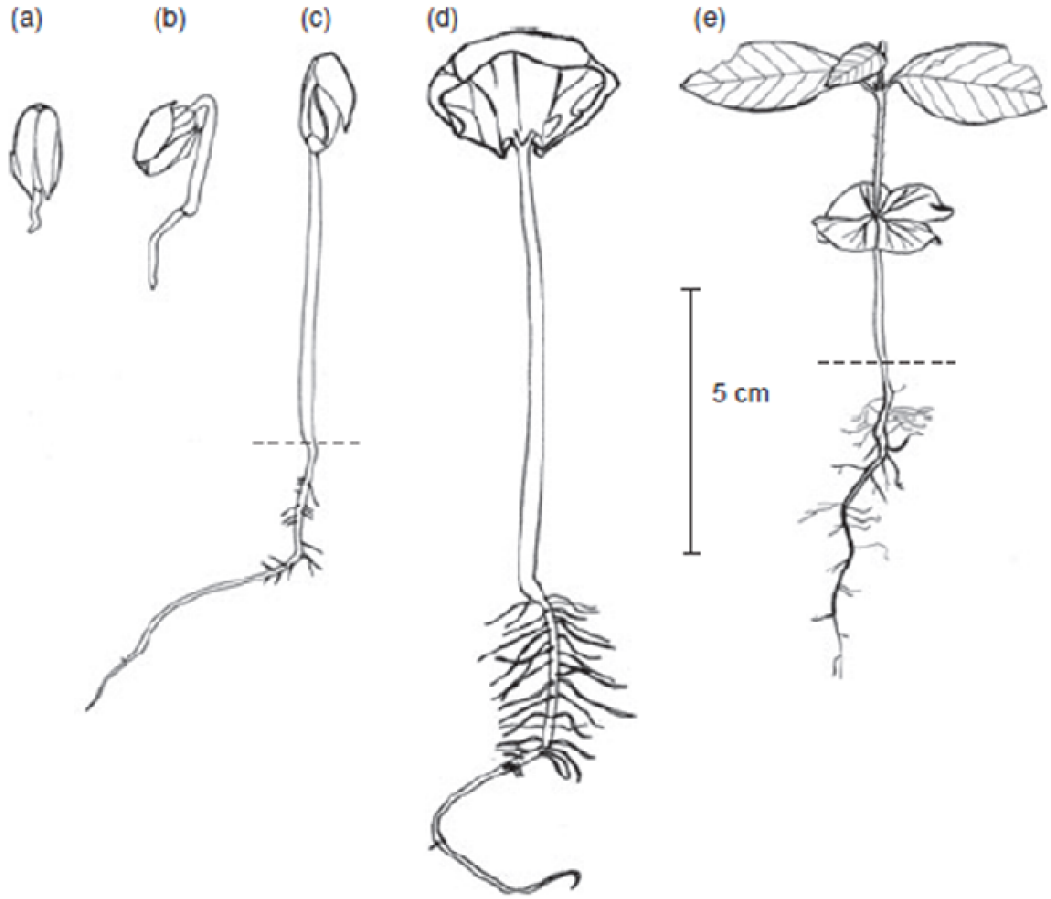

Figure 6: Stages in the germination and early development of *Fagus sylvatica*: (a) Radicle breaking through the nut wall; (b) radicle well established and hypocotyl emerging; (c) hyocotyl fully elongated, developing cotyledons well above the ground; (d) cotyledons, whose lower sides have a white sculpted appearance at this stage, almost fully expanded; (e) vigorous young seedling whose first two leaves have been damaged by insects. R. Packham *et al*., 2012.

The life span of European beech under natural conditions varies between 150 to 300 years (Rameau, Mansion and Dumé 1989), often not exceeding 500 years, but the oldest individuals known are 900 years old (Enzyklopädie der Holzgewächse – 27. Erg.Lfg. 3/02).

F. sylvatica is a very competitive species. It tolerates a wide range of environmental conditions and biotic and abiotic stresses, including poor soil conditions (Grime, Hodgson and Hunt (2007). It is very effective in eliminating growth of other species beneath it, mainly because of its shade (Rodwell, 1991). It competes with several other broadleaved trees, but its

main competitors are oaks and in particular conifers, whose evergreen strategy contrasts with the one possessed by *F. sylvatica* (Packham 2012).

Distribution

Paleontological studies indicate that Fagus originated in the Early Tertiary in the northern Pacific Basin (Denk, 2003). The genus comprehends 13 species and according to Shen (1992), 11 are East Asian; *F. sylvatica* is European and *F. grandifolia* is North American (Packham, 2012).

Fagus sylvatica has been widespread at different times through Europe (Packham, 2012) and appears to have survived the glaciations periods as small scattered populations across Europe (Denk, 2002).

Nowadays, the distribution of European beech covers southern, central and western Europe (Fig. 7), being limited in areas of continental climates (Packham, 2012). In Germany, 17 % of the existing forest area of 11.1 million hectares is dominated by beech forest. This corresponds to a beech forest area of 1.89 million hectares with a standing wood volume of 583 million m³. The largest volumes of beech wood are found in the states Bavaria, Baden-Württemberg and Hessen. Due to its versatile characteristic, the demand of beech wood consumption on the European and the world market has been increased continuously (Hapla, 2004).

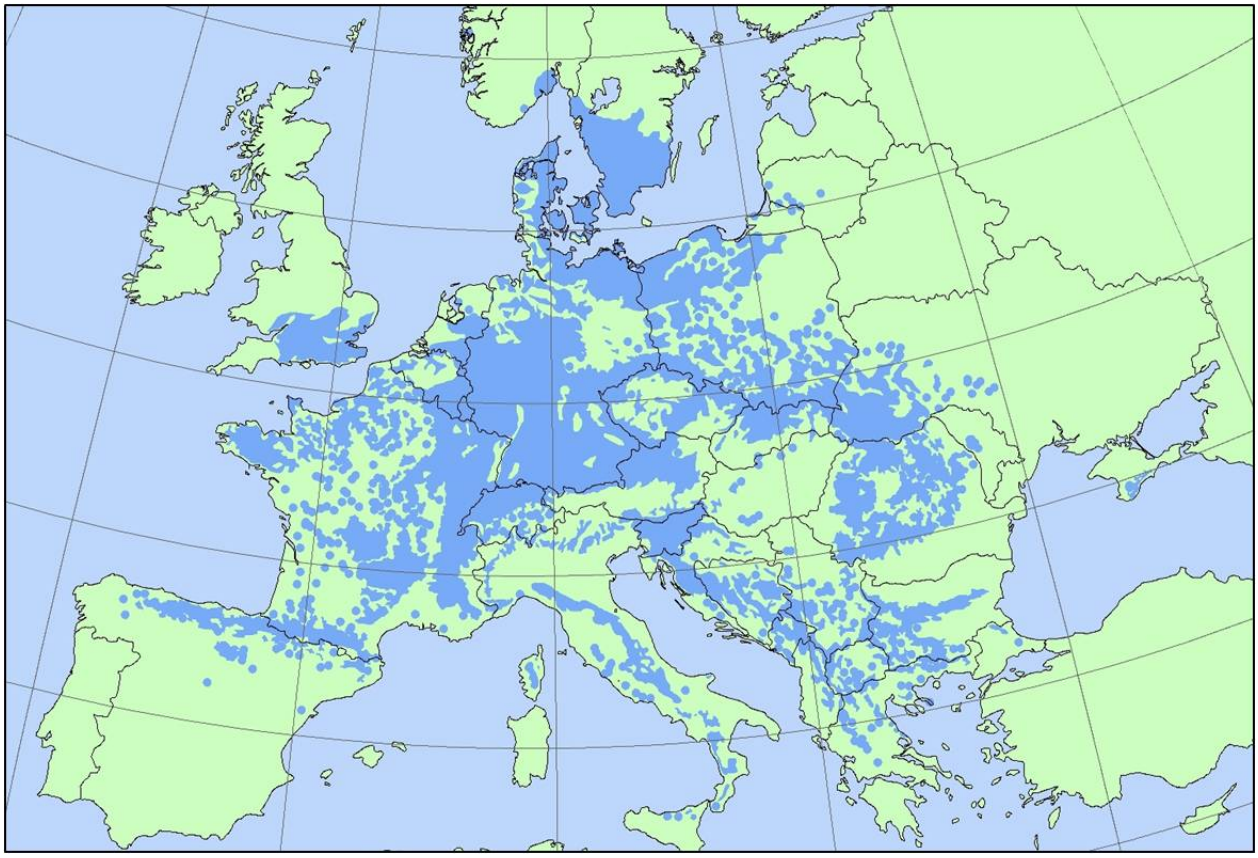

Figure 7: Distribution map of *Fagus sylvatica* in Europe, EUFORGEN, 2009.

After centuries of human influence, beech forests have preserved much of their original characteristics and have continued to appeal to people over the years. In its traditional habitat, beech is often described as the "mother of forests". Beech represents both the mysterious unknown and the established tradition. Therefore, a committee of experts from the United Nations Educational, Scientific and Cultural Organization (UNESCO) has agreed that five German beech forests should be declared World Heritage Sites since 2007 (Unesco, Christchurch, 2007). However, during the past decade, the number of trees and stands of European beech in Central Europe is declining dramatically. Particularly, *Phytophthora* spp. was mentioned to be strongly involved soil-born pathogens in several devastating declines of forests across Europe (Jung *et al*., 2005; Schmitz *et al*., 2007).

Threat

The root system structure of beech is shallow and intensive, exploiting a relative small volume of soil very intensively. In conditions of low availability of water in the soil, the root system is not able to extend the main and lateral roots far enough to reach damper regions, resulting in low tolerance to drought (Packham, 2012). It has a particular importance, since climate change in Europe tends to result in drought and Beech cannot respond to it. Recent publications have highlighted the potential of climatic warming to cause strong directional selection in natural populations, including *F. sylvatica* (Jump and Peñuelas 2006; Thomas, 2000). In addition, Jung (2009) has associated increase in declining of beech in Europe with climatic extremes, such as drought and infection with Phytophthora species.

Several *Phytophthora* species have been reported to infect *F. sylvatica*, the most aggressive species are *P. plurivora* and *P. cambivora* (Fleischmann *et al*., 2005). However other species can also cause serious disease such as: *P. cactorum*, *P. pseudosyringae*, *P. kernoviae*, *P. ramorum* and *P. cryptogea*.

Up to now, there are not many efficient methods to counter-attack diseases caused by *Phytophthora* species in Beech. Deep analysis of the *F. sylvatica* - *Phytophthora* interaction is required to develop effective strategies to manage these diseases.

1.3 Plant-pathogen interactions

Millions of years coexisting in the same environment have developed a complex communication system between plants and microbes. In each plant-microbe interaction exchanging signals represents the earliest and fundamental step. The ability to perceive and respond to environmental signals is fundamental for survival (Tyler *et al*., 2006). In the rhizosphere plants and microbes exchange signals mainly via root exudates and microbe-

associated-molecular-patterns (MAMPs) respectively, which can result in interactions such as symbiosis or pathogenesis, depending on the species involved (Fig. 8).

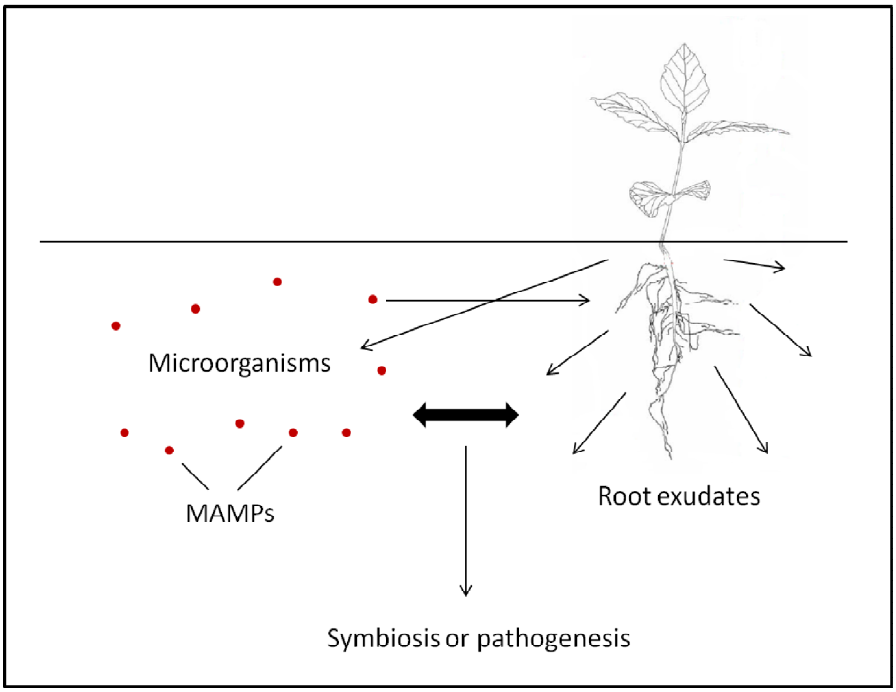

Figure 8: Plants and microorganisms communicate in the rhizosphere trough root exudates and MAMPs respectively. The establishment of interactions such as symbiosis or pathogenesis depends on the species communicating and signaling recognition. Root exudates can attract or repeal growth or movement of microorganisms. On the other hand, MAMPs can burst defense responses or even induce growth of roots facilitating the interaction.

Ultimately, the interplay of signals between plant and microorganisms is fundamental for the adaptability of t species to the environment, therefore being a target for natural selection.

Pathogens have evolved the ability to recognize signals from hosts to start infection. On the other hand, plants have developed an immune system which is triggered by pathogen/microbe-associated molecular patterns (PAMP/MAMP) and is therefore called PAMP-triggered immunity (PTI). This highly effective defence system enables the plant to resist potential attack by microbial pathogens (Hückelhoven, 2007; Boller and He, 2009;

Thomma *et al*., 2011). In order to break these defence barriers, pathogens secrete an arsenal of proteins, termed effectors to the plant cell which suppress PTI, resulting in the so-called effector-triggered-susceptibility (ETS). Although effectors are thought to work primarily in virulence, they are also known to elicit innative immunity in plants that carry disease resistance (R) proteins. They either directly or indirectly ("guard-model") recognize the presence of pathogen effector proteins and in consequence defence responses are activated (effector-triggered immunity = ETI). Figure 9 illustrates PTI, ETS and ETI.

While pathogens are evolving new effectors to manipulate the host to its advantage, plants are evolving new recognition molecules, suggesting a continuous dynamic co-evolution on both sides of plant-pathogen interaction. Jones and Dangl (2006) proposed the "zigzag model" to help understand the action and reaction of both, plant and pathogen in the gene-for-gene arms race (Figure 9). This model is divided into five phases, the three already mentioned above (PTI, ETS and ETI) and two more as follows: if under selection pressure the pathogen is able to alter its effectors in some way as to avoid ETI, this results in the fourth phase, a new ETS phase. This can be achieved by shedding or altering the recognized effector gene, or by acquiring additional effectors that suppress ETI. As a result, by natural selection new R specificities can emerge and ETI can be triggered again resulting in phase 5, where immunity is restored. As a consequence of this confrontation the selection pressure is constantly turning from one side to the other resulting in an arms race (plant vs. pathogens) that will never come to an end (Fig. 9).

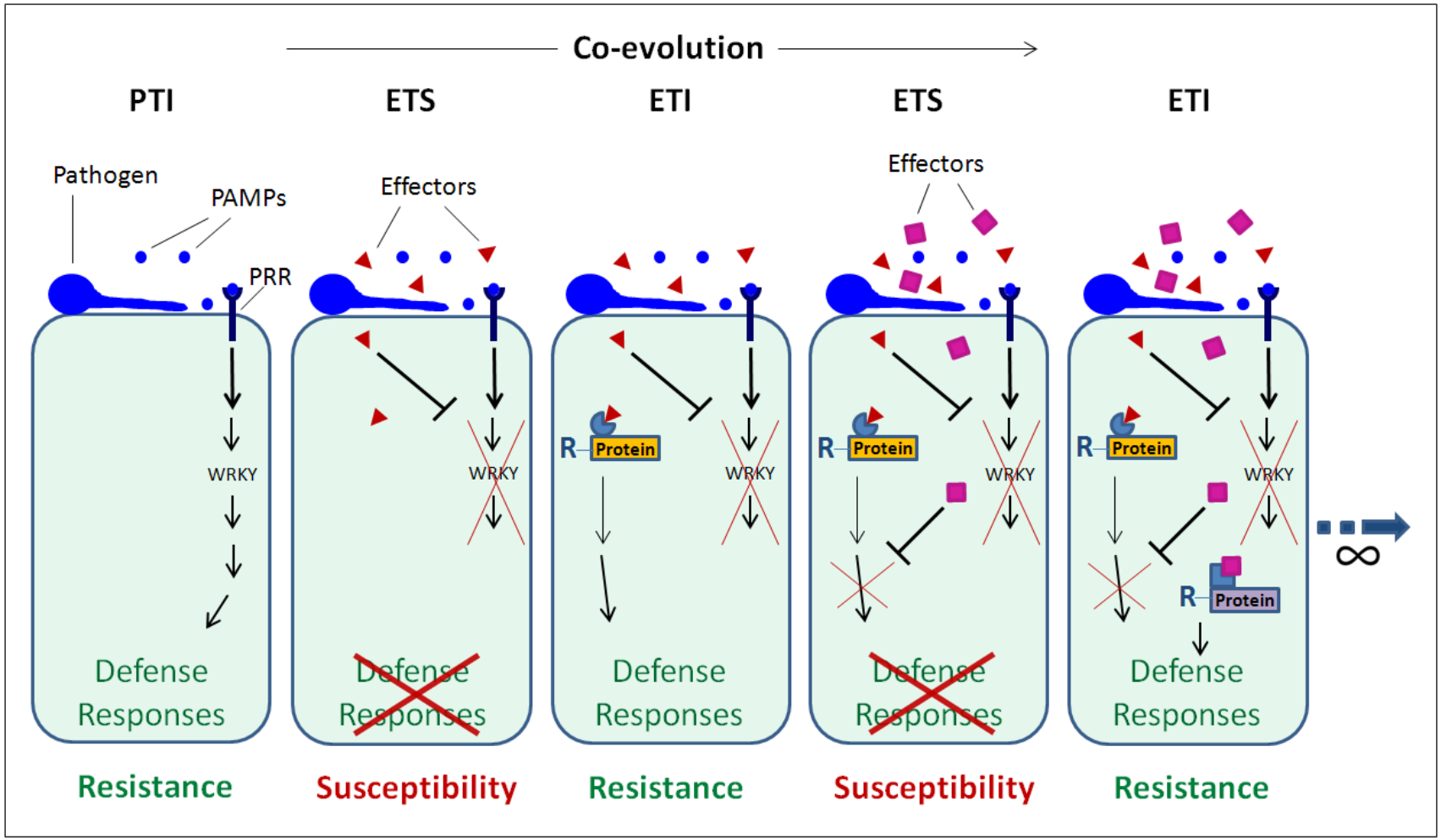

Figure 9: Schematic representation of the co-evolution between plants and pathogens. Upon pathogen attack, pathogen-associated molecular patterns (PAMPs) activate pattern-recognition receptors (PRRs) in the host, resulting in a downstream signaling cascade, usually through WRKY transcription factors that leads to PAMP-triggered immunity (PTI). Virulent pathogens evolved effectors that suppress PTI, resulting in effector-triggered susceptibility (ETS). In turn, plants have acquired resistance (R) proteins that recognize these attacker-specific effectors, resulting in a secondary immune response called effector-triggered immunity (ETI). Under selection pressure the pathogen alters its effectors to avoid ETI, resulting in a new ETS phase. As a consequence, by natural selection new R specificities can emerge and ETI can be triggered again and immunity is restored. This process can be repeated several times, tending to infinite.

PAMPs and PRRs

Plant defense responses can be activated by generic signals indicating pathogen presence. These signals were often referred to as elicitors, and now are named as PAMPs (Pathogen-Associated Molecular Pattern), DAMPs (Damage Associated Molecular Patterns) or WHIMPs (Wound/Herbivory-Induced Molecular Patterns) (depending on their origin or localization) and are recognized by plant receptors (Bent and Mackey, 2007; Boller and Felix 2009).

PAMPs are structures or molecules unique to microbes that are conserved through all classes of microbes including pathogenic and non-adapted microorganisms (Gohre and Robatzek, 2008). The term PAMP was developed for mammalian innate immune system researchers and due to nonpathogenic microorganisms or microorganisms nonpathogenic for many hosts also possess PAMPs, the term MAMP (Microbe-Associated Molecular Pattern) can also be used (Bent and Mackey, 2007). Among the PAMPs that are detected by plants are the bacterial flagellins (the main peptide component of the motility organ) (Gomez-Gomez and Boller, 2000), lipopolysaccharides (LPS, which are the glycolipid component of Gram-negative bacteria external membranes), elongation factor-Tu (an abundant protein involved in translation), peptidoglycans (PGN, an essential component of the microbial cell envelope) and muropeptides released from PGN by the action of lysozyme (reviewed in (Aslam *et al*., 2009) chitin and ergosterol, major constituents of the fungal cell wall (Chisholm *et al*., 2006) and heptaglucan from *P. sojae* (Hahn, 1996).

The recognition of PAMPs is carried out by PAMP receptors also called Pattern Recognition Receptors, PPR (Fig. 9) and a lot of questions remain unanswered about the mechanism of their interaction. Previous studies have shown that these receptors are

trans- membrane proteins with an extracellular LRR (leucine-rich repeat) and an intracellular serine/threonine kinase domain such as FLS2, a receptor from flagellin (Gomez-Gomez and Boller, 2000) and the EFR1 receptor from EF-Tu, both from Arabidopsis (Zipfel et al., 2006).

After PAMP recognition, mitogen-activated protein (MAP) kinases cascades are initiated and regulated through action of transcriptions factors, such as WRKY, resulting in regulation of the gene expression of defense related proteins, continuing the defense process (Qiu *et al*., 2008) (Fig. 9).

WRKY transcription factors

More than 70 members that belong to WRKY family have already identified in *Arabidopsis* and more than 100 in rice (Eulgem and Somssich, 2007; Ross *et al*., 2007), nearly all members respond to diverse biotic stresses (Dong *et al*., 2003; Jaskiewicz *et al*., 2011; Kalde *et al*., 2003).

Several studies have showed increased levels of WRKY mRNA, protein and DNA-binding activities in response to viruses (Wang *et al*., 1998), bacteria or Oomycetes (Eulgem *et al*., 1999), fungal elicitors (Fukuda, 1997; Rushton *et al*., 1996) and signaling substances such as salicylic acid (Yang *et al*., 1999). Furthermore, pathogen-mimicking treatments show selective up regulation of similar genes in rice, potato, sugarcane, and chamomile (reviewed by Ulker and Somssich, 2004). These data present strong evidence for the role of WRKY proteins in the regulation of protective responses (Ravet *et al*., 2012). According to Dong *et al*., (2003), 49 out of 72 WRKY genes tested from *Arabidopsi*s were responsive to bacterial infection or salicylic acid (SA) treatment. Studies with WRKY genes identified them as an important regulatory

component in the cross-talk between SA and JA-signaling during plant defense (Li *et al*., 2004).

Effectors

Effectors are pathogen molecules that manipulate host cell structure and function thereby facilitating infection and/or triggering defense responses. Unlike the terms "avirulence", "elicitor", "toxin", and "virulence", the term effector is neutral and does not imply a negative or positive impact on the outcome of the disease interaction. They are responsible for promoting penetration into host tissues, persistence inside the host tissue, suppression of immune responses, allowing access to nutrients, proliferation, and growth (Gohre and Robatzek, 2008).

Common features from well characterized effectors are used by plant pathologists to search for possible candidates from new and old pathogens. These candidates are normally small, secreted proteins, which are rich in cysteine and show no obvious homology to other known proteins (Gohre and Robatzek, 2008). Secreted effectors reach their cellular target at the intercellular interface between host cells and the pathogen (apoplastic effectors) or inside the host cells (cytoplasmic effectors) (Djamei *et al*., 2011; Kamoun, 2006).

Apoplastic Effectors: Oomycetes secrete large numbers of apoplastic effectors. In most of the cases these apoplastic effectors have the function of inhibiting or degrading defense proteins from the plant basal resistance, like proteases and glucanases. Among the already described apoplastic effectors of *Phytophthora* species are: enzyme inhibitors (such as glucanase and cystein proteases inhibitors), Nep1-like family (such as PiNPP1 and transglutaminases), as well as elicitins, which are small cysteine-rich proteins (Kamoun, 2006).

Elicitins:

Special interest was put to *Phytophthora* elicitins during the last years. These peptides are a family of small peptides (M_r of about 10 kDa) which are secreted to huge amounts into the culture filtrate of any *Phytophthora* pathogen. Active elicitins consist of 98 amino acids and exhibit a highly conserved amino acid sequence with 6 invariant cysteine residues building three disulfide bridges stabilizing the characteristical three-dimensional structure (Fig. 10). Their overall structure comprises 5 α - helices, an antiparallel β – sheet and an Ω - loop building a beaker-like hydrophobic core (Fefeu *et al*., 1997; Boissy *et al*., 1999). Elicitins are classified as basic ß-elicitins (pI > 7; lysine residue at position 13) or acidic α-elicitins (pI < 7; valyl residue at position 13) on the basis of their isoelectric point (pI) (Ponchet *et al*., 1999). The lysine residue at position 13 of basic elicitins was correlated with their high necrotic activity. Additionally the basic elicitin cryptogein of *P. cryptogea* has also been shown to act as a sterol carrier protein in order to transfer sterols from plant plasma membranes to the hyphae. Recently Pleskova *et al*., (2011) proved that the positive lysine residues on the surface of basic elicitins were crucial for that carrier activity. Tavernier *et al*., (1995) and Pugin and Guern (1996) showed that early events induced by cryptogein in tobacco cells are the stimulation of a plasma membrane NADPH oxidase generating superoxide and the activation of glycolysis and the pentose phosphate pathway, that generate NADPH. Reactive oxygen formation was also induced in tobacco by quercinin the basic elicitin of *P. quercina* (Koehl *et al*., 2003). When elicitins, such as cryptogein or capsicein were applied to tobacco plants, they caused hypersensitive cell death (HR) and induced resistance against *Phytophthora nicotianae* (Ricci *et al*., 1989; Bonnet *et al*., 1996). There is also good evidence that this peptide is released into the cytoplasm of infected

root cells, where it might interact with receptor proteins, as it was shown for the INF1-elicitin which recognizes the kinase subdomain of the NbLRK1 gene product, encoding for a lectin-like receptor kinase (Kanzaki *et al*., 2008). All these specific elicitin effects mentioned above could only be measured in *Nicotiana* species and in some *Brassicaceae*. However, from recent results of Manter *et al*., (2010), Horta *et al*., (2010) one can conclude that *Phytophthora* elicitins act differently in woody plants. For example Manter *et al*., (2010) found a positive correlation between the amount of elicitin production and virulence of *P. ramorum* isolates. Horta *et al*., (2010) showed that the ß-cinnamomin knock-down mutant of *P. cinnamomi* exhibited the lowest virulence towards *Quercus suber* as compared to the wild type. Figure 10 shows the 3D structure of an elicitin.

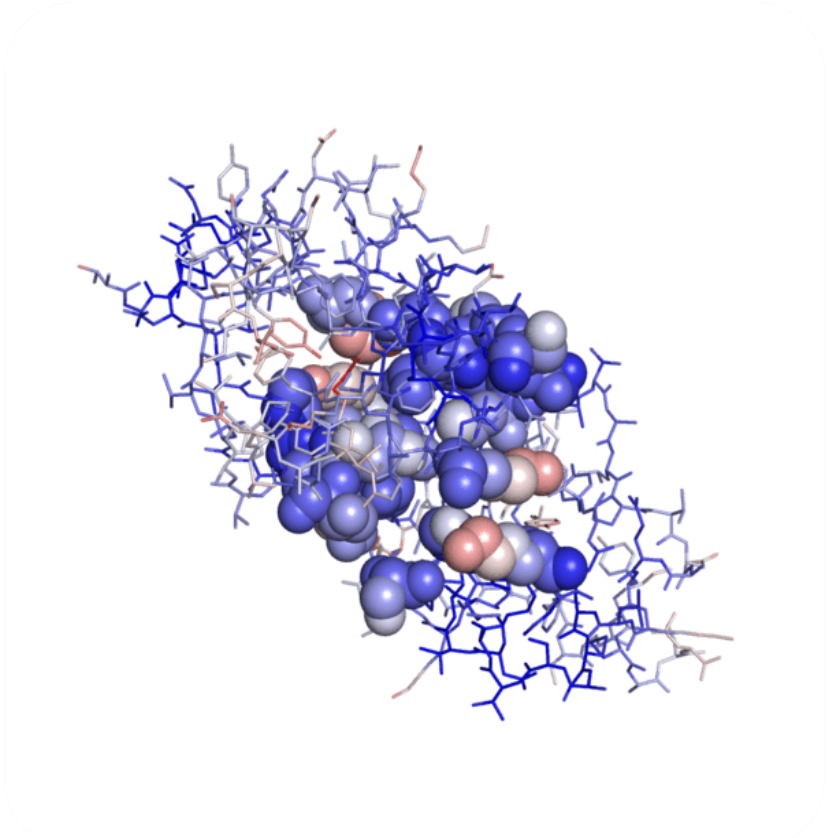

Figure 10: 3D structure of an elicitin molecule. Image was generated using: Poliview 3D (Porolo *et al*., 2004). Hydrophilic chains are represented by sticks and hydrophobic core is represented by balls.

Summarizing these findings one can conclude that elicitins are associated, either directly or indirectly with the infection and the susceptible host-pathogen interaction. The question rises, whether in these cases elicitins might act as suppressors of PAMP triggered immunity (PTI) rather than as inducers of resistance.

Cytoplasmic effectors: Bacteria can deliver effectors into the host cytoplasm by using their needle structure, the type-III secretion system (TTSS) (Zhang *et al*., 2011; Zhao *et al*., 2011; Zhao *et al*., 2003). Knowledge on eukaryotic effectors is sparse in comparison to that available for bacterial effectors; nonetheless Oomycetes are known to deliver two types of intracellular effectors, the RXLR (Morgan and Kamoun 2007) and the Crinkler effectors (CRN like family) (Hass *et al*., 2009).

The RXLR (R means arginine, X any amino acid and L Leucine) motif was used to identify effectors in *Phytophthora sojae*, *Phytophthora ramorum*, and *Hyaloperonospora parasitica* based in draft genomes (Win *et al*., 2007). This motif is also found in effectors from malaria, a human parasite (Bent and Mackey, 2007). The RXLR and CRN motifs are required for effector translocation through the cell membranes (Kamoun, 2006)

R Proteins

R Proteins are products of *R* genes encoded by specific hosts that evolved to recognize effector molecules or their action. Most of these proteins have a leucine-rich repeat (LRR) domain (described previously) as part of intracellular NBS-LRR proteins that also carry a nucleotide binding site (NBS) and other conserved domains, as an extracellular LRR in transmembrane receptor-kinase proteins, or in "receptor-like proteins" that have an extracellular LRR and a transmembrane domain (Bent and

Mackey, 2007; Boller and Felix, 2009; Gohre and Robatzek, 2008). The NBS domain contains sequences conserved in plant and animal proteins (Takken *et al*., 2006; van der Biezen and Jones, 1998).

Several studies show direct interaction between R proteins and effector proteins where the specificity is by physical interaction (Deslandes *et al*., 2003; Dodds *et al*., 2006; Jia *et al*., 2000) and the LRR domains are responsible for determining this specificity (Bent and Mackey, 2007; Boller and Felix, 2009). On the other hand, a number of examples show that the R proteins can monitor the integrity of the host proteins and are activated only in response to an alteration in these proteins, known as indirect detection (Bent *et al*., 1994; Grant *et al*., 1995; Innes *et al*., 1993).

In general, the main domains of these proteins are highly conserved across the taxa most probably by natural selection for maintenance of their function. However, in some *R* proteins, the predicted solvent-exposed residues lie along a concave face and the LRR region shows a lower level of conservation. This probably happens as a consequence of selection pressure caused by changes in the pathogen forcing the plant to adopt new or alter existing interactions in this part of the R proteins, thus allowing the recognition of different or altered pathogen Avr proteins by direct detection (Bent and Mackey, 2007). Consequently these *R* proteins represent a flexible part of the plant immune system. Like effectors, these genes evolve very fast by single mutations and small insertions or deletions, resulting in a *co-evolutionary* conflict where evolutionary selection favors resistance in plants and virulence in their pathogens (Dodds *et al*., 2006).

Chapter 2

The elicitin of *Phytophthora plurivora* is essential for the susceptible interaction with *Fagus sylvatica* (European beech)

The elicitin of *Phytophthora plurivora* is essential for the susceptible interaction with *Fagus sylvatica* (European beech)

Summary:

To manipulate host metabolism during infection, *Phytophthora* species secrete many effectors, including high amounts of elicitins, a small protein family first described to elicit defence responses in tobacco plants. Elicitins trigger a variety of defence responses, including programmed cell death (PCD) in several plants and share many features of pathogen-associated molecular patterns (PAMPs). However, the precise role of elicitins as possible virulence factors has to be clarified.

This study shows for the first time that α-plurivorin, an elicitin secreted from *Phythophthora plurivora*, is essential for its virulence. Blocking of α-plurivorin by incubation with a specific antibody during infection drastically impaired its disposal in host tissue and *P. plurivora* penetration. In consequence all plants treated with the anti-α-plurivorin antibody survived pathogen attack. Furthermore, the lack of α-plurivorin inside the host tissue led to an up-regulation of defence-related genes, suggesting that α-plurivorin might act as a defence suppressor during infection.

Remarkably, given the potential of hundreds of effector genes in the *P. plurivora* genome, inhibition of α-plurivorin compromises *P. plurivora* pathogenicity, suggesting that α-plurivorin acts as a virulence factor for infection.

2.1 Introduction

Among the more than 1000 effectors described for *Phytophthora* species that have the potential to manipulate host metabolism (Vleeshouwers *et al*., 2006), this study focussed on the functions of the elicitin α-plurivorin, an apoplastic effector of *P. plurivora*, in the highly susceptible interaction between European beech (*Fagus sylvatica*) and the oomycete.

To date, most reports describe the effects of effector molecules on plant-pathogen interactions, arguing that an effector cocktail, i.e., a group of effectors secreted by a pathogen, is responsible for the manipulation of the defence responses in plants (Oh *et al*., 2010).

The inherent biological function of *Phytophthora* elicitins and their role in plant-pathogen interactions has long been a subject of examination. Because *Phytophthora*

species cannot synthesise sterols and are, therefore, obliged to assimilate them from hosts, elicitins have been proposed to act as sterol carriers (Mikes *et al*., 1998).

Several studies have shown that elicitins trigger programmed cell death (PCD) in *Nicotiana* spp (Kamoun *et al*., 1998; Shibata *et al*., 2010; Chaparro-Garcia *et al*., 2011). Defence responses in tobacco have also been suggested to be mediated via elicitin-sterol complexes (Osman *et al*., 2001), which would be in agreement with the proposed biological function of elicitins as sterol carriers.

Despite the broad range of compatible *Phytophthora*-host interactions that cause diseases worldwide, the biological functions of elicitins as virulence factors during susceptible infections are barely discussed in literature. Here, it is demonstrated for the first time the importance of elicitins, particularly of α-plurivorin, for pathogen penetration and its involvement in plant-defence suppression.

2.2 Material and methods

Purification of α-plurivorin and treatment of plants with the specific antibody

α-plurivorin was previously isolated from *P. plurivora* M1-liquid culture medium and purified as follows: *P. plurivora* was grown in 0.5 L of M1 liquid culture for 8 days before mycelia were filtered and the flow through was concentrated to 50 mL in a freeze drier. The concentrated medium was dialyzed against a 0.02M phosphate buffer (pH=7.2) for 16 h in a dialysis tube with a molecular weight cut off of 6–8 kDa (Spectra/ PorR 1, Roth, Germany). Afterwards, the medium was concentrated to a volume of 15 mL and separated in an ion-exchange column. The acidic flow-through was collected, concentrated and dialyzed. The volume was collected and separated in portions of 1.5mL on a gel filtration column (Sephacryl S-100HP 16/60, Amersham,

Germany) in 0.02M sodium phosphate buffer containing 0.15M sodium chloride (pH 7.2) with 0.5 mL flow rate. The flow-through containing the α-plurivorin peak was collected in 4.5 mL fractions and their absorbance at 210 nm and protein content was measured. In addition, fractions were analyzed with SDS polyacrylamide electrophoresis using 12.5% horizontal clean gels (ETC, Germany) and isoeletric-focusing (SERVA Electrophoresis GmbH), according to the protocols of the manufacturers. Proteins were stained by Coomassie brilliant blue or silver stained according to the protocols of the manufacturer. Subsequently, an affinity-purified polyclonal IgY antiserum against α-plurivorin (1 mg protein mL^{-1}) was produced by Davids Biotechnologie (Regensburg, Germany) using 1.0 mg of α-plurivorin for the chickens immunisation and 0.6 mg α-plurivorin for the affinity purification. The activity and quality of the antiserum were checked through Western-blot analysis of the SDS gels, following the manufactures protocol.

One hour before the inoculation with zoospores, the antiserum was applied to the root system. A set of experiments were conducted, primarily, with application of 500 μl of the antiserum, which corresponds to the final dilution of 1:100 of the antiserum. The 1:100 dilution of the antiserum was further tested to influence mobility of the zoospores or encystment under the microscope. It was found that the α-plurivorin antiserum did not alter the zoospores behaviour in the first 40 to 50 minutes, which was enough for a successful infestation of roots. Nevertheless, it was also observed that the antiserum dilution of 1:400 had absolutely no effect on the zoospores behaviour, therefore a second set of experiments was carried out using that dilution factor.

In order to check if the antiserum had an unspecific effect to plants or to the pathogen (zoospores), a pre-immune normal-serum (the natural chicken antibodies

before immunization with α-plurivorin) was used as a control treatment to the anti-α-plurivorin antiserum in the second set of experiments. The same dilution factor used for the antiserum at the first set of experiments (1:100) was used in the application of the pre-immune serum, as an additional control treatment.

Plant growth conditions and inoculation:

Seeds of European beech (*Fagus sylvatica L.*) were germinated and grown in root trainers with sterile vermiculite for 3 months at 20 °C and light conditions of 250 μmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD). Three days before initiating the experiment, the seedlings were carefully removed from the containers. The roots were rinsed of the substrate and placed in 50 mL Falcon tubes containing 50 mL of deionized water and sealed with Parafilm in the centre of the tubes.

Phytophthora plurivora T. Jung and T.I. Burgess, isolate CIT55, which was isolated from a declining beech in Southern Bavaria (Germany), was grown on V8 agar in the dark at 20°C. After one week, the cultures were submerged in deionized water. The water was replaced daily to remove the nutrients and to induce sporangia development. Release of the zoospores was induced by incubating the submerged cultures at 4°C for 1 hour. The zoospore concentration in the supernatant of the cultures was quantified using a Thoma counting chamber. The plants were inoculated with 5×10^5 zoospores per plant.

Disease assessment and measurement of plant physiology parameters:

The plants, 6 per treatment, were monitored daily for browning of roots, for the growth of visible mycelia on the root surface, and for the wilting of leaves as well as for mortality.

Gas exchange measurements were conducted using a CO_2/H_2O diffusion porometer equipped with a broad-leaf LED cuvette (LI-6400, LI-COR, Lincoln, Nebraska, USA). All of the measurements were conducted under steady-state conditions of 23 °C (leaf temperature), between 50% and 60% relative humidity and 400 ppm CO_2 concentration (in the reference air), 250 µmol m^{-2} s^{-1} PPFD, and 500 mL min^{-1} air flow.

The maximum efficiency of photosystem II was measured using a MiniPAM yield analyser (Walz, Effeltrich, Germany) under ambient light conditions.

Confocal laser-scanning microscopy:

To prepare the sample after harvesting, the root material was fixed in PBS (pH 7.2) with 3% formaldehyde. The root samples were manually sliced using a razor blade, and the cuttings were washed in PBS/0.2% Tween three times. The samples were then blocked for 30 min with 100 mM glycine in PBS/0.2% Tween. After washing the material again, a protein block was performed with 1% BSA in PBS (pH 7.2) for 30 minutes. The root cuttings were then incubated for 2 h at 37°C with two primary antisera, the purified antibodies against α-plurivorin (chicken anti-α-plurivorin, 1:100 diluted) and the commercial antibody against *P. plurivora* (rabbit anti-*Phytophthora* spp. polyclonal antiserum from Loewe Diagnostica®, diluted 1:400). After incubation, serial washings (two x 10 minutes) were performed with PBS/0.2%Tween and then with PBS. The root cuttings were incubated for 60 minutes at 37°C with secondary antisera (goat anti-chicken conjugated to Alexa Fluor 633, Invitrogen®, diluted 1:200; goat anti-rabbit conjugated to Pacific Blue, Invitrogen®, concentrated at 1:200). Before the confocal laser-scanning microscopy, the samples were washed several times with PBS/0.2% Tween and with PBS (pH 7.2).

The confocal imaging was performed using a Leica TCS SP5 confocal laser-scanning microscope (Leica Microsystems CMS GmbH, Mannheim, Germany). Alexa Fluor 633 was excited with a 633 nm laser-line and detected between 641 and 655 nm. Pacific Blue was excited at 405 nm and detected between 430 and 480 nm. Plant auto-fluorescence was detected between 500 and 550 nm after excitation with a 488 nm laser-line.

qPCR for *P. plurivora* and gene expression:

Genomic DNA was extracted from 20 mg freeze-dried and milled root material (6 plants per treatment) using the DNeasy plant mini kit (Qiagen, Hilden, Germany) and was further purified using the Wizard® DNA clean up system (Promega, Mannheim, Germany) according to the manufacturer's protocols. The DNA was diluted 1:10 in H_2O to prevent the inhibition of the PCR reaction. The amount of *P. plurivora* DNA in 5 µL of root extract was determined by TaqMan quantitative PCR using an SDS7700 sequence detection system (Applied Biosystems, Germany) (Böhm *et al*., 1999) with the primer pair P5 / P6 and the fluorogenic probe F3 labelled with FAM as a reporter dye and TAMRA as a quencher. All of the analyses were performed in three technical repetitions using ABsolute QPCR ROX chemicals (ABgene, Hamburg, Germany) and performing 40 cycles of denaturation at 95°C for 15 s and annealing / extension at 62°C for 60 s. The Ct values of the samples were compared with a standard curve that was generated for pure *P. plurivora* genomic DNA extracted from mycelia grown in M1-liquide culture. The standard curve concentrations ranged from 1 pg DNA mL^{-1} to 10 ng DNA mL^{-1} in five steps.

For the extraction of total RNA, 50 mg of roots (4 per treatment) were ground in liquid nitrogen using a mortar and pestle. The total RNA was extracted using the

MasterPure Plant RNA Purification kit (Epicentre Biotechnologies, Madison, WI, USA) according to the manufacturer's protocol, including a DNAse I treatment. The concentration and quality of extracted RNA was measured using a BioMate 3 Photometer (Thermo Fisher Scientific, Ulm, Germany). cDNA was reverse transcribed using 1 µg of total RNA with oligo-dT primers and the MMLV Reverse Transcriptase 1st strand cDNA Synthesis kit (Epicentre Biotechnologies, Madison, WI, USA). The transcript levels of specific genes were analysed by using 0.05 µg of cDNA by qRT-PCR in three technical replicates using the ABsolute SYBRGreen ROX chemicals (ABgene, Hamburg, Germany) and performing 40 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s. The expression of the genes: ACO, PR1, PR2, PR3, PRP and WRKY of *Fagus sylvatica* under the *P. plurivora* and antibody treatment was calculated relative to the expression levels of the untreated *P. plurivora* using the Relative Expression Software Tool REST 2009 (Pfaffl *et al*., 2002). Actin, tubulin and GAPDH were used as reference genes. The gene expression of α-plurivorin was measured using the primer pair uni1 / uni2 (Colas, V. *et al*., 2001). Its relative gene expression was calculated using REST 2009 with the *P. plurivora* content of the corresponding genomic DNA sample as a reference. The list of oligo-nucleotides used in this study is presented in the Table 1, in the Appendix.

2.3 Results

The purification of α-plurivorin was confirmed by SDS gel electrophoresis and isoeletric focusing electrophoresis (Fig. 1, A and B). The specificity of α-plurivorin antibodies was confirmed by western blot analysis (Fig. 1C). In all three analyses single bands can be visualised, which proves the purity of the α-plurivorin, as well as the quality of the α-plurivorin antiserum.

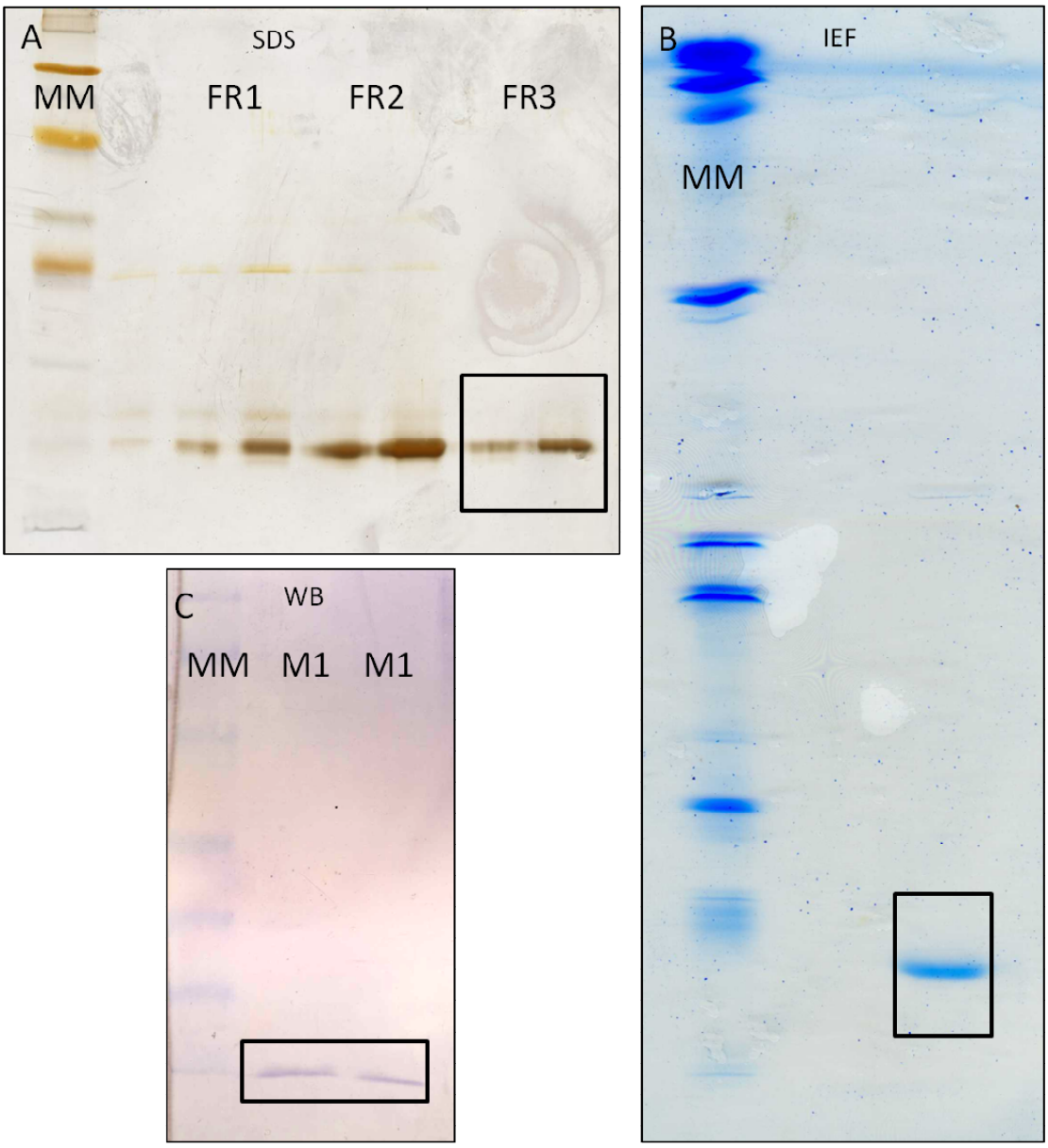

Figure 1: α-plurivorin purification analysis and testing of antibody specificity. A: SDS gel electrophoresis of α-plurivorin peak collected after gel permeation column. The protein bands were silver stained. The peak was divided in three fractions 1, 2 and 3 (FR1, FR2 and FR3). The third and final fraction of the peak showed one single band. B: Isoeletric focusing (IEF) showing only one clear band, proving high purification of α-plurivorin. C: Western-blot (WB) analysis proving high specificity of antiserum against α-plurivorin. The antiserum was tested against the total secretome of *P. plurivora* grown in M1 media for 10 days (M1). MM: molecular markers.

The different concentrations of the antiserum used in the first set of experiments (1:100 dilution factor) and the second set of experiments (1:400 dilution factor) has originated very similar outcome, therefore results from both sets are shown below. Seedlings of *F. sylvatica* infected with *P. plurivora* showed wilting symptoms one day after inoculation, mortality was first recorded after four days. At 192 hpi five out of six

plants died, and one was severely wilted (Fig. 2A and B). On the other hand, plants treated with α-plurivorin antiserum showed no symptoms or mortality throughout the experiment (Fig. 2A and B). The pre-immune normal serum did not have any effect on the pathogen and on the plants, as no differences can be seen when comparing infected plants (Plu) and plants treated with the pre-immune normal serum and infected with *P. plurivora* (N-Plu) and control plants and non-infected plants treated with the pre-immune normal serum (Normal), respectively.

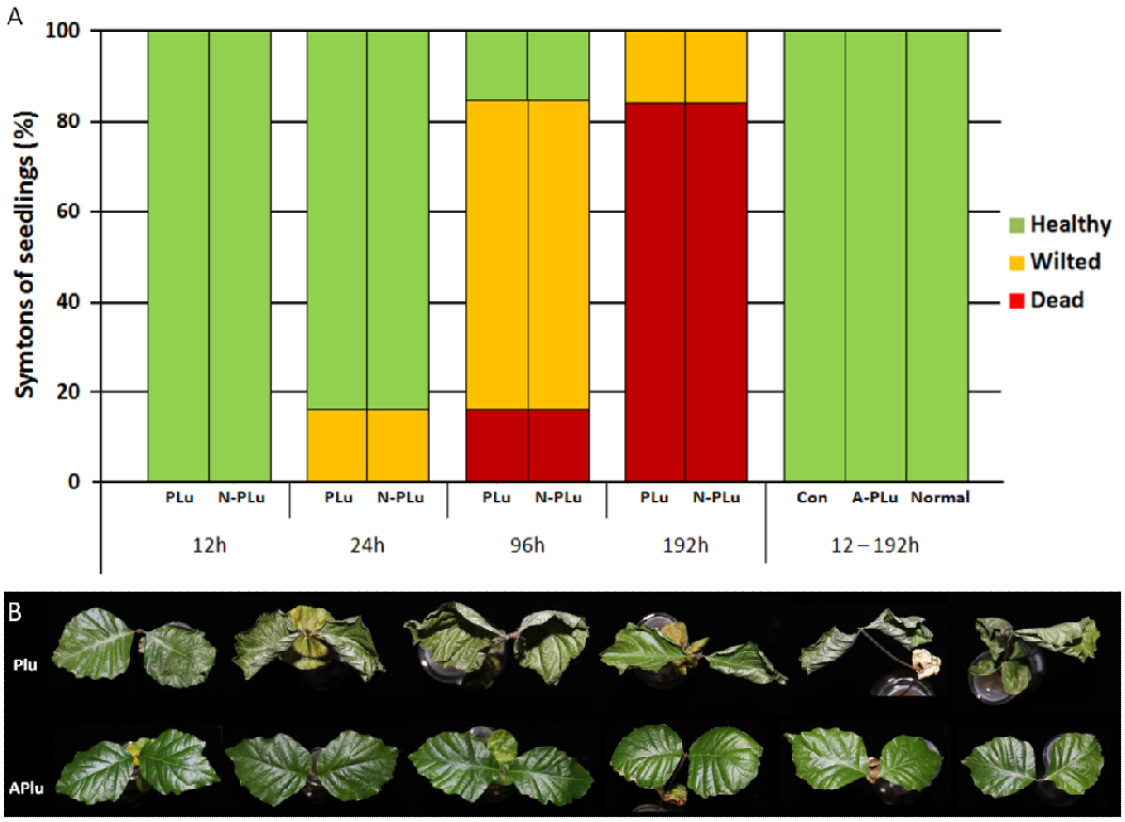

Figure 2: A: symptoms of *F. sylvatica* seedlings (%) over 12, 24, 96 and 192 hours post-inoculation (n=6). Green bars: healthy plants; yellow bars: wilted plants; red Bars: dead plants. Plu: *P. plurivora* infected plants. N-Plu: plants treated with the pre-immune normal serum and infected with *P. plurivora*. Con: control plants (non-treated, non-infected plants); A-Plu: plants treated with α-plurivorin antiserum and infected with *P. plurivora*. Normal: non-infected plants treated with the pre-immune normal serum. B: representative pictures of the upper part of leaves at 192 hours post inoculation. This experiment was repeated 4 times showing similar results.

All the physiological parameters studied were impaired by *P. plurivora* infection (Fig. 3 A, B, C and D). The antibody treatment in non-inoculated plants did not improve or impaired the physiology of the plants as compared to controls. Interestingly, the

plants that were treated with the antibodies and inoculated with zoospores showed no impairment in their physiological status (Fig. 3 A, B, C and D).

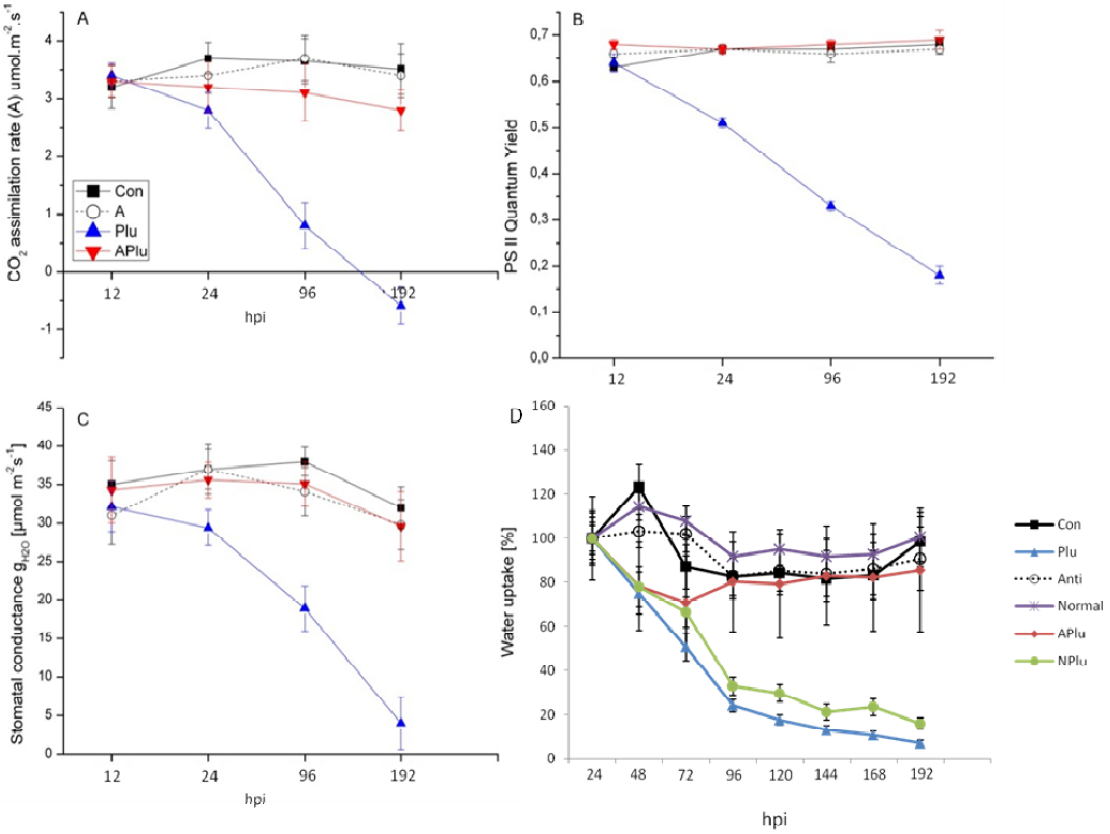

Figure 3: CO_2-assimilation rates (A), PSII Quantum Yield (B), Stomatal conductance (C) and water uptake (% calculated from g) (D) of *F. sylvatica* seedlings after 12, 24, 96 and 192 hours post-inoculation. Beech seedlings infected and treated with the antibody against α-plurivorin exhibited CO_2-uptake rates comparable to those of the control seedlings. "Con", control seedlings, no infection and no antibody treatment; "A", seedlings not infected, but treated with α-plurivorin antiserum; "Plu", seedlings infected with 5×10^5 zoospores of *P. plurivora*; "APlu", seedlings infected with 5×10^5 zoospores of *P. plurivora* and treated with α-plurivorin antiserum. In the Figure 3D, the treatments "Normal": no infection and treatement with the pre-immune normal serum and "NPlu": infection and treatment with the pre-immune normal serum are also shown. (n=6). Error bars represent the s.d. of six replications. "hpi" hours post-inoculation. This experiment was repeated 3 times showing similar results.

Figure 4 shows a confocal image of α-plurivorin production of *P. plurivora* mycelia growing on V8 agar plates. A great portion of the mycelia growing in V8 media

was in the process of producing α-plurivorin, whereas no elicitin was detectable at the surfaces of the zoosporangia and oogonia.

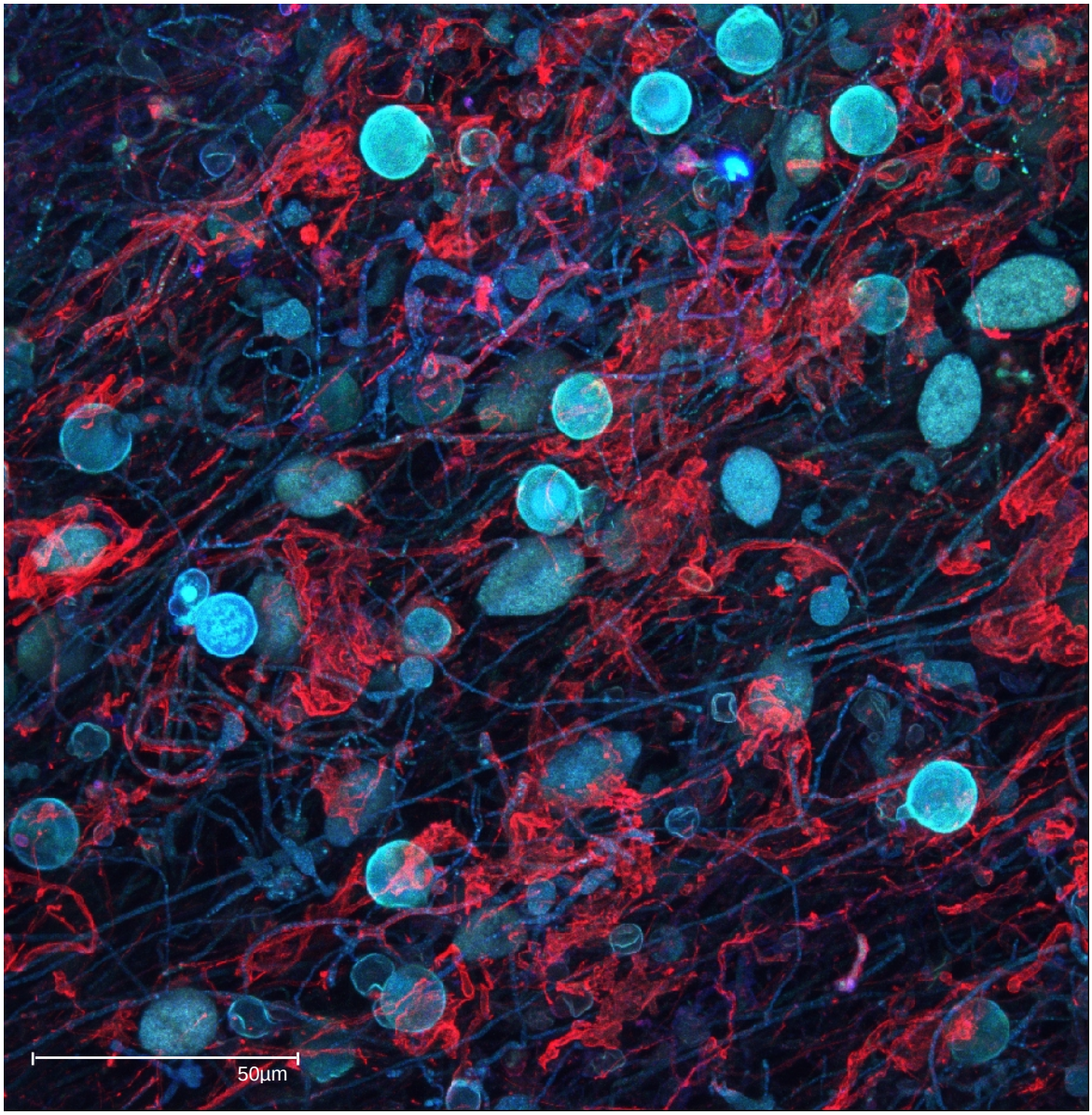

Figure 4: Confocal image of α-plurivorin production of *P. plurivora* mycelia growing on V8 agar plates. The red colour indicates α-plurivorin of *P. plurivora*, and blue indicates the mycelia of the pathogen.

The internalization process and the localisation of both α-plurivorin and *P. plurivora* within the root tissue during infection were further investigated by performing immunofluorescence experiments. To this end, α-plurivorin and *P. plurivora* were

labelled as described in the Methods sections with red and blue tags, respectively. A high amount of the elicitin was secreted during the life cycle of *P. plurivora* (Fig. 4), beginning at the early stages of infection and rapidly spreading throughout the plant tissues. In particular, 6 hours post-inoculation, at the beginning of zoospores germination at the root surface, α-plurivorin was already detected near the central cylinder of infected roots (Figs. 1 and 2, Appendix).

The amount of α-plurivorin detected inside the root tissue increased at later time points (Fig. 5, A, B, C and D), as *P. plurivora* spread throughout the tissue. After 12 hpi (Fig. 5A), the pathogen colonised the epidermis and cortex of the roots, and after 24 hpi (Fig. 5B), the cortex was completely colonised by the pathogen, and hyphae started to enter the central cylinder (first wilt symptoms were already observed at this point of the infection, Fig. 2A). At 96 hpi (Fig. 5C), *P. plurivora* was visible in all of the root tissues and began to damage the cortex cells and central cylinder (most of the plants were wilted and some already died at this time point, Fig. 2A). A devastating situation was observed after 192 hpi when the majority of plants died, the central cylinder collapsed and the cortex was completely damaged (Figs. 5 D and 2B). At this time point, *P. plurivora* was likely in its necrotrophic growth phase (Schlink, 2010).

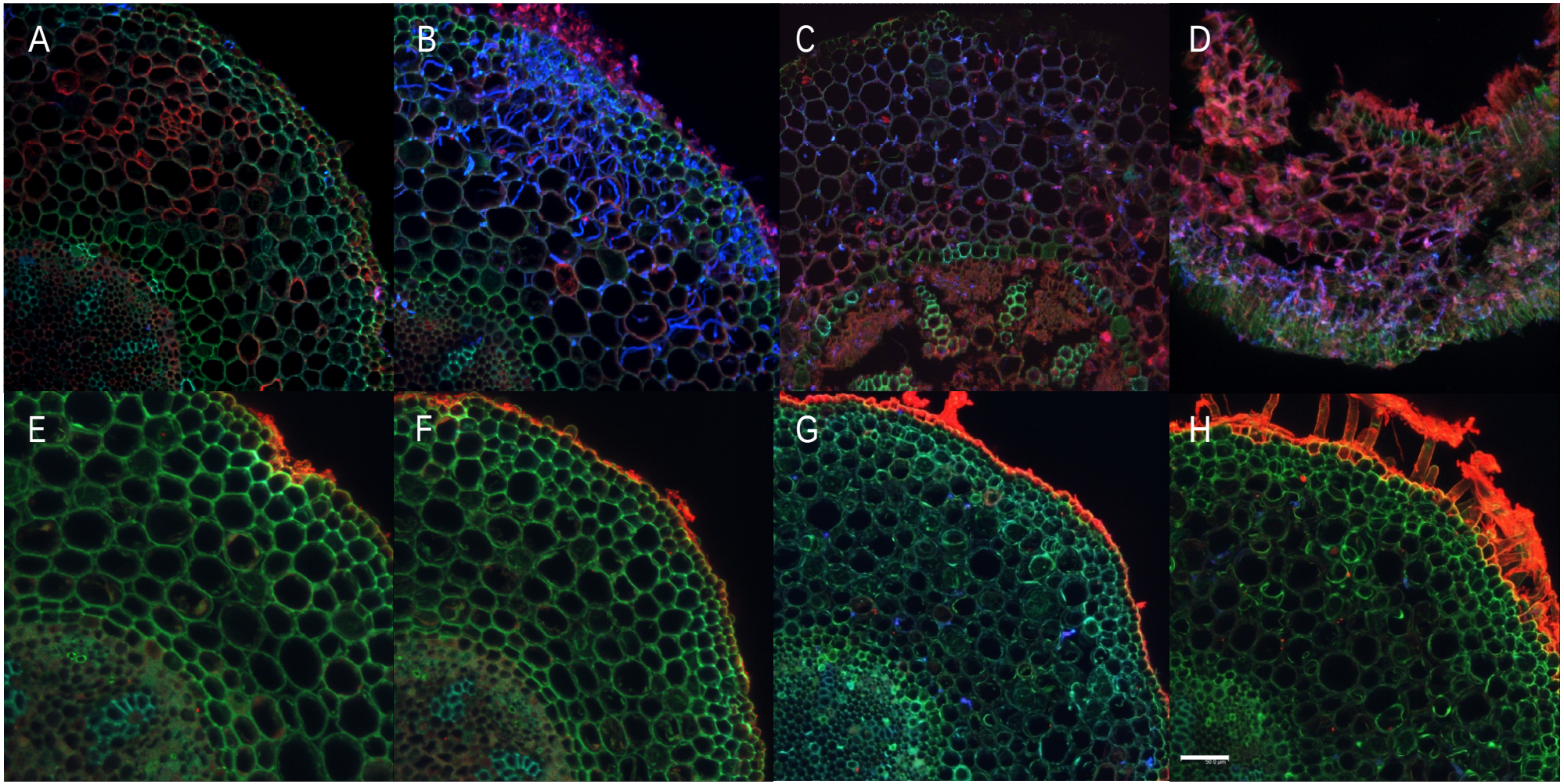

Figure 5: Confocal laser-scanning microscopy images of cross-sectioned roots of *F. sylvatica* seedlings infected with *P. plurivora*. α-plurivorin is immunolabelled with the Alexa Fluor red fluorescent dye, and *P. plurivora* is immunolabelled with the Pacific Blue fluorescent dye. *F. sylvatica* roots autofluorescence in green. Panels A, B, C and D show images of the roots harvested at 12, 24, 96 and 192 hpi, respectively, following the inoculation of 5×10^5 zoospores of *P. plurivora*. α-plurivorin can be observed throughout the entire root tissues from 12 to 192 hpi E, F, G and H show images of roots of seedlings treated with anti-α-plurivorin antibody and inoculated with 5×10^5 zoospores of *P. plurivora* harvested at 12, 24, 96 and 192 hpi, respectively. Controls and the integral view of the images are shown in the Appendix, Figs. 3 to 14. White bar represent 50 µm.

qPCR analyisis showed that *P. plurivora* had an exponential growth pattern in the roots of untreated *F. sylvatica* seedlings. Surprisingly, high amounts of *P. plurivora* DNA were also detected in roots of α-plurivorin antiserum-treated plants, particularly at later time points (96 and 192hpi). No statistically differences were found comparing data from treatment Plu with NPlu (pre-immune normal serum and inoculation), which indicates that the pre-immune normal serum does not have an effect neither on plant defence nor on the pathogen physiology.

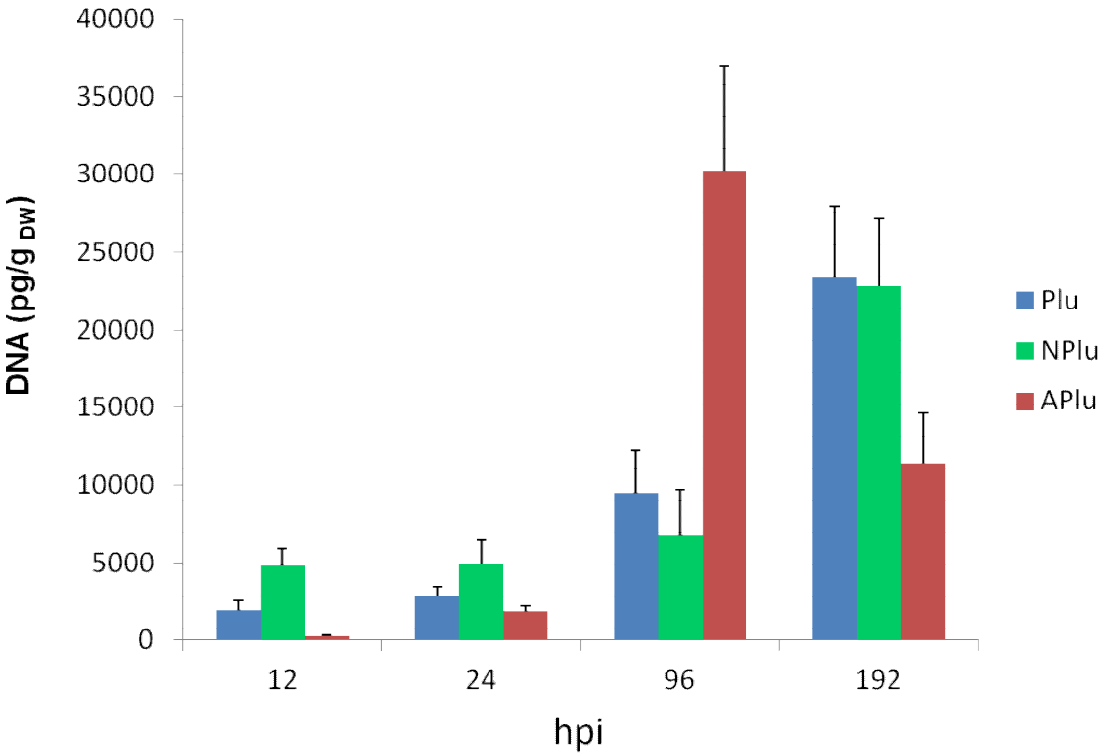

Figure 6: *P. plurivora* genomic DNA content in the roots of *F. sylvatica* seedlings after 12, 24, 96 and 192 hours post-inoculation (hpi). Blue bars: seedlings infected with 5×10^5 zoospores of *P. plurivora* (Plu); green bars: seedlings treated with pre-immune normal serum and infected with 5×10^5 zoospores of *P. plurivora* (NPlu); red bars: seedlings treated with α-plurivorin antiserum and infected with 5×10^5 zoospores of *P. plurivora* (APlu); (n=6). Error bars represent the e.d. of six replications. The experiment was repeated three times showing similar results. Non-infected treatments: Control, seedlings treated with α-plurivorin antiserum (Anti) and plants treated with pre-immune normal serum (Normal), showed no signal for *P. plurivora* genomic DNA, therefore they were not included in the graph.

Figures 7 and 8 show confocal images of integral and sectionated roots at 96 hours post-inoculation. Immunoprecipitation of α-plurivorin prevents penetration of the pathogen inside roots, but did not alter the pathogen grow. High amount of mycelia can be observed growing at the root surface and explains the high level of DNA recorded after qPCR analysis.

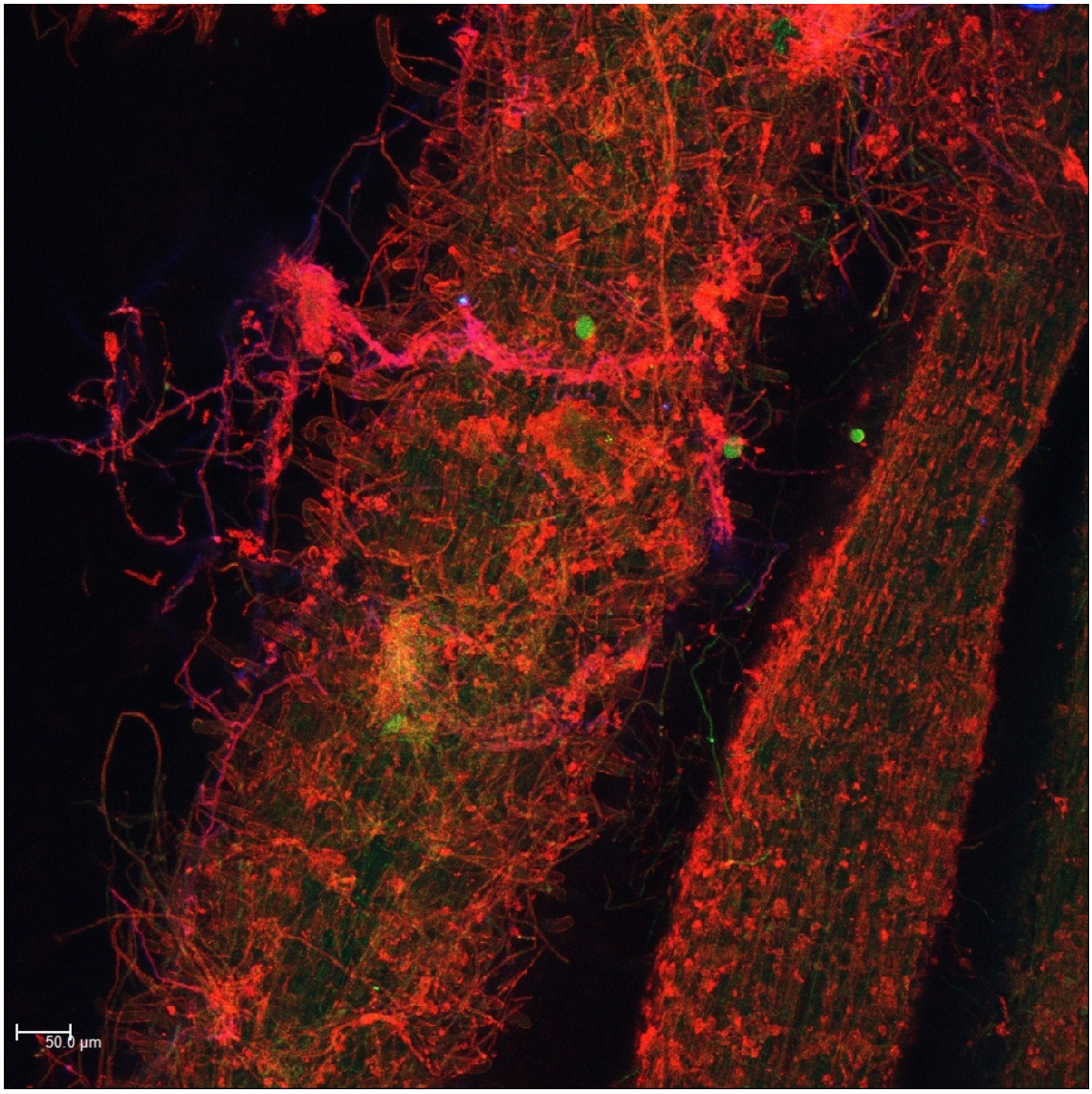

Figure 7: Confocal image of *F. sylvatica* integral roots at 96 hours post-inoculation of 5×10^5 zoospores of *P. plurivora* and prior immunoprecipitation of α-plurivorin. *P. plurivora* is immunolabelled with a blue fluorescence die. α-plurivorin is immunolabeled with a red fluorescence die. Bar represent 50 µm.

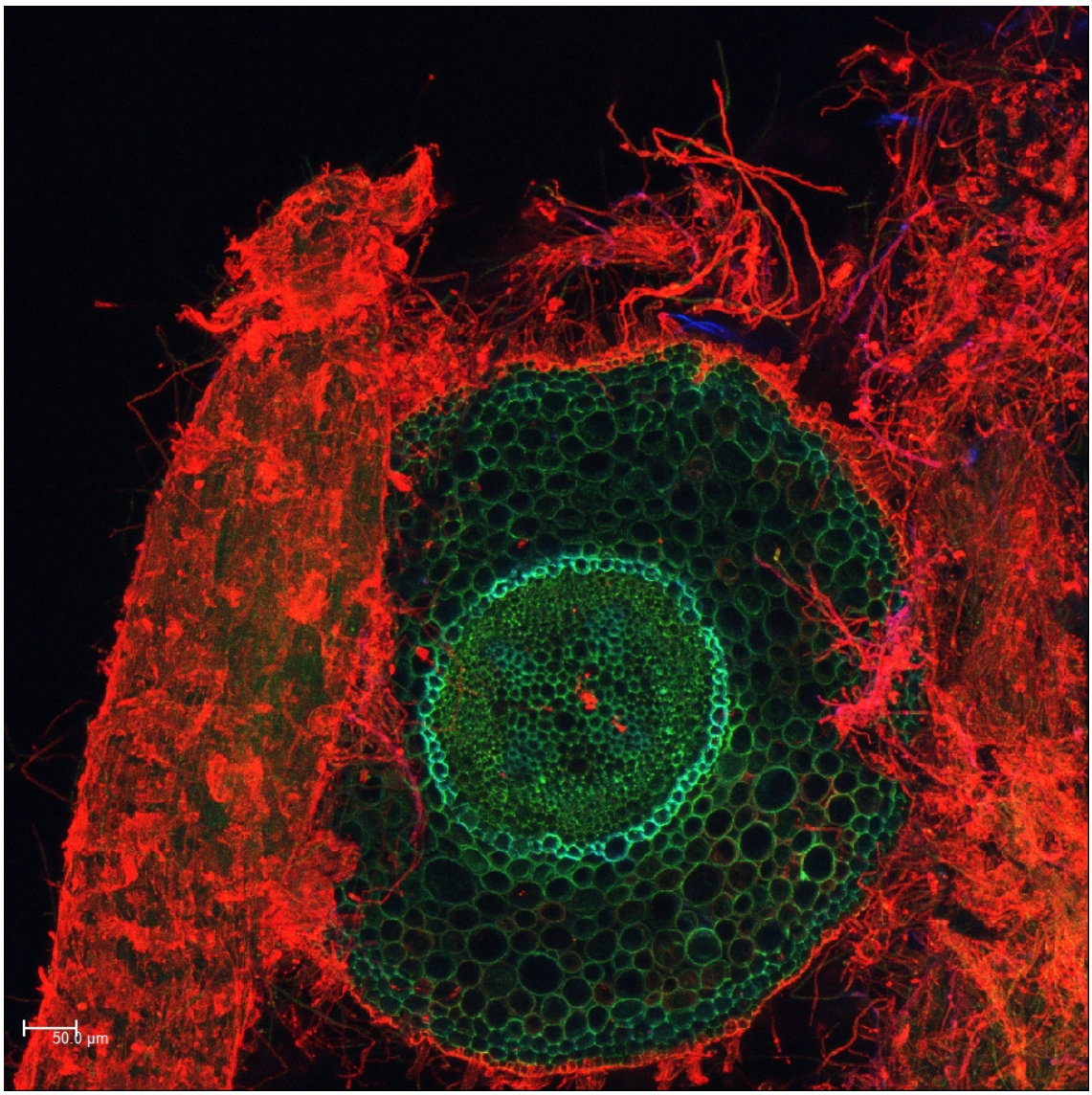

Figure 8: Confocal image of *F. sylvatica* roots, integral and sectionated at 96 hours post-inoculation of 5 × 10^5 zoospores of *P. plurivora* and prior immunoprecipitation of α-plurivorin. *P. plurivora* is immunolabelled with a blue fluorescence die. α-plurivorin is immunolabeled with a red fluorescence die. Bar represent 50 µm.

Gene expression analysis of *P. plurivora* reveals that the treatment with the α-plurivorin antiserum induced a higher α-plurivorin gene expression in comparison with non-treated plants . This gene up-regulation was found to be statistically significant at 24, 96 and 192 hpi (Fig. 9).

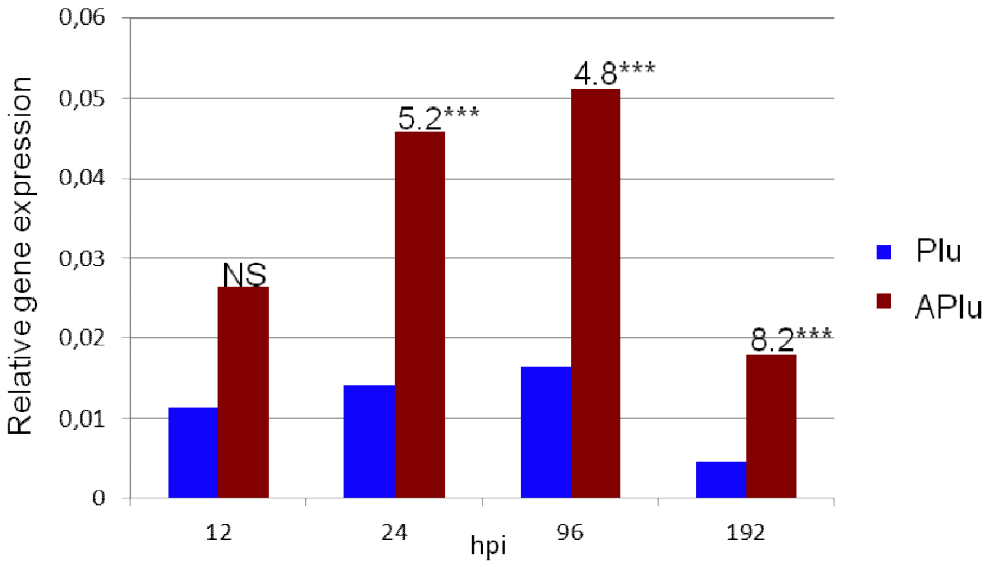

Figure 9: Relative gene expression of the α-plurivorin gene in roots of *F. sylvatica* seedlings after 12, 24, 96 and 192 hours post-inoculation (hpi) (n=4). Blue bars P.plurivora in roots. Red bars P. plurivora in roots treated with the antiserum. Data were normalized against the qPCR of *P. plurivora* in roots. "NS" non-significant. *** P≤0,001.

Figure 10 and Table 1 show gene expression analysis of defence-related genes from *F. sylvatica*. *P. plurivora* infection resulted in a significant down-regulation in at almost all time points of all the genes analysed. On the other hand, blocking α-plurivorin resulted in down-regulation of only PRP and ACO at 96 hpi, all the other genes showed either a constitutive transcription level comparable as compared to controls (PR1 at 12 and 96 hpi, PR2 and PR3 all the time points, PRP at 12 and 24 hpi, and ACO at 24 hpi), or even an up-regulation (WRKY at all the time points, PR1 at 24 hpi and ACO at 12 hpi).

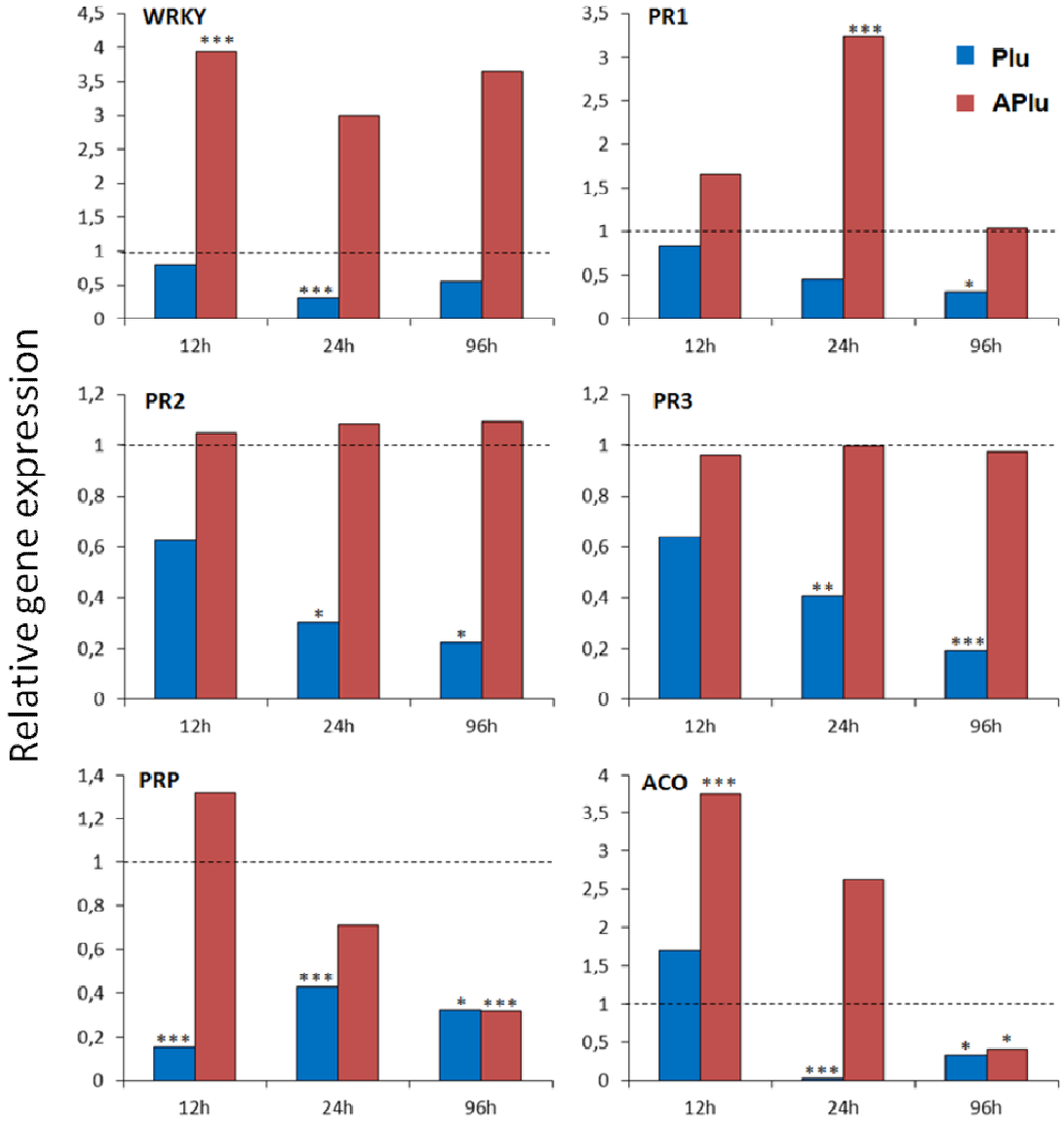

Figure 10: Relative expression of defense related genes in roots of *F. sylvatica* seedlings at 12, 24 and 96 hours post-inoculation (hpi) with 5×10^5 zoosporres of *P. plurivora* (n=4). Graphs show data of infected plants (Plu – blue bars) and plants treated with α-plurivorin antiserum and inoculated with P. plurivora (APlu – red bars). Dashed line represents the relative expression of control plants. The transcript levels were normalized with tubulin and actin as refrence genes. Data showing statistically different relative values above or below the dashed line are up or down-regulated respectively. P≤0,05*; P≤0,01**; P≤0,001***. This experiment was repeated twice showing similar results.

Table 1: Relative expression of defense related genes in roots of *F. sylvatica* seedlings at 12, 24 and 96 hours post-inoculation with 5×10^5 zoosporres of *P. plurivora*. Upper three tables show relative gene expression of plants from treatment with antibody and inoculated (APlu) em relation to non-treated inoculated (Plu). Middle three tables show relative gene expression of plants from treatment with pre-immune normal serum and inoculated (NPlu) in relation to non-treated inoculated (Plu).). Lower three tables show relative gene expression of non-inoculated control plants (Control), non-inoculated antiserum treated plants (Anti) and non-inoculated pre-immune normal serum treated plants (Normal). "Reac. Eff." means reaction efficiency of the analysis. "UP" means statistically up-regulated genes. Data was normalized with Actin and tubulin. (n=4).

Plu Vs A-Plu 12hpi

Gene	Expression	Reac. Eff.	P(H1)	Result
WRKY	4,975	0,997	0	UP
PR1	1,986	0,908	0,111	
PR2	1,678	0,968	0,269	
PR3	1,503	0,999	0,248	
PRP	2,229	0,976	0,003	UP
ACO	2,209	0,725	0,017	UP

24 hpi

Gene	Expression	Reac. Eff	P(H1)	Result
WRKY	7,132	0,997	0	UP
PR1	7,192	0,908	0	UP
PR2	3,578	0,968	0,002	UP
PR3	2,423	0,999	0	UP
PRP	1,648	0,976	0,007	UP
ACO	23,898	0,725	0,001	UP

96 hpi

Gene	Expression	Reac. Eff.	P(H1)	Result
WRKY	3,797	0,997	0,006	UP
PR1	1,938	0,908	0,153	
PR2	2,833	0,968	0,009	UP
PR3	2,912	0,999	0,003	UP
PRP	0,564	0,976	0,277	
ACO	0,728	0,725	0,245	

Plu Vs N-Plu 12 hpi

Gene	Expression	Reac. Eff.	P(H1)	Result
WRKY	0,923	0,997	0,727	
PR1	0,94	0,908	0,892	
PR2	0,952	0,968	0,915	
PR3	0,919	0,999	0,778	
PRP	0,92	0,976	0,675	
ACO	0,877	0,725	0,536	

24 hpi

Gene	Expression	Reac. Eff	P(H1)	Result
WRKY	1,091	0,997	0,647	
PR1	1,052	0,908	0,852	
PR2	0,969	0,968	0,917	
PR3	1,056	0,999	0,792	
PRP	0,991	0,976	0,958	
ACO	1,072	0,725	0,806	

96 hpi

Gene	Expression	Reac. Eff.	P(H1)	Result
WRKY	1,101	0,997	0,855	
PR1	1,067	0,908	0,822	
PR2	1,036	0,968	0,93	
PR3	1,037	0,999	0,898	
PRP	0,611	0,976	0,29	
ACO	0,901	0,725	0,465	

Control Vs Anti 24 hpi

Gene	Expression	Reac. Eff.	P(H1)	Result
WRKY	0,997	0,682	0,353	
PR1	0,908	0,687	0,455	
PR2	0,968	0,73	0,557	
PR3	0,999	0,739	0,412	
PRP	0,976	0,669	0,176	
ACO	0,725	0,756	0,548	

Control Vs Normal 24 hpi

Gene	Expression	Reac. Eff.	P(H1)	Result
WRK	0,997	0,907	0,769	
PR1	0,908	0,768	0,557	
PR2	0,968	1,078	0,852	
PR3	0,999	0,859	0,615	
PRP	0,976	0,882	0,602	
ACO	0,725	0,832	0,612	

Anti Vs Normal 24 hpi

Gene	Expression	Reac. Eff.	P(H1)	Result
WRKY	1,33	0,997	0,452	
PR1	1,118	0,908	0,808	
PR2	1,476	0,968	0,38	
PR3	1,162	0,999	0,636	
PRP	1,319	0,976	0,306	
ACO	1,1	0,725	0,791	

2.4 Discussion

P. plurivora colonisation of beech roots is fast and highly destructive. Three-month-old seedlings showed wilting symptoms one day after inoculation, and mortality was first recorded after four days. Consequently, the CO_2-uptake rates, chlorophyll fluorescence, stomatal conductance and water uptake of the infected seedlings were strongly impaired. At the end of the experiment, five out of six plants died, and one was severely wilted, demonstrating the aggressiveness of *P. plurivora* in beech (Fleischmann *et al*., 2005; Manter *et al*., 2010; Portz *et al*., 2012).

To investigate the role of α-plurivorin in the infection process, the elicitin function was inhibited by performing a blocking experiment with a specific antibody against α-plurivorin. To this aim, the antibody was added to the water covering the root system of the seedlings before the inoculation with zoospores. All plants pre-incubated with the anti-α-plurivorin survived the *P. plurivora* infection. Interestingly, all of these plants remained healthy showing only minor changes in photosynthesis, chlorophyll fluorescence, stomatal conductance and water uptake throughout the entire experiment.

As observed by microscopy, the pattern of root tissue colonisation by *P. plurivora* is in agreement with the results obtained by quantifying the *P. plurivora* genomic DNA content in the roots of *F. sylvatica* seedling by real-time PCR (qRT-PCR) measurements.

Notably, the treatment with the anti-α-plurivorin antibody almost completely prevented the elicitin internalization. Indeed, most of fluorescence signal related to α-plurivorin was detected on the fine root surface, likely due to size and/or conformation changes following the interaction with the antibody (Pellequer *et al*., 1999). Also, the

masking of specific α-plurivorin recognition regions with potential root surface receptors cannot be excluded.

Overall, these findings support the importance of α-plurivorin for the penetration of *P. plurivora* in host tissues, because no hyphae were visible inside the roots tissue at earlier time points and only a few hyphae were detectable at 96 and 192 hpi, compared to control samples.

Interestingly, although a weak colonisation of the root tissue by *P. plurivora* was observed at later time points (i.e. 96 and 192 hpi) in plants treated with anti-α-plurivorin antibody, the corresponding qRT-PCR data indicated a strong infection by the pathogen. At 96 hpi, the amount of *P. plurivora* DNA in the roots treated with the antibody against α-plurivorin was approximately three times higher than the non-treated controls. However, all of the measured physiological parameters of beech were comparable to those of plants not infected but treated with the antibody.

Additionally, at this time point no mortality was observed in treated plants whereas the mortality of infected plants was greater than 80 %. These results clearly demonstrated that the blocking of α-plurivorin function strongly impaired the ability of the pathogen to penetrate inside host tissues, growing almost exclusively on the root surface.

Moreover, to likely counteract the inability to infect host tissue, α-plurivorin gene expression was strongly up-regulated in the pathogen at 24, 96 and 192 hpi (with significant relative expression values on the plants treated with the antibody compared to the untreated plants.

To elucidate the potential role of α-plurivorin in the regulation of plant defence responses, the expression of several defence-related genes, such as ACC-oxidase (ACO, ethylene pathway) (Schäfer *et al*., 2009), PR1 (Mukhtar *et al*., 2009), PR2 (Yu et al., 2010), PR3 (pathogenesis-related proteins) (Veluthakkal *et al*., 2012), PRP (proline-rich protein of cell walls) (Yeom et al., 2011) and the transcription factor WRKY, which is known to be strongly up-regulated after biotic stresses (Oh *et al*., 2008), was analysed. *P. plurivora* infection consistently down-regulated all defence-related genes at almost all time points. Similar down-regulation of defence-related genes resulting in high susceptibility of *F. sylvatica* to *P. plurivora* was previously shown by Schlink (2010); however, the intrinsic mechanisms of this manipulation were still unclear. It was found, after blocking α-plurivorin, that the only genes down-regulated were PRP and ACO at 96 hpi, all the other genes showed either a constitutive transcription level comparable to controls or an up-regulation. These results give strong evidence that α-plurivorin is directly involved in manipulation of plant defences by a broad down-regulation of defence-related genes, independently of the signalling pathways. Furthermore, up-regulation of WRKY, PR1 and ACO after blocking α-plurivorin suggests that the α-plurivorin can be also correlated with suppression of either PTI or ETI, therefore, acting as an effector triggering susceptibility (ETS).

In all parameters investigated, the treatment with pre-immune normal serum, in combination or not with infection, did not change any aspect of plant physiology or pathogenicity of *P. plurivora*. As a consequence, it can be concluded that the results coming from the blocking of α-plurivorin with its antiserum are strictly related to the alteration of the protein functions and not as a secondary effect of foreigner antibodies.

On the basis of our results it can be hypothesized a potential role for α-plurivorin in the highly susceptible interaction between beech and *P. plurivora* (Fig. 11). The elicitin, secreted by *P. plurivora* growing on the root surface, easily penetrates into the apoplast where it inundates the entire root tissue before the pathogen is internalized. The elicitin α-plurivorin seems to have dual functions in the susceptible interaction: it is responsible for the penetration of the pathogen, and it directly or indirectly suppresses the defence responses of beech, thus causing a susceptible host-pathogen interaction (Fig. 11A). The inhibition of α-plurivorin function by anti- α-plurivorin antibody prevented the internalization of *P. plurivora*, and concurrently, allowed the induction of plant defence responses in the beech roots (Fig. 11B), thus blocking the disease development.

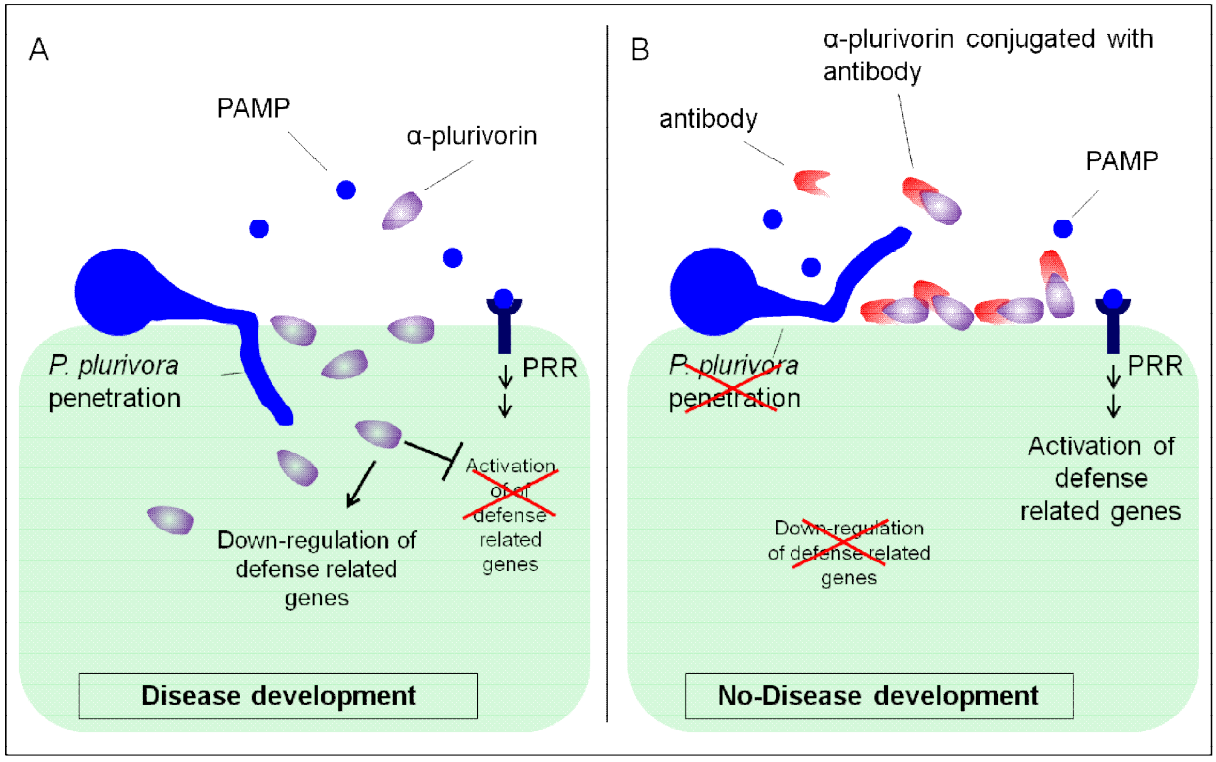

Figure 11: A: α-plurivorin, secreted by *P. plurivora* while the pathogen is growing on the root surface, easily penetrates into the apoplast where it inundates the entire root tissue before the pathogen enters; the elicitin down regulates defence related genes, suppressing the defence responses of beech triggered by *P. plurivora* PAMPs after recognition by pattern-recognition receptor (PRRs), thus causing the disease development. B: The treatment with anti- α-plurivorin antibody prevented the internalization of *P. plurivora*, and concurrently, allowed the induction of plant defence responses, possibly after recognition by PAMPs by the PRRs in the beech roots. In this instance, no disease symptoms are observed.

Our results are in good agreement with previous data showing that a strain of *P. cinnamomi* silenced for the β-cinnamomin gene, which is involved in β-cinnamomin elicitin synthesis, was unable to invade root tissue actively and cause disease symptoms (Horta et al., 2010). The authors conclude that elicitins are either directly or indirectly associated with the infection process of *Phytophthora*, but did not excluded that the transformation process itself generated some collateral disruption of the pathogen genome, reducing its aggressiveness. In addition, strains of *P. parasitica* (P3461) and *P. cryptogea*, lacking any elicitin-coding sequence, has been shown to be completely non-pathogenic in host plants (Kamoun et al., 1993).

Elicitins were first described as an elicitor of defence responses. During decades, elicitins, particularly INF1, were extensively studied as an elicitor triggering programmed cell death (PCD) in *Nicotiana* spp (Kamoun *et al*., 1998; Shibata *et al*., 2010; Chaparro-Garcia *et al*., 2011). INF1 is an alkaline elicitin produced by *Phytophthora infestans*. *P. infestans* share an incompatible interaction with tobacco, where INF1 is suggested as a main component in this non-host resistance (Kamoun, *et al*., 1997). Overall, the functions of elicitins in the Phytophthora/plant interactions can vary depending on the type of protein and hosts, therefore every kind of interaction involving elicitins, phytophthora and plants should be investigated separately.

Remarkably, the data showed in this work demonstrated that, even considering the presence of hundreds of effector genes in the *P. plurivora* genome, the blocking of α-plurivorin function compromises *P. plurivora* pathogenicity, thus suggesting its essential role for virulence. Because elicitins are highly conserved proteins (with high similarity (Yu *et al*., 1995), Fig. 12) and are almost ubiquitously secreted by all *Phytophthora* species (Takemoto *et al*., 2005), it will be of interest to investigate their

role as virulence factors in other *Phytophthora*-susceptible plant interactions. More importantly, the results found in this work open new perspectives towards the use of elicitins as specific targets for protecting plants against Phytophthora infection.

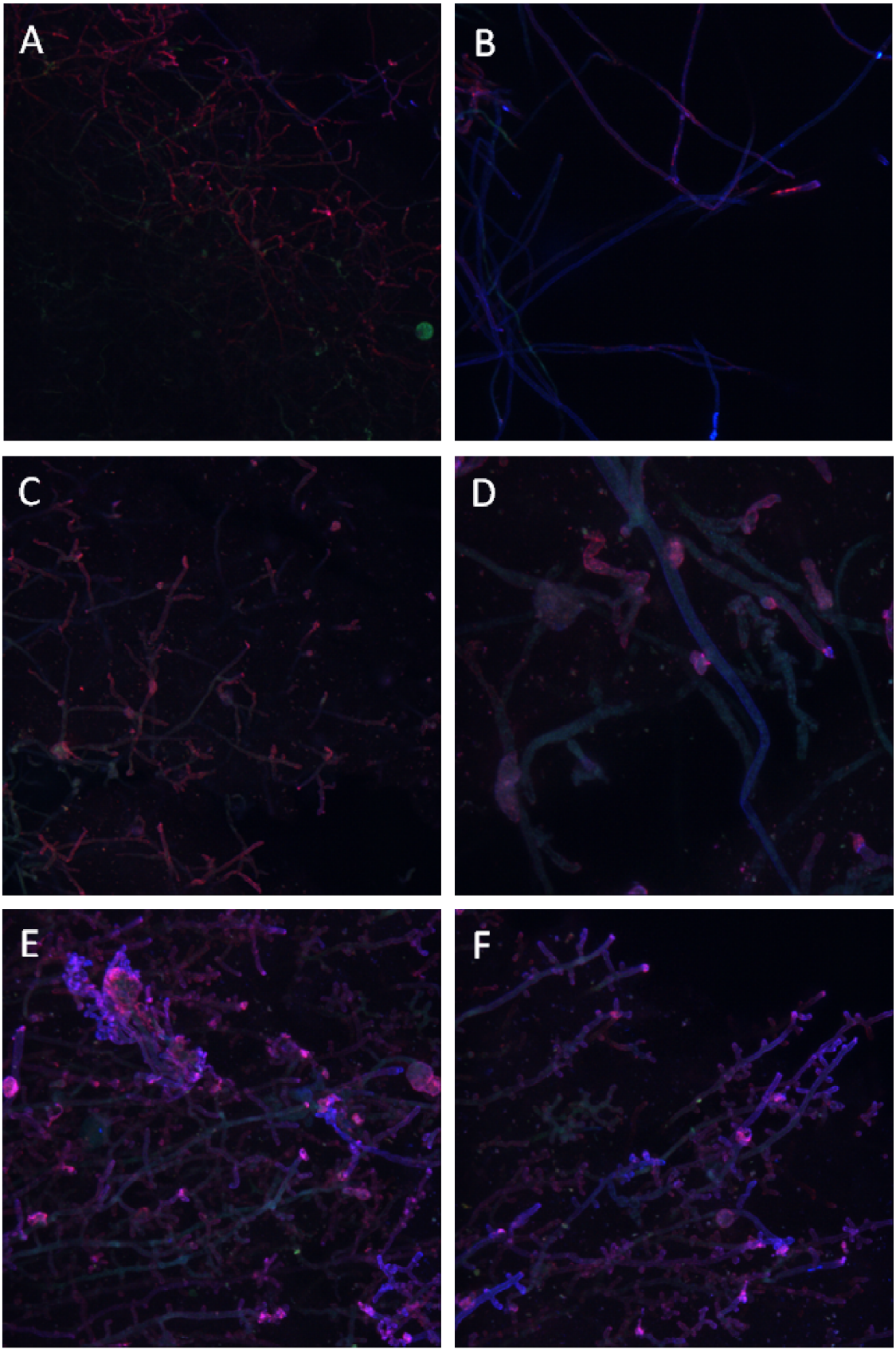

Figure 12: Elicitins from *P. quercina*, *P. nicotianae* and *P. pseudosyringae* share high similarity with α-plurivorin from *P. plurivora*. Confocal image of elicitin production by *Phytophthora spp.* mycelia growing on V8 agar plates. Elicitins were labeled with specific antibodies for α-plurivorin, evidencing high similarity of these proteins among different species. "A and B" *P. quercina*, "C and D" *P. nicotianae*, "E and F" *P. pseudosyringae*.

Chapter 3

Phosphite protects *Fagus sylvatica* seedlings towards *Phytophthora plurivora* via local toxicity, priming and facilitation of pathogen recognition

Phosphite protects *Fagus sylvatica* seedlings towards *Phytophthora plurivora* via local toxicity, priming and facilitation of pathogen recognition

Summary

Phytophthora plurivora causes severe damage on *Fagus sylvatica* and this root pathogen is jointly responsible for the widening decline of European Beech. Unfortunately no efficient treatment against this disease is presently available. Different salts of phosphite (Phi) are known to protect plants against *Phytophthora* species; however their mechanism of action towards *P. plurivora* is still unknown.
To discover the effect of Phi on root infection, leaves were sprayed with Phi and roots were subsequently inoculated with *P. plurivora* zoospores. Seedling physiology, defense responses, colonization of root tissue by the pathogen and seedling mortality was monitored. Additionally the concentration of Phi in roots was quantified. Finally, the effect of Phi on mycelial growth and zoospore formation was recorded.
Phi treatment was remarkably efficient in protecting beech seedlings against *P. plurivora*; all Phi treated plants survived *P. plurivora* infection. Surprisingly, Phi treated and infected seedlings showed a strong up-regulation of several defense genes, however, all physiological parameters measured were comparable to control plants. The local Phi concentration detected in roots was high enough to inhibit pathogen growth. Phi treatment by itself did not either harm seedling physiology nor induces defense responses.
The monitored up-regulation of defense genes could be explained either by priming or by facilitation of pathogen recognition of the host.

3.1 Introduction

Protecting plants from *Phytophthora* species is still a challenge nowadays. Successful pathogens, including *Phytophthora* species, have the ability to subvert plant defense mechanisms either by avoiding their recognition or by re-programming host metabolism (Kamoun, 2006; Win *et al*., 2007). Schlink (2010) has shown that the susceptible interaction between *P. plurivora* and *F. sylvatica* was characterized by a "striking lack of defense gene induction" and she concluded that *P. plurivora* possibly escapes the main plant-recognition systems. Thus, recognition of the invader by the host is a key-factor in defending the pathogen.

Plant recognition of invaders commonly causes a fast flux of ions, the accumulation of reactive oxygen species (ROS), the activation of MAP kinases

signaling cascades as well as specific gene expression and finally the activation of defense pathways. Induced downstream responses rely in most cases on a network of cross-communication between signaling pathways mediated by salicylic acid (SA), jasmonate (JA) or ethylene (ET).

Unfortunately *Phytophthora* pathogens cannot be combated with well-known fungicides, because as Oomycetes they do not synthesize chitin and ergosterol. However, many investigations have shown that different salts of the phosphores acid, the phosphites are effective to control growth of *Phytophthora* pathogens. Daniel and Guest (2006) proved that phosphite treatment of *Arabidopsis thaliana* leaves triggered the release of superoxide, caused localized cell death and enhanced accumulation of phenolics around infected cells. In consequence growth of *P. palmivora* was restricted and the production of sporangia was inhibited. In the presence of the superoxide quencher Mn(II)-desferal, there was no longer hypersensitive cell death and the pathogen was able to grow in phosphite-treated plants. These data prove that inhibition of the pathogen was due to superoxide release rather than a direct effect of the chemical. Recently Eshraghi *et al*. (2011) showed that phosphite treatment of *Arabidopsis thaliana* primed the plant for defense responses of the salicylic acid (SA) and jasmonic acid/ethylene (JA/ET) pathways. Thus phosphite treated plants showed a significant reduced lesion size after infection with *P. cinnamomi*.

Besides interfering with defense pathways of host plants, phosphite was shown to interact directly with *Phytophthora* pathogens. (King *et al.* 2010) published that the chemical caused hyphal distortion and lysis of cell walls in parallel with a down-regulation of many genes encoding proteins involved in cell wall synthesis and cytoskeleton functioning. (Coffey and Joseph, 1985) demonstrated that oospore

production, sporangia formation and mycelial growth of *P. cinnamomi* and *P. citricola* were significantly inhibited by either phosphorous acid or fosetyl-Al. (Jackson *et al.*, 2000) concluded from their data that phosphite acts in a dual way. When phosphite concentrations are low, the chemical induced host defense enzymes such as 4-coumarate coenzyme A ligase (4-CL), cinnamyl alcohol dehydrogenase (CAD). However, at high phosphite concentrations, the chemical acted directly on *P. cinnamomi* and inhibited its growth.

Extensive field studies by (Shearer *et al.*, 2006) proved that phosphite treatment of *Eucalyptus* and *Banksia* species is a practical option to control *P. cinnamomi* root infection over several years. However, glasshouse studies of (Wilkinson *et al.*, 2001) showed that phosphite treatment reduced but did not prevent the production of viable zoospores on infected trees. Thus the authors concluded that phosphite application will lower the amount of infection by *Phytophthoras,* but may not remove the risk of *Phytophthora* spreading from already infected trees.

The aim of this study has been to elucidate the mechanisms of potassium phosphite on plant physiology and gene regulation of treated beech saplings in order to understand the protective effect of this chemical on the highly susceptible interaction between *F. sylvatica* and *P. plurivora*.

3.2 Material and Methods

In vivo:

Phytophthora plurivora T. Jung and T.I. Burgess, isolate CIT55, which was isolated from a declining beech in Southern Bavaria (Germany), was grown on V8 agar in the dark at 20°C.

Radial growth: Petri dishes containing V8 media with addition of 0, 5, 10, 50 and 100 µg/mL of phosphite (5 plates per phi concentration) were prepared and *P. plurivora* was transferred. Radial growth of the mycelia was recorded throughout 5 days.

Sporulation: *P. plurivora* was transferred to Petri dishes containing V8 media. After the colony reached up to 80% of the plates, sporangia development was induced by pouring a solution of 0, 5, 10, 50 and 100 µg/mL of phosphite (5 plates per phi concentration) on the plate. The solutions were replaced each day throughout one week. Zoospores release was induced by placing the Petri dishes at 4°C for one hour. The zoospores concentration was recorded using a Thoma chamber.

In Planta:

Plant growth conditions: Seeds of European beech (*Fagus sylvatica L.*) were germinated and grown in root trainers with sterile vermiculite for 3 months at 20 °C and light conditions of 250 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD). Three days before initiating the experiment, the seedlings were carefully removed from the containers. The roots were rinsed of the substrate and placed in 50 mL Falcon tubes containing 50 mL of distilled water and sealed with Parafilm.

Phosphite treatment: Plants were treated with phosphite 4 days before inoculation with *P. plurivora* zoospores (T=-4). The leaves were sprayed until run-off with potassium-phosphite using a plastic spray bottle. The phosphite solution was prepared by mixing KOH with H_3PO_3, pH 3.0, with final concentration of 0.5%. The plants were inoculated with 5×10^5 zoospores per plant.

Experimental design and inoculation of plants with zoospores: Four months old *F. sylvatica* seedlings were treated as follows:(a) control: not Phi-treated / not-inoculated (Con); (b) phosphite treated / not-inoculated (Phi); (c) not phosphite-treated / *P. plurivora* inoculated (Plu); (d) phosphite treated / inoculated with *P. plurivora* (Phi-Plu). Six plants were used for each treatment.

Quantification of phosphite in roots

For the determination of phosphite within different plant organs, beech seedlings were harvested, washed with deionized water, dried with cellulose tissue, separated into leaves, stems and roots and freeze dried for 24 hours. Fresh and dry weight of each organ were determined before and after freeze drying. All samples were grained to a fine powder using a ball mill and phosphite was extracted from 50 mg of powdered plant tissue according to Roos et al. (1999) in 500 µL HPLC-water. The samples were vortexed vigorously and incubated over night at RT in the rotary overhead-shaker. Afterwards samples were centrifuged for 10 minutes at 15,000. The supernatant was filtered through a 0.45 µm nylon filter and stored at -20°C until analysis. Ion chromatography of phosphite ($H_2PO_3^-$) was performed according to Ryder (1986) using 20 mM succinic acid as mobile phase at a flow rate of 0.8 mL per minute. The HPCL system consisted of a L-6200 A Pump (Hitachi), a Vidac 302 Anion Column (250 x 2.1

mm; 10 µm, Grace) tempered at 40° C in a HIC-6 A Column oven (Shimadzu), and of a CDD-6 A Conductivity detector (Shimadzu) tempered to 43° C.

Disease assessment and plant physiology:

The plants were monitored daily for root necrosis, for growth of visible mycelia on the root surface, and for the wilting of leaves as well as for mortality.

Gas exchange measurements were conducted using a CO_2/H_2O diffusion porometer equipped with a broad-leaf LED cuvette (LI-6400, LI-COR, Lincoln, Nebraska, USA). All of the measurements were conducted under steady-state conditions of 23 °C (leaf temperature), between 50% and 60% relative humidity and 400 ppm CO_2 concentration (in the reference air), 250 µmol m^{-2} s^{-1} PPFD, and 500 mL min^{-1} air flow.

To analyze the activity of the enzyme RuBisCO, an A/C_i-Curve has been performed with the LI-COR LI6400 using the default program of the device. Leaf CO_2 uptake (A) versus intercellular CO_2 concentration (C_i) curves provide information about the limitation of photosynthesis.

The slope of an A/C_i-curve was used for calculation of the RuBisCO activity (V_{cmax}), the maximum rate of electron transport (J_{max}) and mitochondrial respiration due to phosphorylative oxidation (R_d). The data was analyzed with a Microsoft Excel based macro (http://landflux.org/Tools.php) on the models of Farquhar *et al*., (1980); Ethier and Livingston (2004) and Elsworth *et al*., (2004).

qRT-PCR and gene expression:

Genomic DNA was extracted from 20 mg freeze-dried and milled root material using the DNeasy plant mini kit (Qiagen, Hilden, Germany) and was further purified using the Wizard® DNA clean up system (Promega, Mannheim, Germany) according to

the manufacturer's protocols. The DNA was diluted 1:10 in H_2O to prevent the inhibition of the PCR reaction. The amount of *P. plurivora* DNA in 5 µL of root extract was determined by TaqMan quantitative PCR using an SDS7700 sequence detection system (Applied Biosystems, Germany), with the primer pair P5 / P6 and the fluorogenic probe F3 labelled with FAM as a reporter dye and TAMRA as a quencher. All of the analyses were performed in three technical repetitions using ABsolute QPCR ROX chemicals (ABgene, Hamburg, Germany) and performing 40 cycles of denaturation at 95°C for 15 s and annealing / extension at 62°C for 60 s. The Ct values of the samples were compared with a standard curve that was generated for pure *P. plurivora* genomic DNA extracted from mycelia grown in M1-liquide culture. The standard curve concentrations ranged from 1 pg DNA mL^{-1} to 10 ng DNA mL^{-1} in five steps.

For the extraction of total RNA, 50 mg of roots were ground in liquid nitrogen using a mortar and pestle. The total RNA was extracted using the MasterPure Plant RNA Purification kit (Epicentre Biotechnologies, Madison, WI, USA) according to the manufacturer's protocol, including a DNAse I treatment. The concentration and quality of extracted RNA was measured using a BioMate 3 Photometer (Thermo Fisher Scientific, Ulm, Germany). cDNA was reverse transcribed using 1 µg of total RNA with oligo-dT primers and the MMLV Reverse Transcriptase 1st strand cDNA Synthesis kit (Epicentre Biotechnologies, Madison, WI, USA). The transcript levels of specific genes were analysed by using 0.05 µg of cDNA by qRT-PCR in three technical replicates using the ABsolute SYBRGreen ROX chemicals (ABgene, Hamburg, Germany) and performing 40 cycles of denaturation at 94°C for 30 s, annealing at 60°C for 30 s, and extension at 72°C for 30 s (Böhm et al., 1999).

In order to shed a light on the phi-induced gene defense responses and elucidate whether these responses are mediated via SA or JA/ET signal transduction, we performed transcriptional analysis of defensive genes related to both pathways. The induction of defense related genes by Phi without inoculation (T=0 and T=6) and after *P. plurivora* inoculation (T=6) was examined. The transcript level of defense genes in the SA (PR1, PR2, PRP and WRKY) and JA/ET (PR3, and ACO) pathways was quantitatively measured using real-time reverse-transcription polymerase chain reaction (qRT-PCR). The relative expression levels were calculated comparing Phi treated and inoculated plants with the untreated *P. plurivora* using the Relative Expression Software Tool REST 2009 (Pfaffl *et al*., 2002) (Qiagen, Hilden, Germany). Actin, tubulin and GAPDH were used as reference genes. A list with the primers and sequences used in this study can be found in the Appendix.

Confocal laser-scanning microscopy:

To prepare the sample after harvesting, the root material was fixed in PBS (pH 7.2) with 3% formaldehyde. The root samples were manually sliced using a razor blade, and the cuttings were washed in PBS/0.2% Tween three times. The samples were then blocked for 30 min with 100 mM glycine in PBS/0.2% Tween. After washing the material again, a protein block was performed with 1% BSA in PBS (pH 7.2) for 30 minutes.

The root cuttings were then incubated for 2 h at 37°C with the primary antisera, the commercial antibody against *P. plurivora* (rabbit anti-*Phytophthora* spp. polyclonal antiserum from Loewe Diagnostica®, diluted 1:400). After incubation, serial washings (two x 10 minutes) were performed with PBS/0.2%Tween and then with PBS.

The root cuttings were incubated for 60 minutes at 37°C with secondary antisera (goat anti-rabbit conjugated to Pacific Blue, Invitrogen®, concentrated at 1:200). Before the confocal laser-scanning microscopy, the samples were washed several times with PBS/0.2% Tween and with PBS (pH 7.2).

The confocal imaging was performed using a Leica TCS SP5 confocal laser-scanning microscope (Leica Microsystems CMS GmbH, Mannheim, Germany). Pacific Blue was excited at 405 nm and detected between 430 and 480 nm. Plant auto-fluorescence was detected between 500 and 550 nm after excitation with a 488 nm laser-line.

Statistics:

The data were analysed using the Statistics software SPSS. A time series factorial was used to analyse the interaction of Phosphite and *P. plurivora* along time. In case of no interaction an Anova one-Way was a conducted comparing data at each time point with 5% significance ($P \geq 0.05$).

3.3 Results:

***In vitro* inhibition of mycelial growth and sporulation of *P. plurivora* by phosphite**

In figures 1A and B the effect of increasing Phi concentrations on mycelial growth of *P. plurivora* are shown. Inhibition of growth started at 5µg/mL and reached 65% inhibition with 100µg/mL of phosphite. The EC50 was calculated as 34µg/mL. Sporangia development was also affected by phosphite treatment (Fig. 1C). The number of zoospores released was significantly lower for all phosphite treatments. The EC 50 was determined as 2.9 µg/mL, being about ten times lower than for inhibition of mycelial growth.

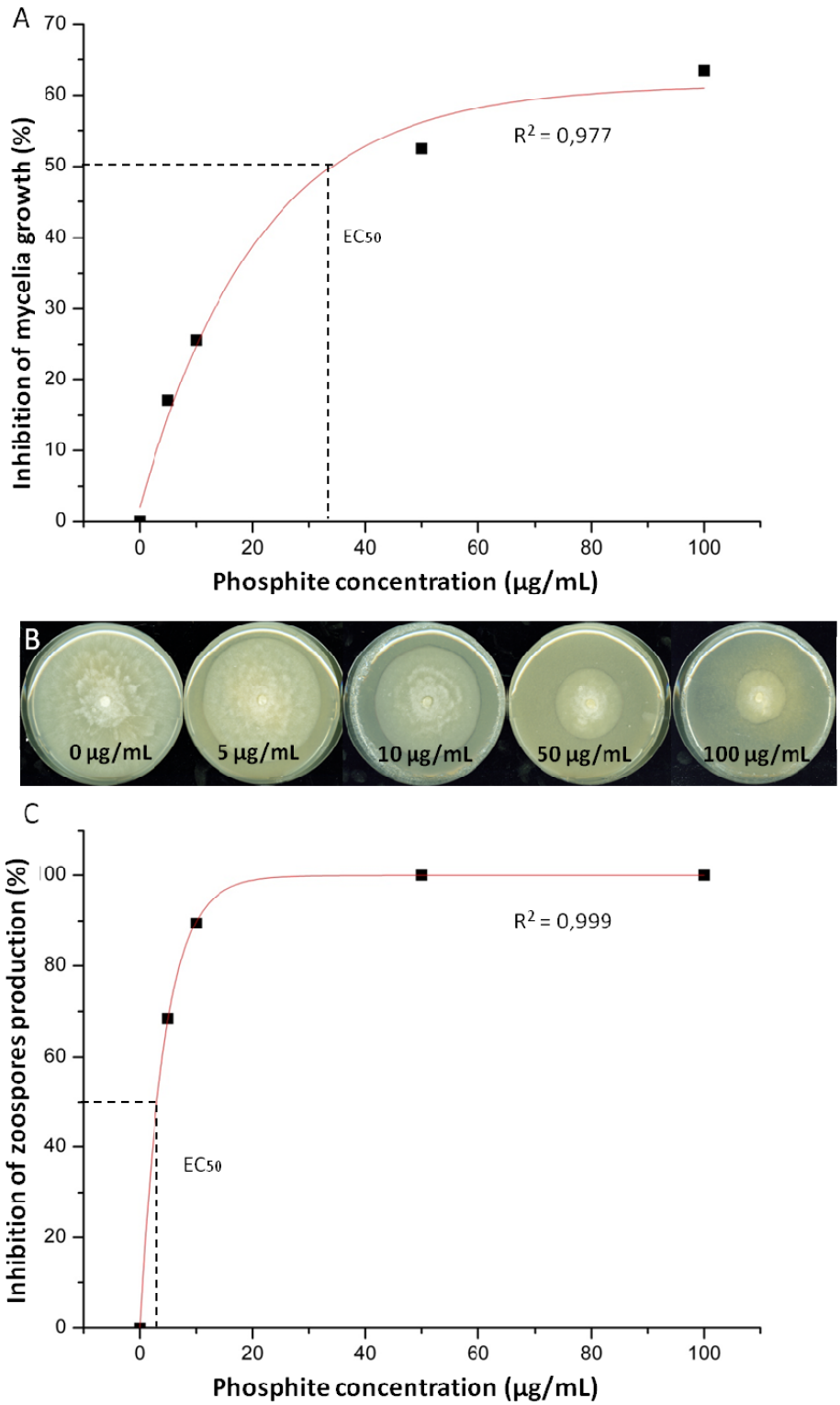

Figure 1: Effect of phosphite on in vitro growth and zoospore production of *P. plurivora*. (a) Inhibition of *P. plurivora* mycelial growth using different phosphite concentrations. (b) *P. plurivora* cultures in Petri dishes illustrating the inhibition of mycelia radial growth with increasing phosphite concentrations (c) Inhibition of *P. plurivora* zoospores production with different phosphite concentrations. Ec50 shows the concentration that inhibits 50% of growth or sporulation. Trend-lines were fitted using a logarithmic function. These assays were independently repeated three times showing similar results. n=5 for each assay.

Physiological plant responses to phosphite treatment and infection

Phosphite treatment did not show any beneficial or adverse effect on physiological parameters of not-inoculated plants. No significant differences were found for net CO_2 assimilation, water uptake, Jmax or Vcmax in comparison with control plants (Fig. 2 a, b, and Table 1).

However, *P. plurivora* infection strongly affected the physiology of not phosphite -treated plants. Values for net CO_2 assimilation, and water uptake strongly decreased during the experiment, reaching almost zero values at 10 dpi (Fig. 2 a,b). VcMax and Jmax were also affected, showing significantly lower values as compared to control plants (Table 1).

Phosphite treatment converted the susceptible interaction between *F. sylvatica* and *P. plurivora* into a resistant one. All physiological parameters analyzed did not differ to control plants (Fig. 2a, b; and Table 1).

Table 1: Rubisco activity (Vcmax) and maximum rate of electron transport (Jmax) data fitted from A/ci curves of *Fagus sylvatica* seedlings at 8 days post inoculation.

	A/ci curves fitted parameters	
Treatment	V_{cmax} [µmol CO_2 $m^{-2}s^{-1}$]	J_{max} [µmol e^- $m^{-2}s^{-1}$]
Control	12,17 a*	24,10 a
Phi	10,22 a	19,46 ab
Plu	4,99 b	13,94 b
Phi-Plu	10,42 a	26,01 a

*Same letters at same numbers represent no statistically difference, Tukey test (P≤0,05).

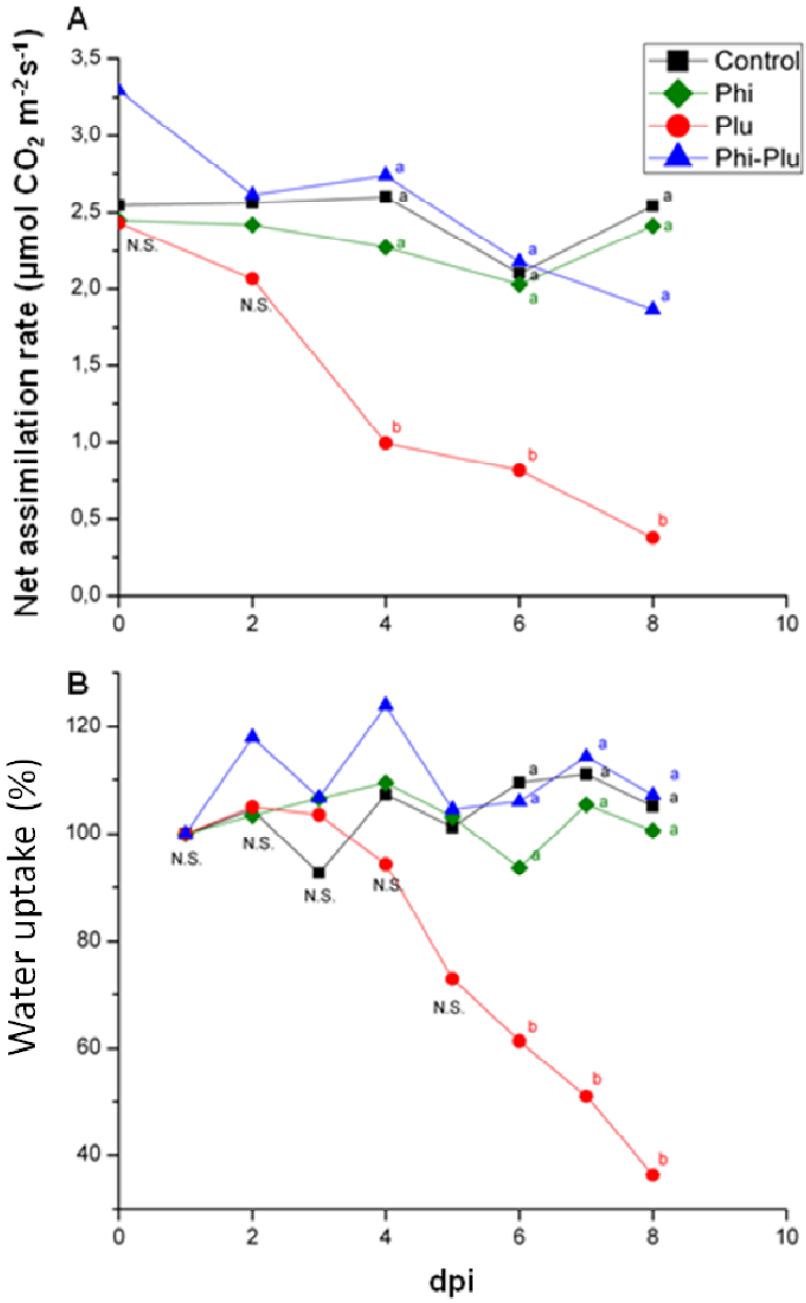

Figure 2: Effect of phosphite treatment and *P. plurivora* infection on net CO_2 assimilation rate (µmol CO_2 $m^{-2}s^{-1}$) (A) and water uptake, which was calculated as % of g/cm^2 leaf surface (B) of 4 months old beech saplings. Treatments: Control: not infected and not phi treated plants; Phi: phi treatment; Plu: roots were inoculated with zoospores of *P. plurivora*; Phi-Plu: foliar application of 0,5% phi in plants prior to inoculation with zoospores of *P. plurivora*. The experiment was repeated 3 times showing similar results. N=6 plants per treatment. Dpi- days post inoculation. Different letters at the same time points show statistical differences (P≤0,05), N.S.= Not-significant.

Symptoms and mortality of plants

No symptoms or mortality were recorded for not-inoculated control plants and those treated with phosphite (Fig. 3). Wilting symptoms were recorded for inoculated plants after 4 dpi. At the end of the experiment, 83% of infected plants had died. Remarkably, plants treated with Phi and inoculated with *P. plurivora* showed no symptoms and no mortality during the whole experiment.

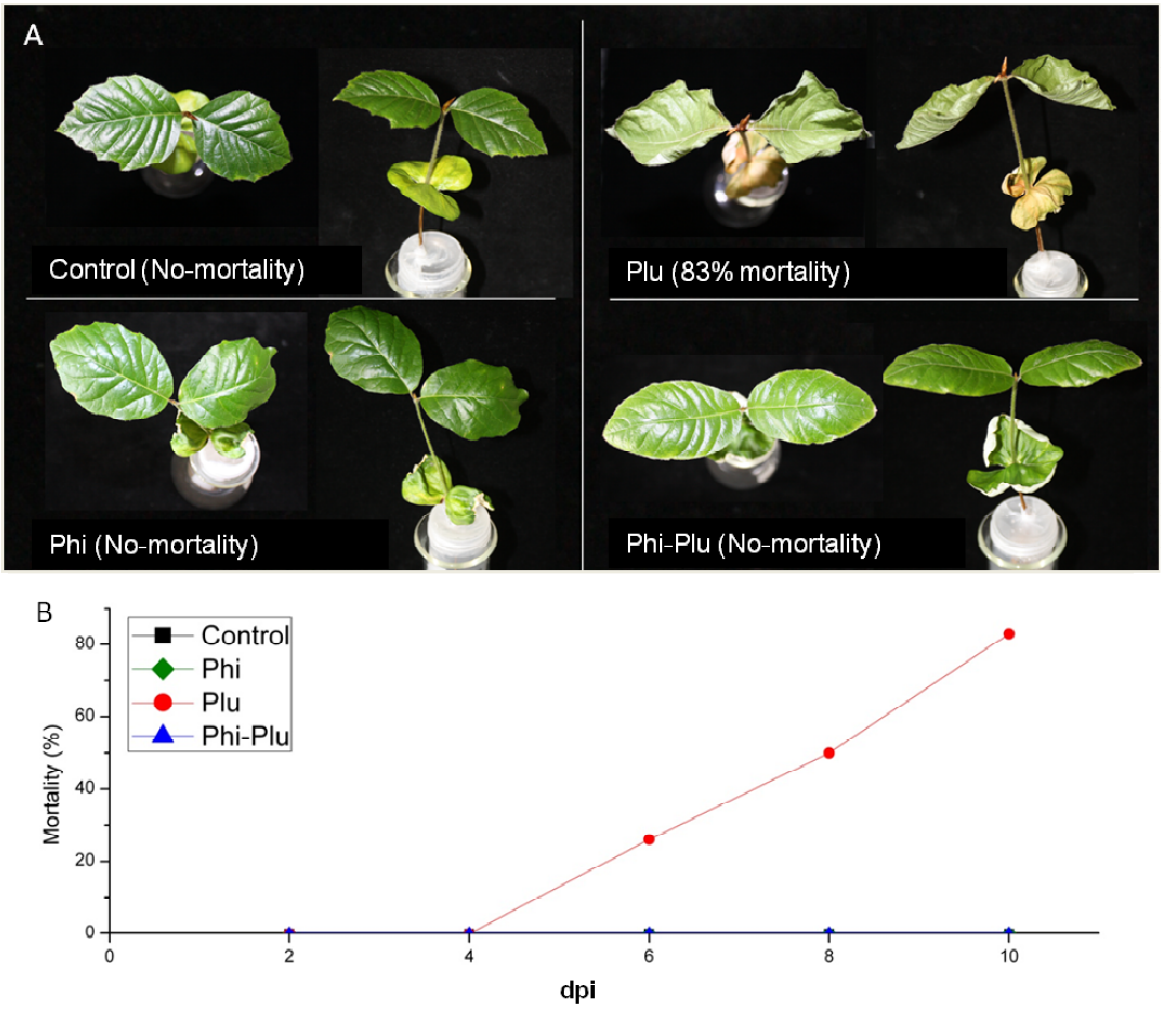

Figure 3: (A) Symptoms of leaves from plants of all treatments at the end of the experiment (10 dpi). (B) Mortality of plants of all treatments along time. Treatments: Con (black square) : not phosphite treated and not inoculated control plants; Phi (green square): plants sprayed with phosphite (0.5%) on leaves until run off Plu (red circle): roots infected with *P. plurivora*; Phi-Plu (blue triangle): plants sprayed with phosphite (0.5%) on leaves until run off four days prior to infection with *P. plurivora*. n=6 plants per treatment. The experiment was repeated 3 times showing similar results. Dpi: days post inoculation.

Quantification of phosphite and *P. plurivora* in roots

The concentration of phosphite of infected roots ranged from 370 to 510µg/mL during the experiment (Fig. 4a). These concentrations are about ten times higher than those necessary to inhibit mycelial growth of *P. plurivora* to 50% (compare fig. 1a and 4a). Similar phosphite concentrations were recorded for *P. plurivora* inoculated and not inoculated plants. No phosphite was detected in the roots of control plants.

The *P. plurivora* DNA contents of infected plants increased steadily throw-out the whole experiment and reached the highest values after ten days (fig. 4b). However, the correspondent DNA values of roots of phosphite treated and infected plants were much lower, indicating the powerful action of this compound to protect beech from *P. plurivora*. No DNA of *P. plurivora* was recorded in control and phosphite-treated not-inoculated plants (data not shown).

***P. plurivora* colonization of plant tissues with or without phosphite treatment**

In figure 5 confocal laser scanning microscopy images of root cross sections of beech plants infected with *P. plurivora* and treated or not treated with phosphite are shown. Two days after infection mycelia of the pathogen was already visible throughout the whole cortex tissue (fig. 5a). Four dpi the pathogen had reached the central cylinder and 10 dpi the phloem tissue as well as the pith was severely destroyed by *P. plurivora* (fig. 5 b and c). In contrast in beech plants treated with phosphite, *P. plurivora* mycelia were only visualized in the outer cortex tissue after 2 dpi. However, no mycelia were found in the central cylinder and the pith even not after 10 dpi at the end of the experiment (Fig. 5 d to f).

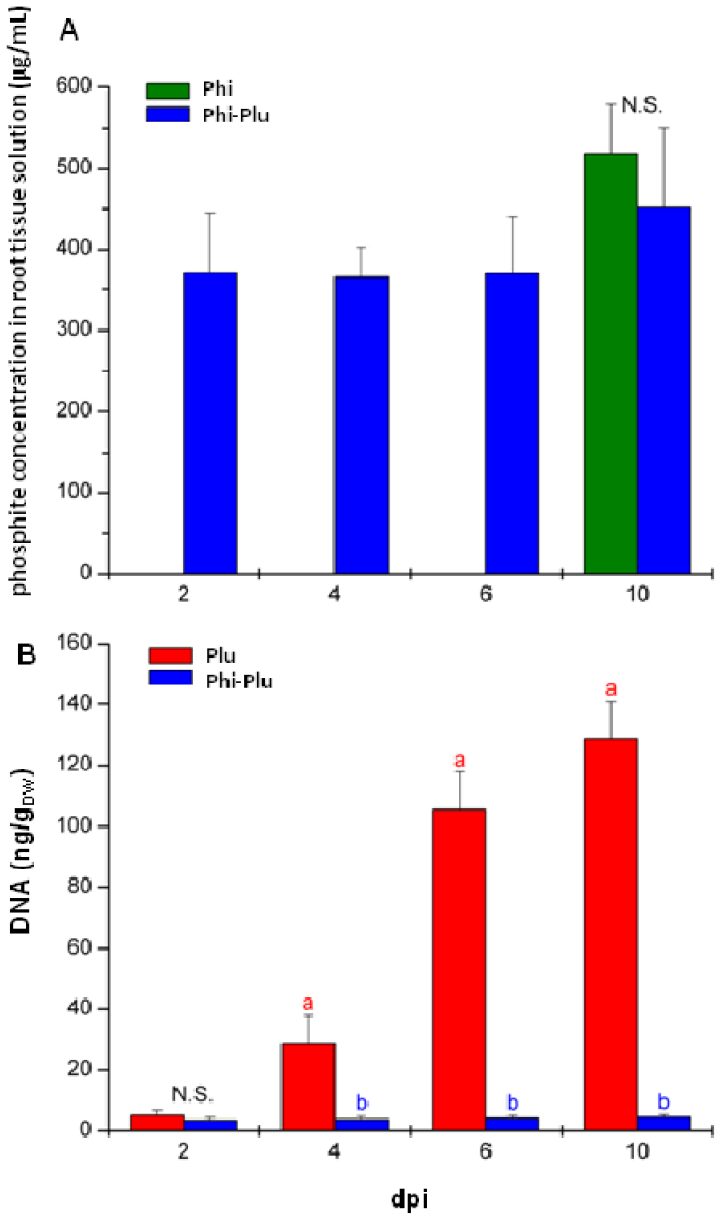

Figure 4: (A) Phosphite concentrations in different root tissue. (B) qPCR analysis of *P. plurivora* DNA along time (ng/gDW, DW: dry weight). Red bars: Plants infected with *P. plurivora*; blue bars: Plants infected with *P. plurivora* and treated with phosphite (0.5%). Grenn bars: plants treated with Phi. n=6 plants per treatment. The experiments were repeated 3 times showing similar results. Dpi: days post inoculation. Different letters at the same time points show statistical differences (P≤0,05), N.S.= Not-significant.

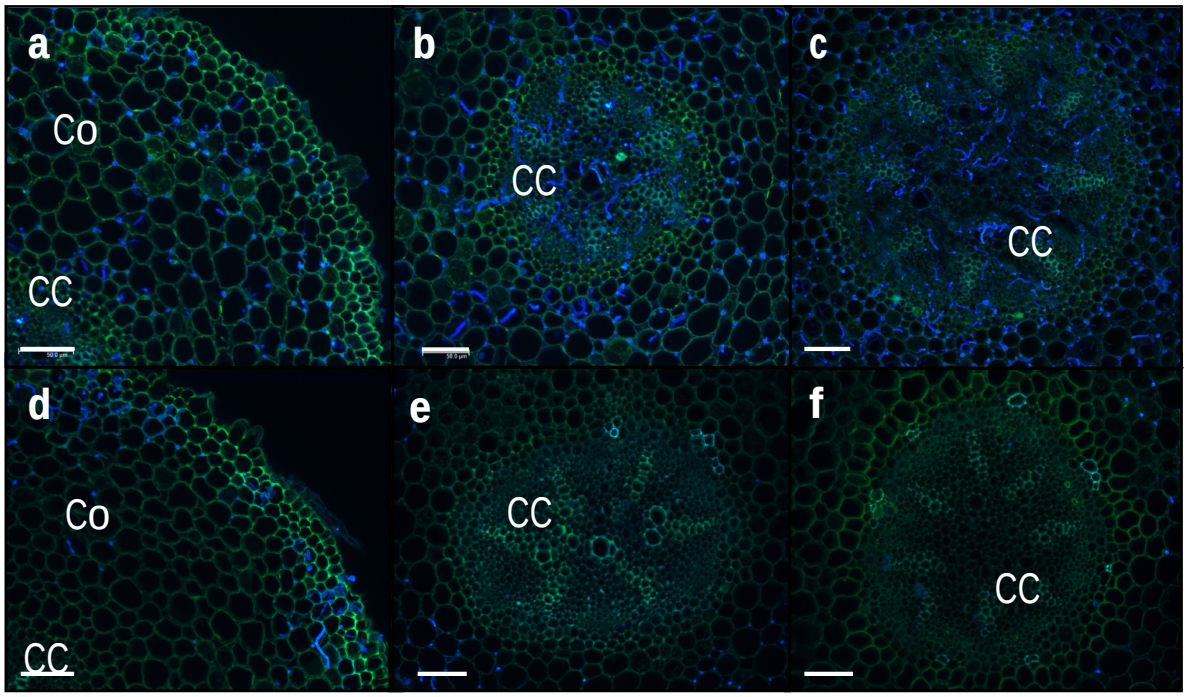

Figure 5: Confocal laser scanning microscopy images of cross-sections of roots infected and treated or not with phosphite. A, B and C: Plants infected with *P. plurivora* after 2, 4 and 10 dpi. D, E and F: Beech plants infected with P. plurivora and treated with phosphite (0.5%9 after 2, 4 and 10 dpi. (Co) cortex, (CC) central cylinder, White bars represent 50 µm.

Expression of defense genes of beech plants treated with or without phosphite and infected with *P. plurivora*.

Figure 6 shows that phosphite treatment did not affect gene expression in beech roots as compared to control plants, neither at time 0 nor at at 6 dpi (purple and black; yellow and green bars respectively). When comparing control plants with infected ones at 6 dpi (yellow and blue bars) it was also found no statistic differences. However, strong up-regulation of all analyzed genes was measured for plants treated with phosphite and infected with the pathogen six days post inoculation.

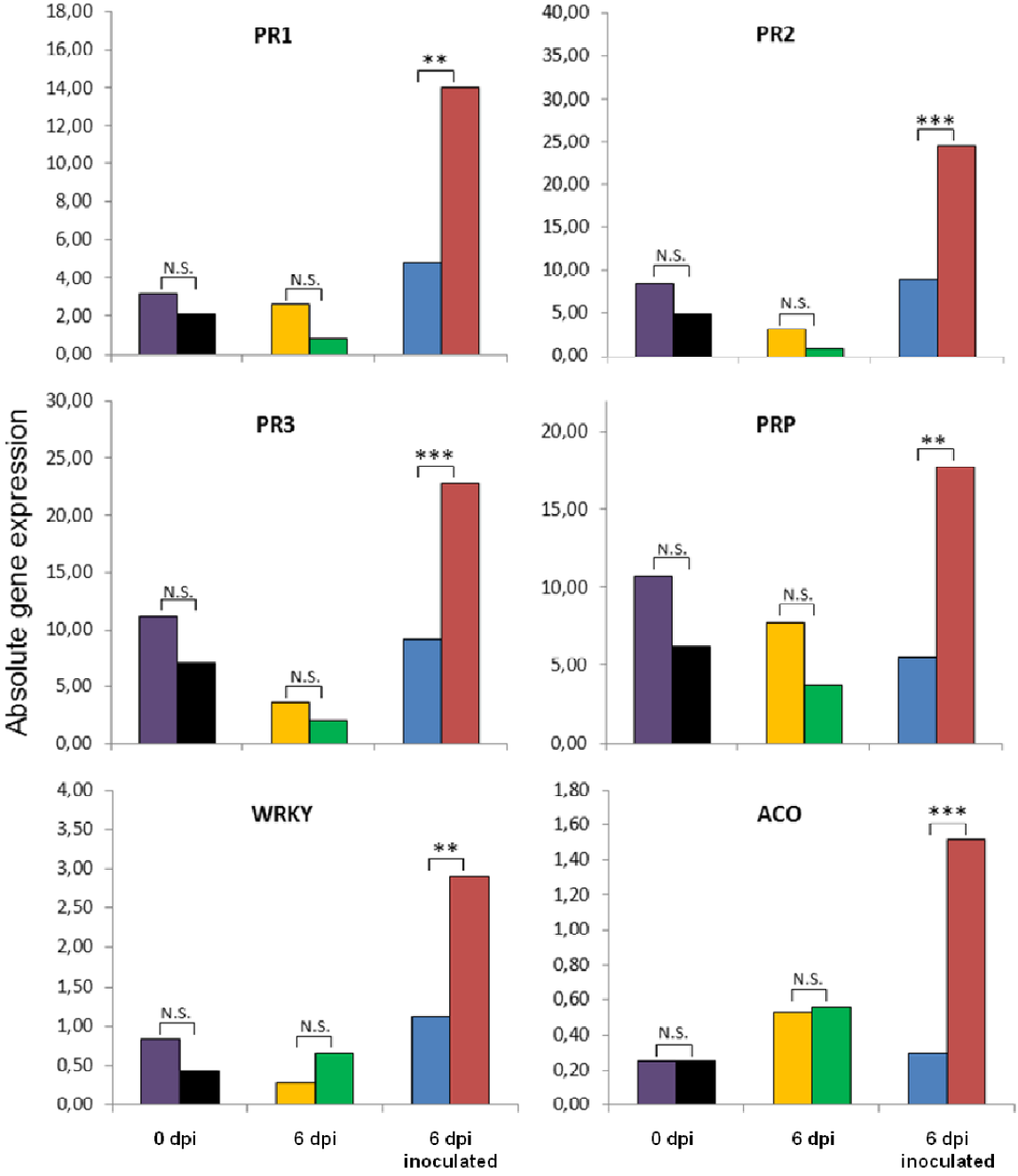

Figure 6: Absolute gene expression for PR1, PR2, PRP and WRKY (SA signaling pathway) as well as for PR3 and ACO (JA/ET signaling pathway) of beech saplings of all treatments. Purple and yellow bars represent control plants at time zero and after six days. . Black and green bars represent Phi treated plants at time zero and after six days. Blue bars represent infected plants at 6dpi. Red bars represent phi-treated and infected plants at time 6dpi. Asterisks show level of significance (P≤0,05*; P≤0,01**; P≤0,001***); N.S. Not-significant; n=4. The experiment was independently repeated three times showing similar results.

3.4 Discussion

In this study the toxic effects of Phi on *P. plurivora*, a highly virulent root pathogen of *F. sylvatica* was investigated, as well as the ability of the chemical to protect the host towards root infection by the pathogen. *In vitro* assays proved that mycelia growth was inhibited in a dose-dependent manner and the EC_{50} value was calculated as 34 µg/ml of Phi. Surprisingly growth of *P. plurivora* could not be inhibited completely even not with the highest Phi concentrations, possibly indicating that the *P. plurivora* isolate used in our experiments was able to detoxify phosphite to a certain amount. Similar results were also reported by (Dobrowolski *et al*., 2008) who showed that some *P. cinnamomi* isolates were less sensitive to phosphite than others. The authors concluded that sensitivity to Phi may vary within species or even within isolates. Phi was also very sufficient in inhibiting sporangia formation. Less than 5µg/ml of Phi were sufficient to stop sporangia formation to 50 per cent. (Coffey and Joseph, 1985) calculated similar EC-values for the effects of phosphorous acid, fosetyl-Al, fosetyl-Ca as well as fosetyl-Na on sporangia formation and zoospore release of *P. cinnamomi* and *P. citricola*.

It was proved that spraying leaves with 0.5% Phi, four days before root infection started, was sufficient to convert the highly susceptible interaction between beech and *P. plurivora* into a resistant one. No mortality of any seedlings was recorded after 10 days of infection at the end of the experiment. This is in good agreement with the qPCR data for root infection and the laser scanning microscopy images showing the spread of *P. plurivora* in root tissue. Both data sets revealed only a very weak colonization of the pathogen in roots of Phi treated plants as compared to not-treated infected saplings. One explanation for the high protective effect of Phi on infected plants could be its

concentration in root tissue which was calculated to be about ten times higher than the EC50 value to inhibit growth of *P. plurivora.* King *et al*., (2010) also suggested that the degree of resistance of host plants correlates with the Phi concentration within plant tissue and that the exposure of hyphae to Phi contributes to the resistance of Phi-treated plants. The reason why the Phi concentration was expressed as µg/ml and not as µg/gDW, as often reported, was to compare the actual concentrations of infected and not infected tissue with data calculated for the *in vitro* experiments mentioned above. The Phi data of roots also implicate that the chemical was transported fast from leaves into roots. Already six days after leaf spray the Phi concentrations were as high as at the end of the experiment. These data are in good agreement with those of (Ouimette and Coffey, 1985, Guest and Grant, 1991 and Jackson *et al*., 2000) who also showed that Phi freely translocates in association with photo assimilates throughout the plant in a source-sink relationship and that it accumulates in the tissue mainly in cell vacuoles.

The Phi concentration used in the experiments (0.5%) to induce resistance did not impair physiological parameters of beech saplings, such as net assimilation rate, Jmax, VcMax and water uptake. However, some phytotoxicity was reported by (Pilbeam *et al.* 2011) for *Eucalyptus marginata* plants when treated with Phi in the rage of 0.25 to 1%. This difference in susceptibility to Phi might by typal.

Besides local toxicity, restriction of growth of *P. plurivora* could also be explained with direct or indirect stimulation of plant defense responses by Phi. Up to now, the molecular mechanisms underlying Phi-activation of SA or JA/ET signaling pathways are poorly understood. In order to shed light on this, the expression of defense-related genes, which are used as markers for "SA" and "JA/ET" defense pathways, was examined. It was shown that up-regulation of defense genes, independent

of the signaling pathway, followed a typical priming pattern (Conrath *et al.*, 2002). Gene up-regulation was only measured in plants treated with Phi and inoculated with *P. plurivora*, whereas there was no response at all in Phi treated as well as in inoculated beech saplings. By definition, the priming phenomenon occurs when a plant, prior exposed to some compound or microorganism, exhibit an augmented defense response under pathogen attack (Conrath *et al.*, 2002). Comparable to our data, Eshraghi *et al.* (2011) also found an up-regulation of genes of both the SA and the JA/ET signaling pathways after Phi treatment. In contrast, (Massoud *et al.,* 2012) and (Machinandiarena *et al.,* 2012), also investigating Phi effects on Oomycete-challenged plants, found that Phi was only related with the regulation of genes of the SA signaling pathway.

Today there is no conclusive explanation regarding the mode of action of Phi and its potential targets in plants. From the results obtained in this study it is possible to conclude that Phi might act in a dual way. In a sub-toxic concentration for the pathogen, the plant might response via priming, as it was shown several times by Eshraghi *et al*., (2011); Machinandiarena *et al*., (2012); Massoud *et al*., (2012). However, if the Phi concentration reaches the toxic threshold inside infected host tissue, *Phytophthora* effectors and/or PAMPs might be released due to hyphae disruption, as it was recently shown by King *et al*., (2010), which facilitate pathogen recognition and the burst of defense reactions of the host via SA and JA/ET signaling pathways (Fig. 7). Further experiments are necessary to clarify, whether this hypothesis will also prove to be true for other Phi treated host plants.

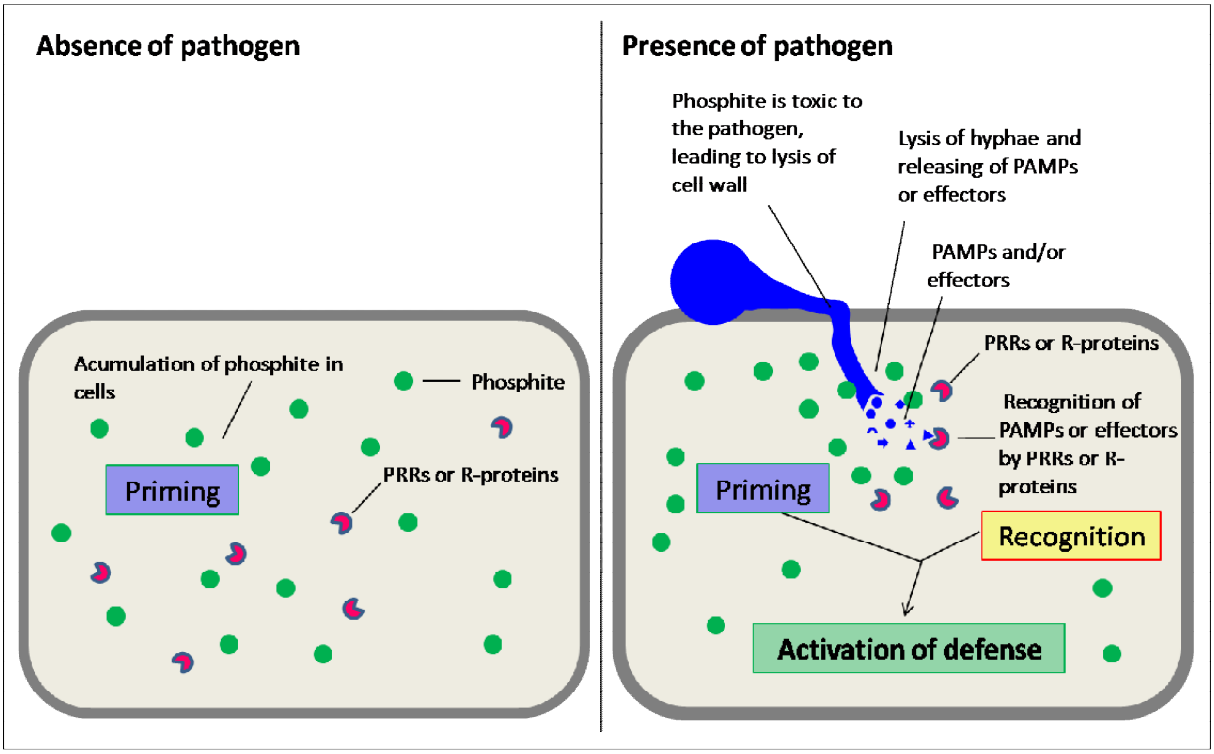

Figure 7: Schematic representation of the proposed phosphite mode of action. After treatment, phosphite accumulates inside cells, priming plants to a subsequent pathogen challenge (absence of pathogen). At the time of infection (presence of pathogen), phosphite accumulated in toxic concentration inside cells leads to lysis of hypha, which results in releasing of PAMPs (Pathogen associated molecular patterns) and effectors. PRRs (Pattern-recognition receptors) or R-proteins present in the cells recognize these molecules from the pathogen. As a consequence, together with priming, a fast and strong defense reaction is activated.

Chapter 4

Zoospore attraction by root exudates - development of a new trap to quantify attraction.

Zoospore attraction by root exudates - development of a new trap to quantify attraction.

Summary:

Phytophthora species release zoospores which are, in case of soil born species, attracted by root exudates, before they encyst and infect root tissue. To test the activity of root exudates towards zoospores and to quantify zoospore attraction a device to test zoospore attraction was developed. It consists of three plastic flasks that are connected to each other at the bottom with two tubes holding a dialysis membrane in the middle position. The left or the right flask is either filled with water or with the test solution (e.g. root exudate) and the central flask holds the zoospore suspension. Attracted by the test solution the zoospores swim towards the attracting solution and are finally trapped at the dialysis membrane where they encyst. The activity of different attractants can be compared and quantified by counting the zoospores cysts on the membrane under the microscope or by quantitative real-time PCR in combination with *Phytophthora* primers. Using the zoospore trap, in combination with qPCR it was proved that *Phytophthora* zoospores were attracted by root exudates of their host plants, however to different extents.

4.1 Introduction

In each plant-microbe interaction exchanging signals represents the earliest and fundamental step. In a complex environment like the rhizosphere soil, the colonization of potential hosts depends on the detection of specific plant molecules by microbes (Hirsch *et al*., (2003). Many pathogens possess ingenious machineries to effectively locate their hosts. Soil-borne *Phytophthora* zoospores have evolved the ability to perceive and swim towards an attractant and use it as a guide to encounter the roots and to start infection.

Invariably, terrestrial plants exudate a variety of compounds into the rhizosphere soil. These compounds can either inhibit growth of competitive plant species (Alelopathy), or change physic-chemical properties of the soil and interfere with the microorganism's community in their surrounding area (Bais *et al*., 2003).

Root exudation varies in quantity and quality depending on species, developmental stage of the plant and environmental conditions. Table 1 shows the different compounds already identified from different plant species and their functions.

The compounds from root exudates can be chemo-attractant or repellent signals for microorganisms. There are a variety of plant-pathogens that reproduce and spread in the environment through motile zoospores, in particularly soil-borne Phytophthora species (Tyler, 2006). It is well known that the zoospores from many important pathogens are attracted by host root exudates. However, the mechanisms underlying these interactions are poorly understood. Furthermore, most of the test systems to investigate chemo-attraction are mainly qualitative, such as the widely used capillarity methods, where the attraction of zoospores is checked by immersing a capillary glass containing a test-compound in a drop of zoospore suspension.

In the case of many Plant-Phytophthora interactions, the size of the initial inoculum can determine the success of an infection. Bigger initial inoculum tends to result in more successful infections because the plants do not have time to defend the rapid spread of the pathogen. Some root exudates from a given host, or even some compounds from a single root exudate are more attractant to the zoospores than others. Selecting hosts that produces less-attractant root exudates can be a very useful management strategy to prevent disease.

The aim of this study was to develop an innovative, cheap and easy-to-built method to quantify chemo-attraction of *Phytophthora* zoospores to any compound or root exudate.

Table 1: Components of root exudates and their function in the rizhosphere.

Component	Function
Phenolics	nutrient source chemoattractant signals to microbes* microbial growth promoters *nod* gene inducers in rhizobia *nod* gene inhibitors in rhizobia resistance inducers against phytoalexins chelators of poorly soluble mineral nutrients detoxifiers of Al phytoalexins against soil pathogens
Organic acids	nutrient source chemoattractant signals to microbes* chelators of poorly soluble mineral nutrients acidifiers of soil detoxifiers of Al *nod* gene inducers
Amino acids	nutrient source phytosiderophores chelators of poorly soluble mineral nutrients chemoattractant signals to microbes*
Sugars	nutrient source chemoattractant signals to microbes*
Inorganic Ions and gaseous molecules	promoters of plant and microbial growth Nutrient source Changes physic-chemical characteristics of the soil
Vitamins	promoters of plant and microbial growth nutrient source
Purines	nutrient source
Proteins/Enzymes	catalysts for P release from organic molecules biocatalysts for organic matter transformation in soil
Root border cells	produce signals that control mitosis produce signals controlling gene expression stimulate microbial growth release chemoattractants* synthesize defense molecules for the rhizosphere act as decoys that keep root cap infection-free release mucilage and proteins

*Functions involved in chemotaxis of zoospores. The table was modified from Dakota and Philips (2012).

4.2 Material and methods

Plant and Phytophthora growing conditions

Seeds of European beech, oak and two varieties of citrus: Sunki and Swingle, were germinated and grown in root trainers with sterile vermiculite for 3 months at 20 °C and light conditions of 250 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD).

Phytophthora plurivora, isolate CIT55, *Phytophthora quercina*, Isolate 1, and *Phytophthora nicotianae* Isolate 1, were grown on V8 agar, in the dark at 20°C.

Zoospores production

All isolates received the same treatment to develop sporangia and to release zoospores. After the agar plates were covered with mycel to about 80%, the cultures were submerged with distilled water, which was replaced daily to remove the nutrients and to induce sporangia development. The release of the zoospores was induced by incubating the submerged cultures at 4°C for 1 hour. The zoospore concentration of the supernatant was quantified using a Thoma counting chamber.

Root exudate production

Beech or citrus seedlings were carefully removed from the containers. The vermiculite was washed off and the plants were incubated in a beaker with 50mL 18 MΩ water at 20 °C for two days at 12 hours photoperiod (light condition: 250 µmol m^{-2} s^{-1} photosynthetic photon flux density (PPFD). In the case of Sunki and Swingle, the mass and surface area of the roots were measured and equalized to gain similar concentrations of root exudates.

After 2 days, the plants were discharged and the solution containing the root exudates were filtered (Whatman paper filters, 150 mm) and stored at -20°C for posterior analysis.

The capillary tube assay

The activity of small amounts of root exudates can be tested by a capillary tube assay. It consists of the following components: Cover glass with a cavity, pipet tip (200µl) and modeling clay to fasten the tip (Fig. 1).

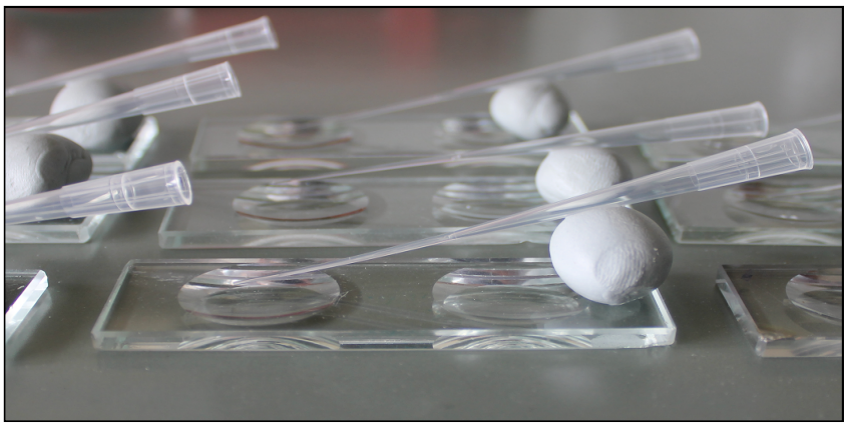

Figure 1: Components of the capillary tube assay.

This assay was first described by Zentmeyer (1961) and was slightly modified as follows. The cavity of the cover glass is filled with 200µL of zoospores suspension (1×10^5 zoospore per mL) and the two pipet tips containing either root exudate or water are dipped into the suspension. The zoospore attraction is visualized under the microscope and can be taped as a movie.

The "Zoospore Trap"

Assembling the trap:

The following components are needed to assemble the trap: Three plastic Falcon tubes (50 mL) (Tubes A, B and C), 1 mL pipette tips, dialyses membrane (molecular weight cut off: 6-8 Kda) (Fig 2).

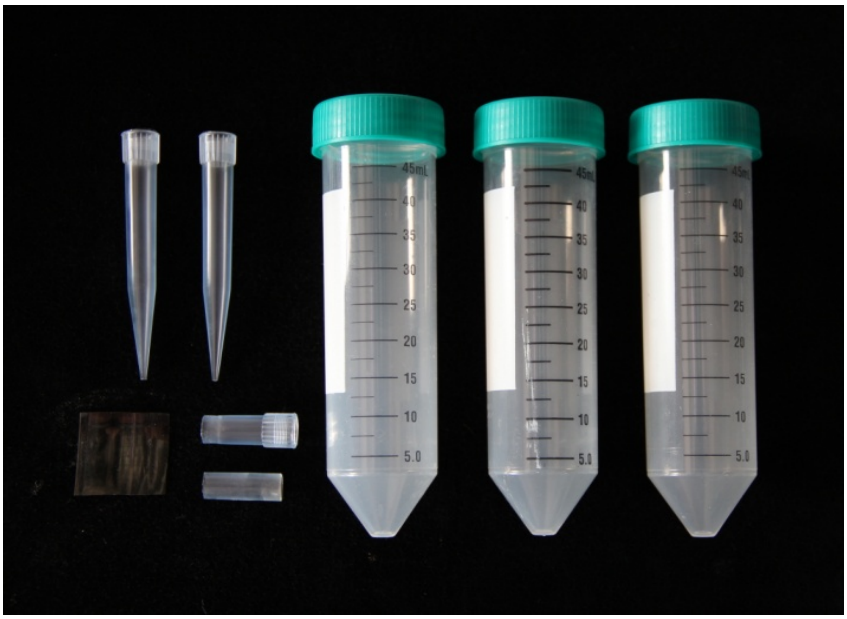

Figure 2: Material used to assemble the "Zoospore Trap": 1mL pipette tips, 50mL essay tubes, and 2cm^2 dialyze membrane (molecular weight cut off: 6-8 Kda).

The three plastic tubes are perforated at 2cm from their bottom (diameter 7mm); tube B is perforated twice. The pipette tips were cut off as follows: base to middle 23 mm, middle to tip 18 mm, the remaining tip of the original pipette tip was discharged. Dialyses membranes were cut in 2 x 2 cm size. The pipette tip pieces were inserted in the tubes holes. The membrane pieces were inserted between the pipette pieces and carefully, avoiding disruption of the membranes, the tubes were connected (Fig. 3).

There is also a possibility to increase the number of channels, i.e. instead of only two connectors and tubes (A and C), one or two more connections can be added to the central tube, increasing the number of compounds to be tested at the same time.

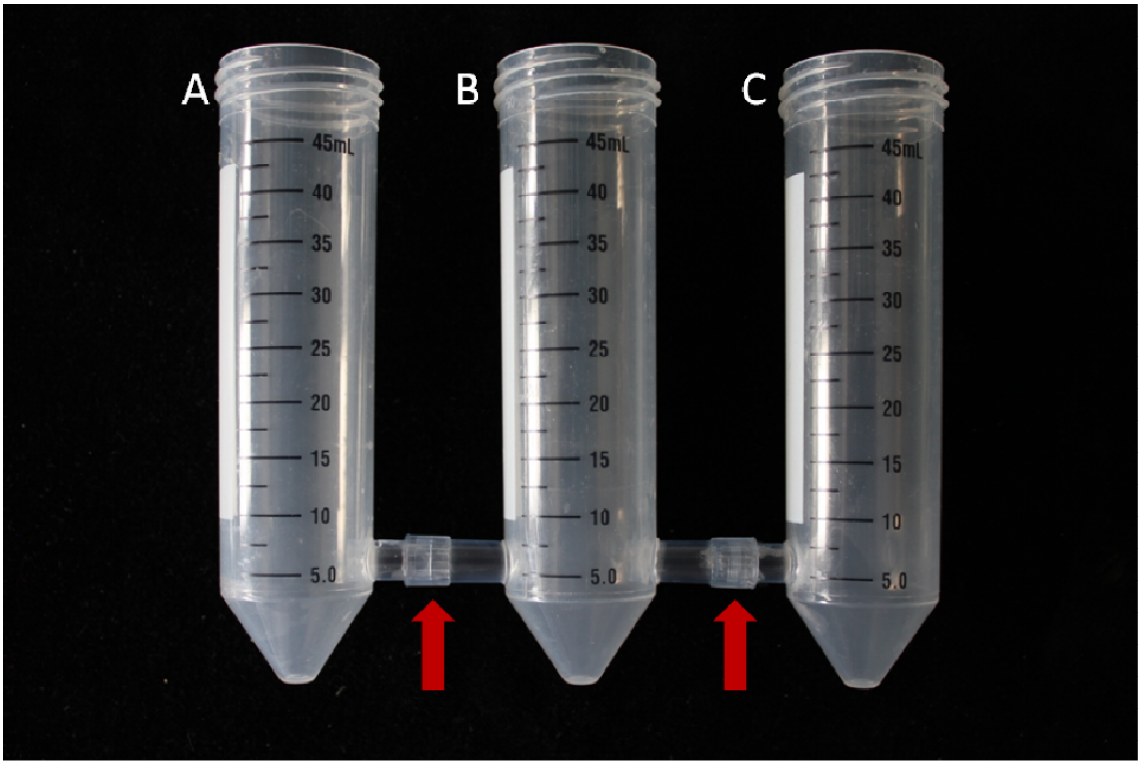

Figure 3: The "Zoospore Trap": 50mL essay tubes (A, B, and C) connected with pipette tip pieces. Red arrows shows the connection pipette tips and where the dialyze membranes were placed, between the connectors.

Running the Zoospore Trap assay

Tubes A and B are filled with 10ml 18 MΩ water, whereas tube C contains 10ml test solution (e.g. root exudate). After equilibration of 30 minutes 1ml of a zoospore suspension (5×10^5 in total) is pipetted into the central tube B. After 2 hours the experiment is terminated and the membranes can be either analyzed under the microscope or the amount of zoospores attracted to the membranes is quantified using qPCR in combination with *Phytophthora* primers. To ensure proper functioning of the assay, leakage as well as air bubbles in the tubing should be avoided and there should be no vibration or turbulence on the bench while the essay is running. For more details see figure 4.

Tubes A and C are filled with the test solutions (10 mL each) and central tube B filled with H_2O. The tube B was filled with 9mL water. After 30 minutes, molecules diffused between the membranes creating a gradient from lateral tubes towards central tube. Zoospores suspension was pipetted (1 mL) in the tube B. The zoospores swim towards the attractant until it encountered the membranes, where they encysted and germinated. After 1-2 hours, the membranes were collected and analyzed in the microscope or by qPCR (Fig. 6).

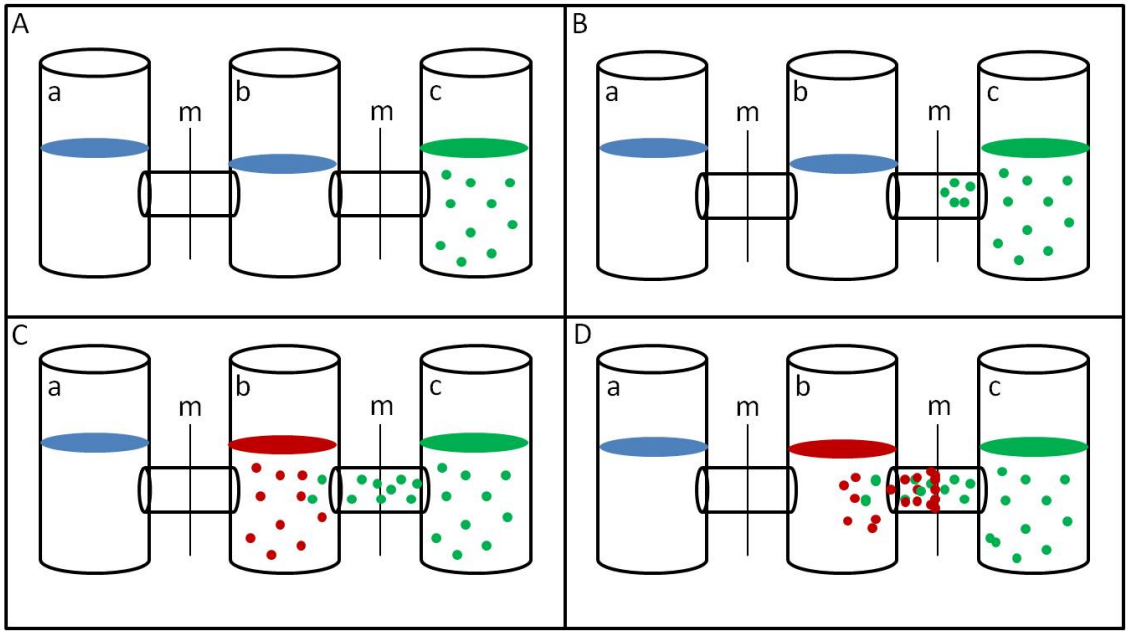

Figure 4: Schematic representation of the "Zoospore Trap" assay: Tubes "a", "b" and "c" are connected by pipette tips. Dialyze membranes "m" are placed between the connections. A: Tubes "a" and "b" are filled with H_2Obidest, "c" is filled with the test solution (e.g. root exudate; represented as green dots); B: The compounds from the test solution smaller than 6KDa diffuse throughout the membranes and the connections and create a gradient between tubes "b" and "c"; C: zoospores suspension is pipette into tube "b" (zoospores are represented as red dots); D: zoospores are attracted and swim towards the gradient and are trapped at the membrane; zoospores encyst and germinate.

qPCR

Genomic DNA was extracted directly from fresh membranes, carefully taken off from the zoospores trap. The DNeasy plant mini kit (Qiagen, Hilden, Germany) was

used according to the manufacturer's protocols. The DNA was diluted 1:10 in H_2O to prevent the inhibition of the PCR reaction. The amount of *P. plurivora* DNA in 5 µL of root extract was determined by TaqMan quantitative PCR using an SDS7700 sequence detection system (Applied Biosystems, Germany) (Böhm et al., 1999), with the primer pair P5 / P6 and the fluorogenic probe F3 labelled with FAM as a reporter dye and TAMRA as a quencher. All of the analyses were performed in three technical repetitions using ABsolute QPCR ROX chemicals (ABgene, Hamburg, Germany) and performing 40 cycles of denaturation at 95°C for 15 s and annealing / extension at 62°C for 60 s.

The Ct values of the samples were compared with a standard curve that was generated for pure *P. plurivora* genomic DNA extracted from mycelia grown in M1-liquide culture. The standard curve concentrations ranged from 1 pg DNA mL^{-1} to 10 ng DNA mL^{-1} in five steps.

4.3 Results

The capillary tube assay:

This modified assay can be used to visualize the activity of small amounts of root exudates towards *Phytophthora* zoospores (Fig. 5).

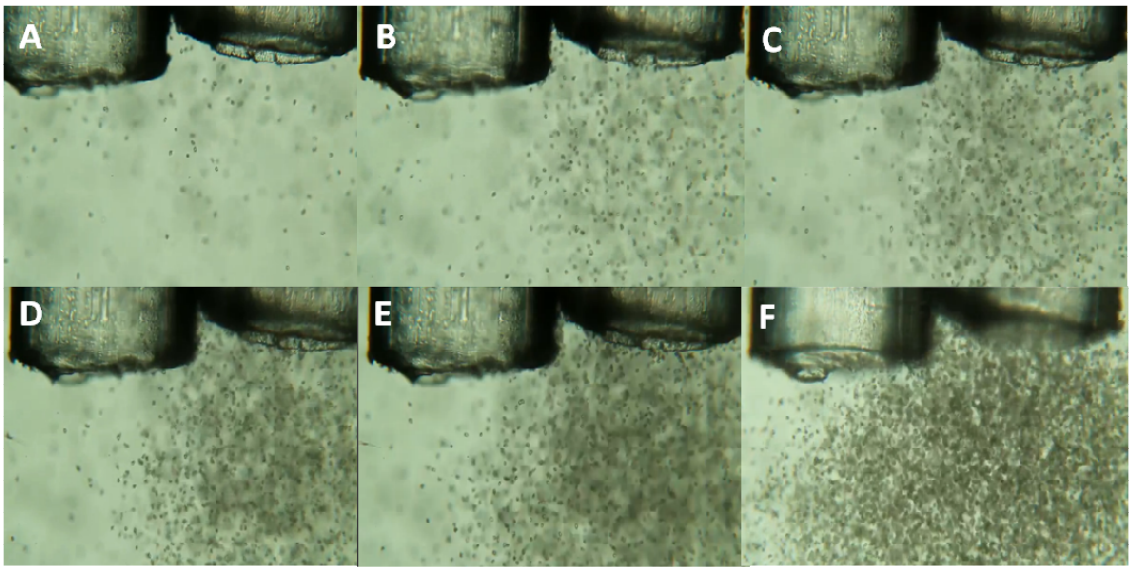

Figure 5: Time-series pictures of the capillarity essay, showing two pipette tips, the left one contained pure water and the right one contained *F. sylvatica* root-exudates. Both pipettes were immersed at the same time in a 200µL *P. plurivora*-zoospores suspension. Time that the pictures were taken after submerging the pipettes: A: 5 seconds; B: 20 seconds, C: 40 seconds; D: 60 seconds; E: 90 seconds and F: 150 seconds. Zoospores are attracted and concentrate in front of the pipette tip containing root exudates (see video on http://www.youtube.com/watch?v=F4slTLkhwuY).

The zoospore trap assay:

This newly developed assay is used to quantify the activity of root exudates of different sources towards *Phytophthora* zoospores.

Quantification of *P. plurivora* zoospore attraction by *Fagus sylvatica* root exudate.

Figure 6 shows the high activity of beech root exudate to attract zoospores of *P. plurivora*. With the exception of some few zoospores almost all were trapped on the membrane which was adjacent to the flask that was filled with *F. sylvatica* root exudate solution. Surprisingly the components of the root exudate also stimulated encysted zoospores to germinate. In order to quantify the activity of the beech root exudate regarding zoospore attraction, the total DNA content of the germinated *P. plurivora* zoospores on membranes was extracted. qPCR in combination with specific *P. plurivora* primers proved again the high activity of the tested beech root exudate (Fig. 7). The total DNA content of the membrane saturated with the root exudate was about 40 times higher as compared to the water control.

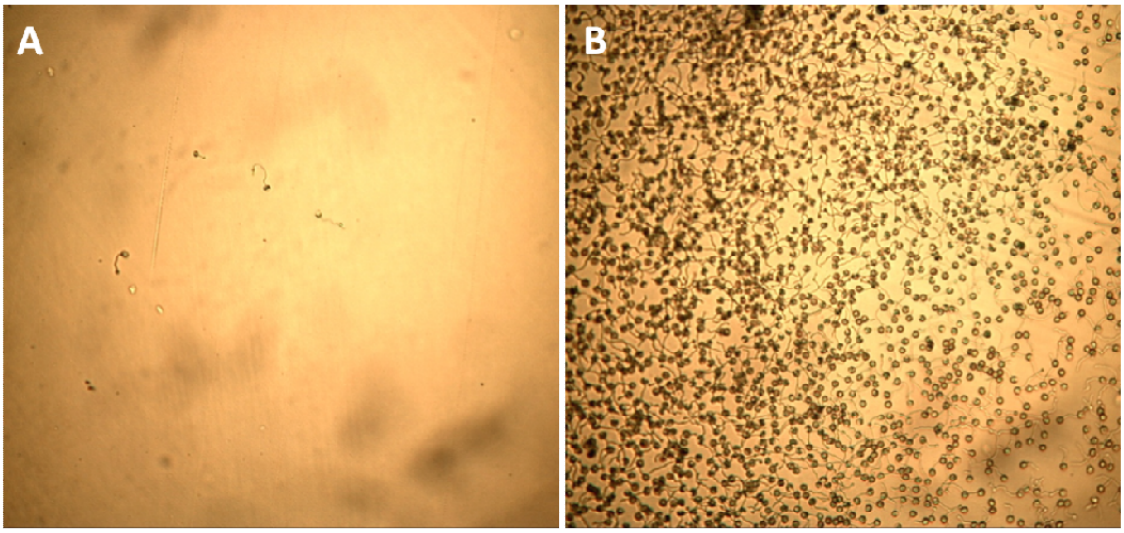

Figure 6: A, shows a picture of a membrane that were between tubes "A", containing water and the tube "B", containing *P. plurivora* zoospores suspension. B, shows the membranes that were between tubes "C", containing root exudates of *F. sylvatica* and the tube B, containing the zoospores suspension. Pictures represent the outcome from one of four zoospore trap essays.

Counting high number of zoospores encysted in membranes can be a very time-consuming and difficult work. Therefore it is recommend extracting the genomic DNA of the encysted zosspores in the membranes and analyze it by qPCR. Fig. 8 shows a qPCR analysis of the membranes showed above.

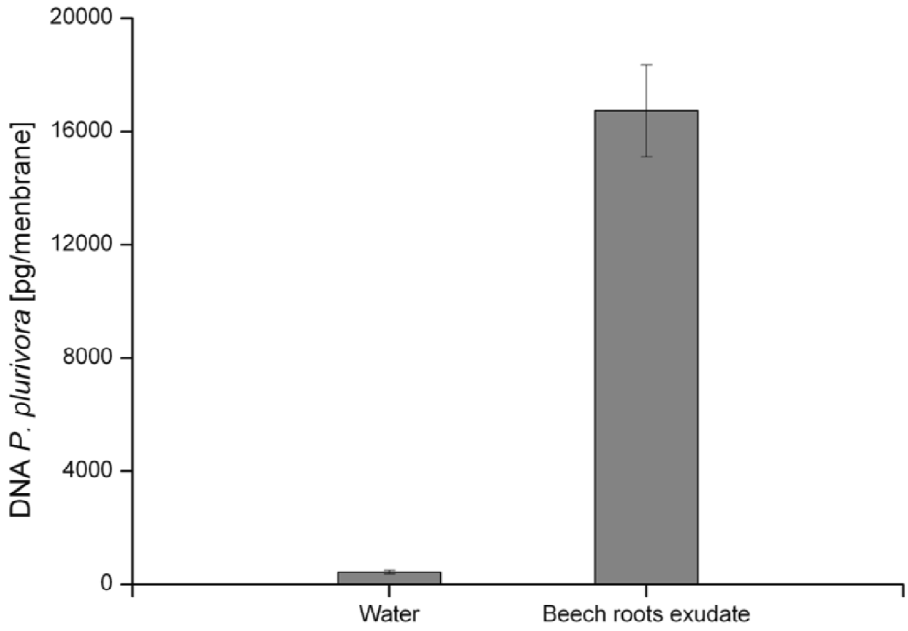

Figure 7: *P. plurivora* DNA quantification of the zoospores that encysted at the surface of the membranes. N=4. Vertical bars represent standard deviation.

The high amount of zoospores found in the membranes of the root exudates channel (Fig. 6B) are confirmed by the qPCR data.

Quantification of *P. quercina* zoospore attraction by *Quercus robur* root exudate.

Figure 8 shows the activity of oak root exudates to attract zoospores of *P. quercina*. Some few zoospores were trapped in the membrane adjacent to the water channel, but the majority of them were trapped in the membrane adjacent to the tube filled with oak root exudates. The components of the root exudate also stimulated encysted zoospores to germinate.

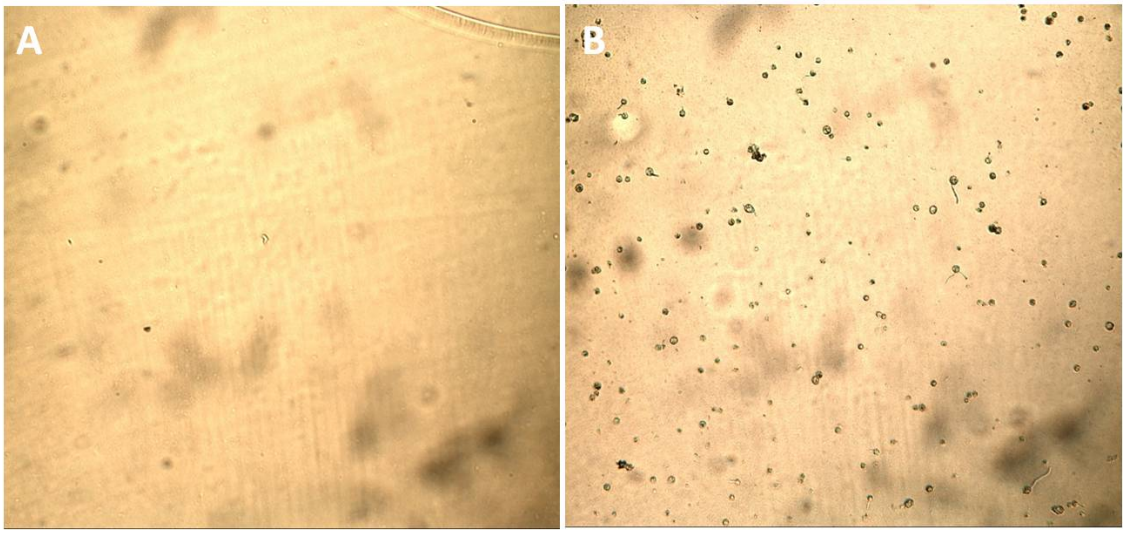

Figure 8: A, shows a picture of a membrane that were between tubes "A", containing water and the tube "B", containing *P. quercin*- zoospores suspension. B, shows the membranes that were between tubes "C", containing root exudates from *Quercus robur* and the tube B, containing *P. quercina*-zoospores suspension. Pictures represent the outcome from one of four zoospore trap essays.

Quantification of *P. nicotianae* zoospore attraction by root exudates of Citrus sunki and Swingle Citromelo two varieties of Citrus.

Figure 9 shows pictures of the membranes after the "zoospore Trap" essay testing attraction of *P. nicotianae* zoospores to Sunki and Swingle root exudates. In average 7,5 zoospores were attracted to the Swingle (resistant cultivar) channel, while the number of zoospores attracted to the root exudates of Sunki (susceptible cultivar) channel was estimated to be 42,1 in average.

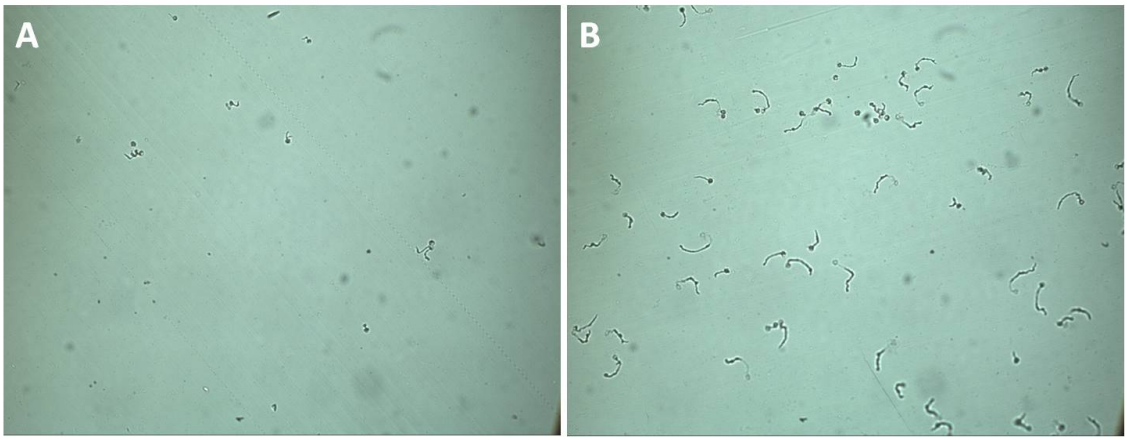

Figure 9: A, shows a picture of a membrane that were between tubes "A", containing Swingle-root exudates and the tube "B", containing *P. nicotianae*-zoospores suspension,"B" shows the membranes that were between tubes "C", containing Sunki-root exudates and the tube B, containing the *P. nicotianae*-zoospores suspension. Pictures represent the outcome from one of four zoospore trap essays.

4.4 Discussion

This study presents an innovative, cheap and easy-to-built device to test and quantify zoospore attraction to root exudates or any other compound. There has been considerable research on root exudates regarding chemotropism of fungal species, where attraction is demonstrated for mycelial growth (Brand and gow, 2009), but little attention has been given to *Phytophthora* species which spread in the environment and infect plants through mobile zoospores mainly in the rhizosphere.

The "Zoospore Trap" presented here, has been shown to be very effective to quantify zoospore chemotaxis. The root exudates of *F. sylvatica* were about 400 times more attractive to zoospores of *P. plurivora* than water. The visual estimation was proved by qPCR analysis of trapped zoospores. It was also showed that zoospores of *P. quercina* were about 10-times less attracted by root exudate of *Quercus robur* saplings as it was shown for the *P. plurivora* – beech host-pathogen interaction. The discrepancy between the above values may be related to differences in the composition of the root exudates or even related to the degree that each pathogen is able to respond to the environmental signals.

One of the first works reporting chemotaxis of *Phytophthora* zoospores to root exudates was published by Zentmyer (1961), working with *P. cinnamomi* and avocado. They made the observation that *P. cinnamomi* zoospores were "strikingly attracted to young, vigorously growing roots of avocado seedlings".

According to Tyler (1996), most of the Phytophthora zoospores show attraction to amino acids, particularly aspartate, glutamate, asparagine, glutamine, arginine, and methionine. As these amino acids are present in almost all root exudates, Phytophthora

zoospores tend to show a nonspecific attraction to many plants. However, some Phytophthora and Pythium species with restricted host range appear to exhibit more specificity in their attraction to root exudates. For instance, *P. sojae* zoospores were attracted only to legume root exudates, *P. graminicola* zoospores, which characteristically infects just graminaceous hosts, were preferentially accumulated behind root tips of grasses compared to dicots roots (Mitchel and Deacon, 1986; Tyler, 1996).

Differences in chemotaxis were also found when testing the preference of zoospores to root exudates obtained from either a susceptible or a resistant host species. E.g. Zentmyer (1961) reported that zoospores of *P. cinnamomi* were less attracted to roots of resistant avocado cultivars than to roots of susceptible ones. These observations were confirmed later by Aveling (1989), Botha and Kotzé (1989) and Chi and Sabo (1978) working with *P. megasperma* and *Medicago sativa* as well as by Marais and Hattingh (1985) working with grapevine and *P. cinnamomi*. All these authors found out that root exudates from susceptible cultivars were more attractant to the zoospores as the root exudates from the resistance cultivars. The results found in this study corroborate with the studies mentioned above. It was also showed that *P. nicotianae* zoospores were about 6 times more attracted by root exudates of Citrus Sunki (susceptible) when compared to attraction by root exudates of the resistant cultivar "Swingle". The preference of zoospores to one cultivar in detriment of another could be explained by differences in the composition of the root exudates. If a cultivar exudates more attractive compounds, it will invariably result in more attraction. Botha and Kotzé (1989) argued that the exudation of different amino acids in different concentrations

could be an important factor in determining tolerance or susceptibility of different avocado rootstocks to *P. cinnamomi*.

Cameron and Carlile (1978) reported that several compounds from root exudates were attractive to zoospores of *P. cambivora*, however, none of those substances were found to be as powerful as the total root exudate. Thus they concluded that the root exudate attractiveness must be the result of a synergistic or additive effect of several components. Dakota and Philips (2002) pointed out that a large number of different types of organic compounds of root exudates are attractants to microbes in the rizhosphere (Table 1).

Tyler et al., (1996) are the only one, who have elucidated the specific structure of active soybean root exudates. They reported that the both isoflavones genistein and daidzein were highly active to attract zoospores of *P. sojae*. Later, Hua *et al*., (2008) elucidated that a G-protein from *P. sojae* was essential for zoospore chemotaxis. However, six other Phytophthora zoospores tested showed no attraction to both genistein and daidzein, suggesting that each species could have evolved their own profile of attractive compounds. Morris and Ward (1992) argued that the specific attraction of zoospores of *P. sojae* to isoflavones may explain the specificity of their attraction to soya bean root exudates.

Table 2 gives a comparison of some methods available and their advantages and disadvantages for analyzing chemotaxis of zoospores. All methods have their peculiarities, advantages and disadvantages. However, a combination of two or more methods can fulfill all the needs for a complete and successful analysis of zoospore chemotaxis.

Table 2: Available methods used to analyze zoospore chemotaxis.

	Capillary tube[1]	**DM-Eppendorf**[2]	**Agar pieces**[3]	**Zoospore trap**
Description	Capillaries containing test solutions immersed in zoospores suspension	Dialyze membrane between a piece of fresh root and a Eppendorf cap filled with zoospores suspension	Agar pieces soaked in test solution are merged in zoospores suspension	Multi-channel structure that traps zoospores on dialyze membranes after chemo-attraction
Type	qualitative and quantitative	qualitative	qualitative	qualitative and quantitative
Advantages	can test all compounds; for small amounts of test solutions	Fast	can test all compounds	Test of high amounts of zoospores; qPCR can validate results from visual estimation,
Disadvantages	only visual estimation, not possible to quantify high amounts of zoospores	only test fresh roots	only visual estimation	gives results only for molecules smaller than 6KDa
Sources of zoospores to be tested simultaneously	1	1	several	Up to 4
Number of compounds to be tested simultaneously	several	-	several	Up to 4

[1]: Tyler et al 1996; [2]:Botha *et al*., 1989 and [3]: Heugens and Parke (2000).

Overall, the "zoospore trap" presented here has been shown to be very effective in testing and quantifying chemo-attraction of zoospores. This device has little disadvantages in comparison with other methods and can deliver important information about the very early interactions between soil borne pathogens and their hosts in the rhizosphere.

Chapter 5

Controlling root colonization of *Fagus sylvatica* seedlings by *Phytophthora plurivora* and *P. pseudosyringae* using specially designed thermo boxes

Controlling root colonization of *Fagus sylvatica* seedlings by *Phytophthora plurivora* and *P. pseudosyringae* using specially designed thermo boxes

Summary

Special thermo boxes were designed to adjust root incubation temperatures over time in order to control root infection and growth of *P. plurivora* and *P. pseudosyringae* on beech seedlings. Quantitative PCR analysis of infected beech roots proved that both pathogens colonized roots to similar amounts when the temperature was set to 14° C for *P. plurivora* and to 20° C for *P. pseudosyringae*. However, *P. plurivora* grew about 6-times faster than *P. pseudosyringae* in beech roots when the root incubation temperatures were set to 20 or 14°C, respectively. In case of *P. pseudosyringae* beech successfully defended the pathogen and all plants survived, whereas *P. plurivora* caused death of 62,5% of infected saplings. These macroscopic data are in accordance with photosynthesis measurements. *P. plurivora* strongly reduced CO_2-uptake rates of infected beech saplings, whereas a *P. pseudosyringae* infection did not impair photosynthesis of beech leaves. The contrasting effect of both pathogens on leaf physiology and mortality of beech seedlings was not affected by different root temperatures or growth rates of the pathogens, respectively. Thus, the amount of Phytophthora infection cannot explain the observed differences, and the reasons are still unknown.

5.1 Introduction:

Phytophthora diseases are a threat for many crops and forests worldwide. Approximately one hundred species were already identified and the majority of them are able to attack woody plants (Brasier, 2009).

One of the most important deciduous trees in Central Europe – European beech (*F. sylvatica* L.) – is known to be infested by several *Phytophthora* species (Jung *et al*., 2005). These soil borne pathogens colonize the root systems of trees and grow from there into the collar and the trunk of beech. Typical symptoms of adult trees are crown transparency, crown dieback as well as bleeding cankers on the trunk (Jung, 2009). As

Phytophthora species infect their host via zoospores, soil moisture is important for infection (Pettitt *et al*., 2002).

P. citricola isolates of European deciduous trees were recently described as a new species called *P. plurivora* by Jung and Burgess (2009). This soil-borne *Phytophthora* is known to be highly aggressive towards *F. sylvatica*, while *P. pseudosyringae* causes a resistant interaction with it (Fleischmann *et al*., 2002, 2004; Jung *et al*., 2003). Both pathogens caused root rot and strongly decreased fine root length as well as the number of root tips. However, *P. plurivora* caused high mortality of beech, while all saplings survived an infection with *P. pseudosyringae*. Furthermore, infection with *P. plurivora* reduced net photosynthesis rates and stomatal conductance of beech leaves as well as nutrient uptake, while saplings infected with *P. pseudosyringae* performed as good or even better as healthy control plants.

The understanding of the mechanisms responsible for such contrasting interactions between *F. sylvatica* and both pathogens could be extremely important to provide new strategies to manage and protect forests.

Since the growth of both pathogens on agar plates and the formation of zoospores are temperature dependent and differ between *P. plurivora* and *P. pseudosyringae*, thermo boxes were developed to guarantee comparable infection and growth rates for both pathogens. These thermo boxes were used to control root colonization and to analyse the susceptible and resistant interaction between beech and both pathogens in more detail.

The main question to be answered was if *P. plurivora* will still be more aggressive to beech seedlings than *P. pseudosyringae* after matching their growth at the

roots to the same rates. The hypothesis is that the *P. plurivora* bigger aggressiveness to beech cannot be only associated to its faster growth rate. So even with the same amount of DNA from both pathogens in roots, *P. plurivora* infected plants will still show more susceptibility than those infected with *P. pseudosyringae*.

5.2 Material and Methods

In vitro growth and zoospores formation of *Phytophthora* isolates

P. plurivora, isolate CIT55, and *P. pseudosyringae*, isolate PSEU1 were grown in Petri dishes on V8 juice agar at 20° C in the dark. After initial growth, five replicates per isolate were prepared and incubated at 10, 14, 17, 20 and 24° C. Radial growth was recorded daily over one week. Afterwards, sporangia formation was induced by washing the plates with sterile water for seven days. In order to induce zoospores release, the plates were incubated for one hour at 4°C. Zoospore concentration was quantified using a Thoma counting chamber.

Design of the thermo boxes

Figure 1 shows a detailed diagram of the thermo boxes. Two plastic (PVC) boxes (height 13 cm, length 37 cm, width 17 cm) were coated with Styrofoam (size 3,5cm) on each side, including the lids. Twenty-eight holes, with a diameter of 3cm were drilled into the lid to fit the Falcon tubes. Inside the boxes a flexible PCV-tube (19 / 25 mm inside / outside diameter, 1.7 m in length) was installed in curves onto the bottom and connected to an external integrated cooling device (Lauda, UKT 350), circulating tempered water through the tube. The inside of the box was filled with deionised water. In order to avoid temperature gradients, an aquarium pump (Eheim compact 1000) was fixed at the bottom of each thermo box. The box water temperatures

were adjusted to 14 and 20° C, respectively. Temperatures inside the falcon tube were checked manually with a thermometer. Both boxes were placed into phytotrons which were set up to long-day conditions, temperature of 20° C, relative humidity 65% and photosynthetic photon flux density (PPFD) to 250µmol m^{-2} sec^{-1}.

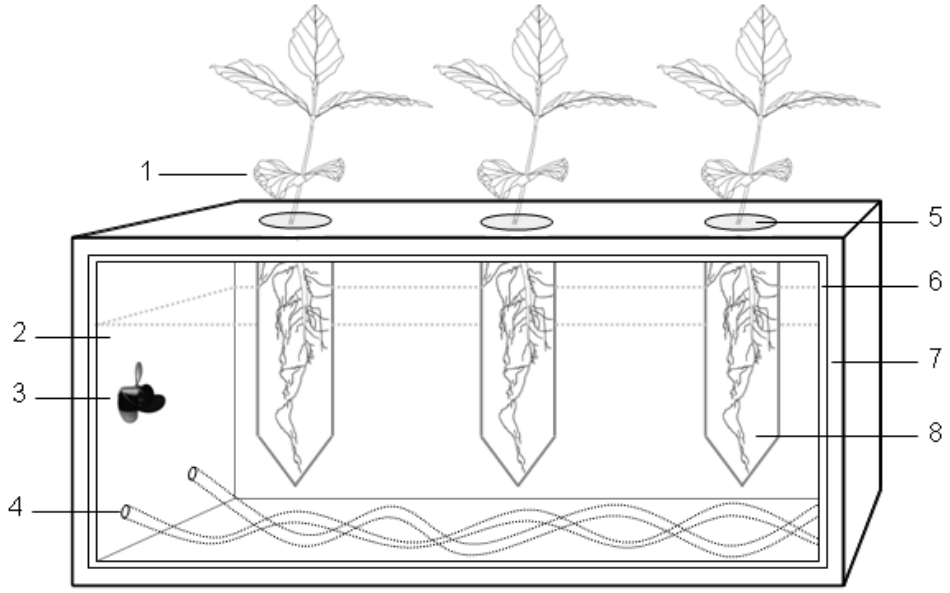

Figure 1: Technical diagram of a thermo box (not in scale). 1: *F. sylvatica* seedlings in 50 mL Falcon tubes. 2: inside of the box filled with water. 3: aquarium pump. 4: tube connected with an integrated cooling device. 5: Para-film sealing holes between shoot and root system. 6: PVC layer. 7: Styrofoam insulation. 8: water inside the falcon tubes in contact with the root system.

Infection of *Fagus sylvatica* seedlings

Three months old beech seedlings were placed into 50 mL-Falcon tubes with 45 ml distilled water. The tubes were transferred to two thermo boxes adjusted to 14 and 20° C root temperature, respectively. After one day of acclimatization, eight seedlings

per thermo box were inoculated with 500.000 zoospores of either *P. plurivora* or *P. pseudosyringae*. Another eight plants were used as healthy controls.

Gas exchange parameters were recorded using a LI-6400 portable photosynthesis system (Licor) at 250 µmol m^{-2} s^{-1} photosynthetic photon flux density and 400 ppm CO_2. Eight days after inoculation the plants were harvested and the amount of infection was quantified using real-time quantitative PCR-techniques. DNA was extracted from 20 mg of freeze dried and milled root material using the Plant DNeasy Mini-kit (Qiagen, Hilden, Germany). Subsequently DNA extracts were further purified by sodium acetate / ethanol precipitation. The amount of *P. plurivora* DNA within the DNA extracts was quantified on a SDS 7700 Sequence Detection System (Applied Biosystems, Frankfurt, Germany) using the ABSOLUTE qPCR ROX chemicals (ABgene, Hamburg, Germany). The qPCR was performed in three technical replicates. For the *P. plurivora* and *P. pseudosyringae quantification* the primer sets and the fluorogenic probes of Böhm *et al*., (1999) and Tooley *et al*., (2006), respectively, were used. Dilution series of genomic DNA extracted of pure *P. plurivora* or *P. pseudosyringae* mycelium was used to calculate a linear standard curve of log [*Phytophthora* DNA] vs. CT-value.

Analyses of variance (ANOVA) were carried out and the Tukeys tests at 5% error probability were performed to test difference among mean values.

5.3 Results

Figure 2 shows the growth rate and zoospore formation for both pathogens at five different temperatures. Growth rates of *P. plurivora* were higher as compared to *P. pseudosyringae* at all the temperatures tested here (Fig. 2A).

With the exception that there was no sporulation at 24°C, different zoospores releasing rate was measured at all tested temperatures of both pathogens. Zoospore formation of *P. plurivora* increased with temperature and reached its highest value at 20°C, while that of *P. pseudosyringae* showed its optimum already at 17°C (Fig. 2B). Comparing data of figures 2A and 2B similar growth and zoospore formation was measured for *P. plurivora* at 14°C and for *P. pseudosyringae* at 20° C, respectively.

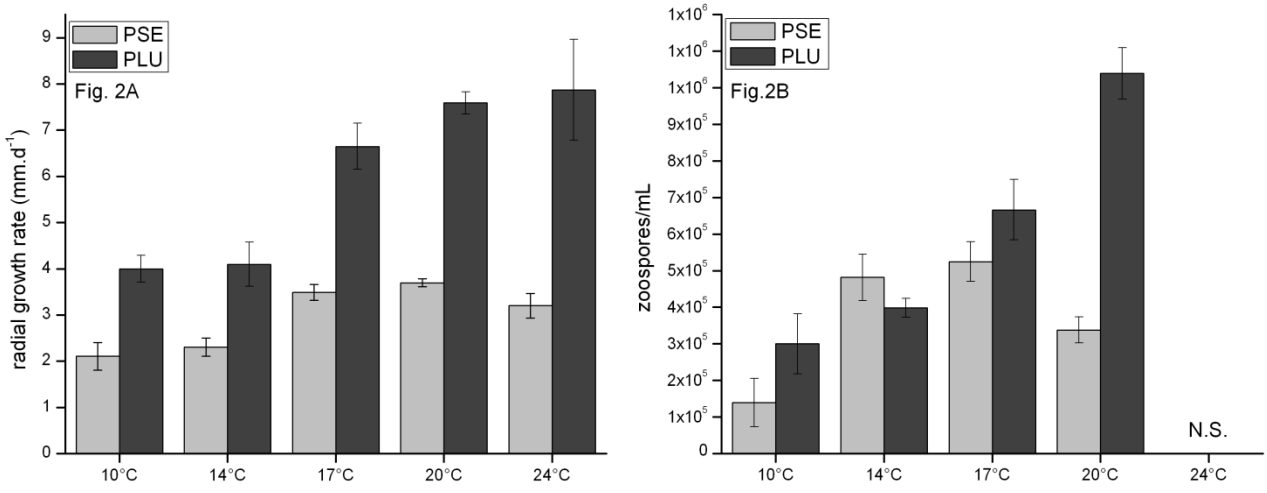

Figure 2: A: Growth rate of *P. plurivora* (PLU) and *P. pseudosyringae* (PSE) on V8-agar plates at different temperatures. B: Zoospore release of *P. plurivora* (PLU) and *P. pseudosyringae* (PSE) at different temperatures. N.S.: No sporulation.

Eight days after inoculation 5 seedlings infected with *P. plurivora* had died (62,5% mortality at 14°C; 87,5% mortality at 20°C, data not shown) and exhibited severe wilting of leaves, whereas all *P. pseudosyringae* infected plants showed no visible symptoms and all had survived. As compared to controls, photosynthesis of *P. plurivora* infected beech was significantly reduced four days after inoculation (table 1). By contrast, *P. pseudosyringae* infected plants showed significantly higher photosynthetic rates (table 1) when compared to control seedlings. Table 1 also shows

that the decrease of the root incubation temperature from 20 to 14°C did not significantly change the photosynthetic rate of non-infected beech seedlings.

Table 1. Net CO_2 assimilation rates (µmol m^{-2} s^{-1}) of infected and control *F. sylvatica* saplings at different temperatures after 1, 4, 6, and 8 days post-inoculation.

	CON 14°C	CON 20°C	PLU 14°C	PSE 20°C
1 dpi	1,101 a*	1,821 a	1,435 a	1,406 a
4dpi	1,454 b	1,633 b	0,490 c	2,254 a
6dpi	1,335 b	1,880 b	0,157 c	2,506 a
8dpi	1,337 b	1,922 b	0,544 c	2,519 a

*The mean values labelled with different letters differed significantly among the treatments at each time point (Tukey test, $p < 0.05$). CON = control; PLU = *P. plurivora*; PSE = *P. pseudosyringae*.

The successful use of thermo boxes to control the growth of *Phytophthora* pathogens is demonstrated in figure 3. Quantitative PCR proved that *P. plurivora* growing at 14°C and *P. pseudosyringae* at 20°C had colonized the roots to a similar amount at the end of the experiment.

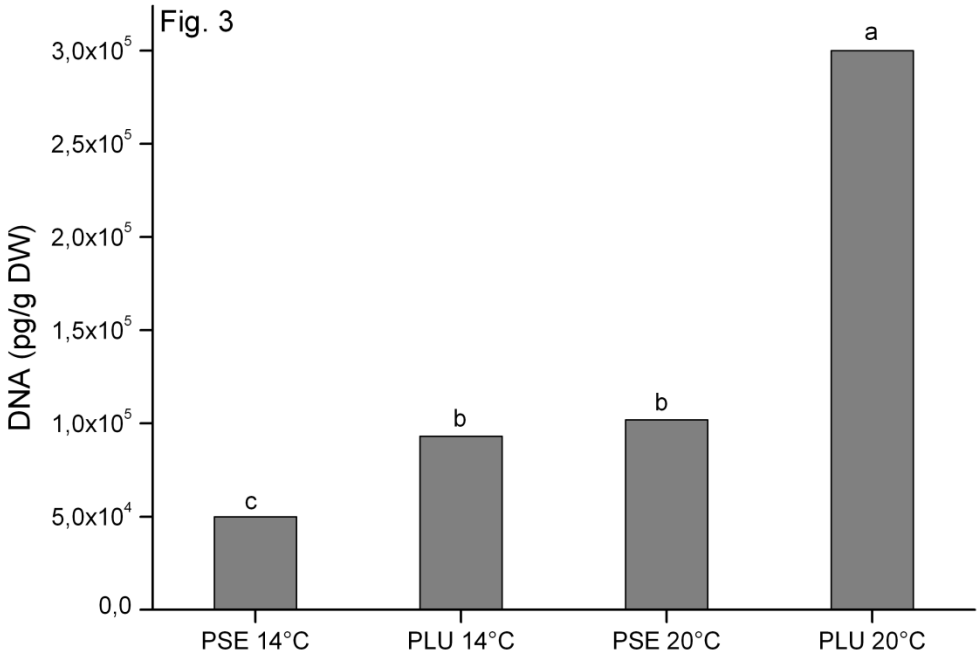

Figure 3: qPCR of *F. sylvatica* roots infected with *P. plurivora* (PLU) and *P. pseudosyringae* (PSE) at 14 and 20°C.

P. plurivora infection was about three-times higher than *P. pseudosyringae* in beech roots when the root incubation temperature was set to 20° C, and about six-times higher when comparing with *P. plurivora* at 20° C and *P. pseudosyringae* at 14° C, indicating a strong influence of the temperature on pathogenesis.

5.4 Discussion:

In order to allow comparable growth and sporulation rates for both pathogens after inoculation on beech roots a special thermo box was designed to control root temperatures. An infection experiment with beech seedlings was conducted in two separate thermo boxes at root temperatures of 14° C and 20 °C, respectively. To our knowledge, that is the first time that a specific device is used to control root temperatures and disease caused by *Phytophthora* species. Gunning and Cahill (2009) (although the work was not focusing root-temperature control) also published a soil-free system to facilitate analysis of plant pathogen interactions in roots. They tested the model legume *Lupinus angustifolius* under infection with *P. cinnamomi* and conclude that this new system facilitates access to the root system, being efficient for the analysis of root and pathogen interactions with no interference from soil or adhering particulate matter.

In vitro analysis showed that the radial growth rate of 7.5 ± 0,24 mm d^{-1} measured for *P. plurivora* at 20° C was slightly higher as recently published by Jung and Burguess (2009) with 6.3 ± 0.1 mm d^{-1}. It was not possible to examine the optimum growth rate for *P. plurivora* as it is beyond the temperature range used. The optimum growth rate of *P. pseudosyringae* was determined at 20° C with 3.6 ± 0,08 mm d^{-1}. These results are in accordance with those published by Jung *et al*, (2003).

Infection with *P. plurivora* resulted in severe wilting of leaves, decrease in photosynthesis and high mortality; whereas *P. pseudosyringae* infected plants showed no visible symptoms, high photosynthetic rates and no mortality was recorded. These contrasting effects of the infection with *P. plurivora* and *P. pseudosyringae* in seedlings of *F. sylvatica* are in agreement with the results reported by Fleischmann *et al*., (2002).

In general, changes in root temperature often affect the whole metabolism of plants. Yet, in this work, decreasing the root incubation temperature of *F. sylvatica* seedlings from 20 to 14°C did not significantly change the photosynthetic rate of non-infected seedlings. These data are in accordance with results of Lyr and Garbe (1995), who did not find any significant difference regarding the growth of *F. sylvatica* saplings, when the temperature of the root system was set to 15 or 20°C. However, for other plants decreasing the temperature of the root system is known to influence whole plant metabolism, and in particular leaf gas exchange (Mangat, 1982; Delucia, 1986).

In conclusion, it is possible to say that the contrasting effect of both pathogens on leaf physiology and mortality of beech seedlings was not affected by different root temperatures or growth rates of the pathogens, respectively. Thus, the amount of *Phytophthora* infection cannot explain the observed differences, and the reasons are still unknown. Experiments are still ongoing to elucidate different growth strategies within the root systems, or different defense mechanisms explaining the observed differences of beech towards both pathogens.

Chapter 6

Physiological investigations and allocation of C- and N-metabolites in European beech (*Fagus sylvatica* L.) infected with *Phytophthora plurivora* or *Phytophthora pseudosyringae*

Physiological investigations and allocation of C- and N-metabolites in European beech (*Fagus sylvatica* L.) infected with *Phytophthora plurivora* or *Phytophthora pseudosyringae*

Abstract:

How do plants allocate carbon and nitrogen during infection is still a question nowadays. The use of stable isotopes labeling to investigate carbon and nitrogen allocation is widely used in ecology but scarce in phytopathology. The aim of this study has been to investigate the patterns of carbon and nitrogen allocation of 3 years-old beech under infection with *P. plurivora* (susceptible interaction) and *P. pseudosyringae* (tolerant interaction). Plants were labeled with ^{13}C and ^{15}N and harvested during two growth phases, spring and midsummer. qPCR analysis was used to confirm infection. Biomass, symptoms and gas exchange were recorded. Allocation and partitioning of carbon and nitrogen was investigated. qPCR analysis confirmed infection of both pathogens. The DNA content of both pathogens did not differ statistically. The net CO_2 assimilation rate was decreased under infection of *P. plurivora* and *P. pseudosyringae*; however it did not altered plants biomass. No mortality or symptoms were recorded. The allocation pattern of carbon and nitrogen did not differ from control plants. Partitioning of carbon was decreased in coarse roots of *P. pseudosyringae* during spring. During summer, newly assimilated carbon were less allocated in twigs of plants infected with both pathogens when compared to control plants, possibly as an attempt to recover infection in the root system. Further experiments and repetitions should be carried out to validate these results.

6.1 Introduction:

The analysis of stable isotopes – that is the analysis of the relative abundance of different isotopes within an element (e.g. abundance of ^{12}C and ^{13}C within carbon) – in plant science can be used either at natural abundance level or by feeding plants with element sources of artificially changed isotopic ratios, which than can be traced as label within the plant.

The natural variation of the isotopic rations of carbon ($^{13}C/^{12}C$; ($\delta^{13}C$)) and oxygen ($^{18}O/^{16}O$; ($\delta^{18}O$)) within plant organic matter allows some conclusions on biochemical and physiological processes of plants. The $\delta^{13}C$ is manly influenced during photosynthesis due to discrimination of ^{13}C by ribulose-bisphosphate-carboxylase/oxygenase (rubisco) and diffusion through stomata (Farquhar et al. 1989;

Farquhar et al. 1982). Thus, $\delta^{13}C$ of plant biomass can be used as an integrative indicator of water-use-efficiency (Farquhar and Richards 1984). The isotopic composition of oxygen in leaf water is mainly influenced by transpiration and therefore depends on water-use-efficiency, too (Farquhar and Gan 2003; Flanagan et al. 1991). This is reflected in the $\delta^{18}O$ of primary assimilates as well. The variations of $\delta^{13}C$ and $\delta^{18}O$ in leaf matter can be studied best in cellulose, which is formed of primary carbohydrates and possesses a low turn-over rate (Barbour et al. 2002; Saurer et al. 1997). Analysis of these variations led to a semi-quantitative model describing the relationship of $\delta^{13}C$ and $\delta^{18}O$ with photosynthetic activity and stomatal conductance (Grams et al. 2007; Scheidegger et al. 2000).

Besides its use as indicator for biochemical and physiological processes in plants, stable isotope analysis can be used to study allocation and partitioning processes within plants by feeding plants with e.g. carbon or nitrogen sources which are enriched or depleted for their heavy stable isotope (^{13}C and ^{15}N respectively) (Deleens et al. 1994; Schnyder 1992). By measuring the isotopic signature of different plant organs or metabolites, respectively, after labeling with^{13}C (or ^{15}N), the fraction of labeled C (N) (that means newly incorporated C (N)) can be deduced applying a simple two component mixing model for labeled and unlabeled carbon (nitrogen). From this the relative specific allocation and the partitioning within the plant can be calculated (Dyckmans et al. 2000a). The allocation describes the fraction of new C (N) within a sample, while partitioning means the amount of new C (N) in an organ relative to the amount of new C (N) on whole plant level.

Within this work, root infections of three year old beech saplings with either *P. plurivora* (susceptible interaction) or *P. pseudosyringae* (tolerant interaction)

respectively (Figure 1) was compared. It was particularly focused on how beech saplings allocate carbon and nitrogen during two growth phases (spring and summer), under Phytophthora root-infection.

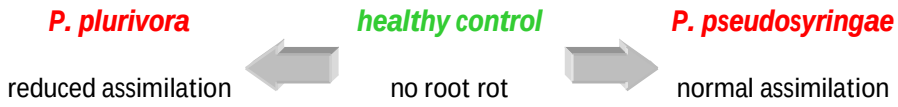

Figure 1: *Phytophthora* treatments and expected effects on beech

6.2 Materials and Methods:

Experiment description:

Labeling took place on the one hand right after inoculation in spring to study the impact on the early stages of infestation and on the other hand mid of summer when the root infections are well established (Fig. 2). During the first labeling period relative growth rate of beech is low and most of the newly assimilated carbon might be used for leaf differentiation. In contrast, during the second labeling period relative growth rate of beech is high and most of the newly assimilated carbon might be allocated to stem and roots. Therefore the impact of *Phytophthora* infestation on carbon allocation within beech should be different between spring and summer.

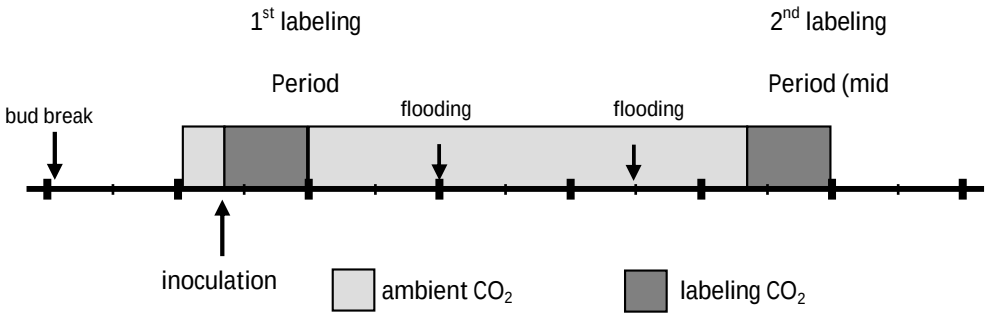

Figure 2: Time scheme for the stable isotope labeling

Plant growth and inoculum production

One year prior to the start of the corresponding experiment, two year old beech saplings were obtained from a nursery and replaced in pots (7 L volume) containing natural forest soil. They were grown under natural climatic conditions for one year to develop new roots inside the pots. For the actual experiment plants were transferred to the phytotrons of the research unit environmental engineering of the Helmhotz Centre Munich (http://www.helmholtz-muenchen.de/en/eus/facilities/phytotron/index.html) after budbreak. The phytotrons simulated the local weather of Freising, Germany of the year 2005, which was close to the long-time average, with 0.5 x of the natural PPFD. Drip irrigation of plant containers was controlled automatically with use of tensiometer sensors (four sensors per treatment) (Fig. 3) to maintain the soil water-potential around zero. Temperature of the root system was maintained at 20°C. During the two labeling periods, climate was set to constant parameters with temperature 20 °C (const.), relative humidity rH = 65 % (const.) and 14 hours of light period (PAR = 250 µmol m^{-2} s^{-1}).

Isolates of *P. plurivora* (Isolate 55) and *P. pseudosyringae* (Isolate Bu3), both obtained from mature declining beech trees in southern Germany, were grown on V8-culture media at 20°C in the dark. An inoculum was produced by mixing pieces of the agar plates containing mycelia in sterilized vermiculite amended with V8-broth. For inoculation a 25 mL hole was drilled into the soil right aside the main root of each plant, and filled with the inoculum and closed again with a thin layer of soil. Subsequent the containers were flooded with deionized water to soil level for two days to induce the production of sporangia and the release of zoospores. Inoculation with the root pathogens was performed ten days after the end of leaf flush. Between the two labeling

periods plants were flooded twice for two days each to trigger the production of new zoospores.

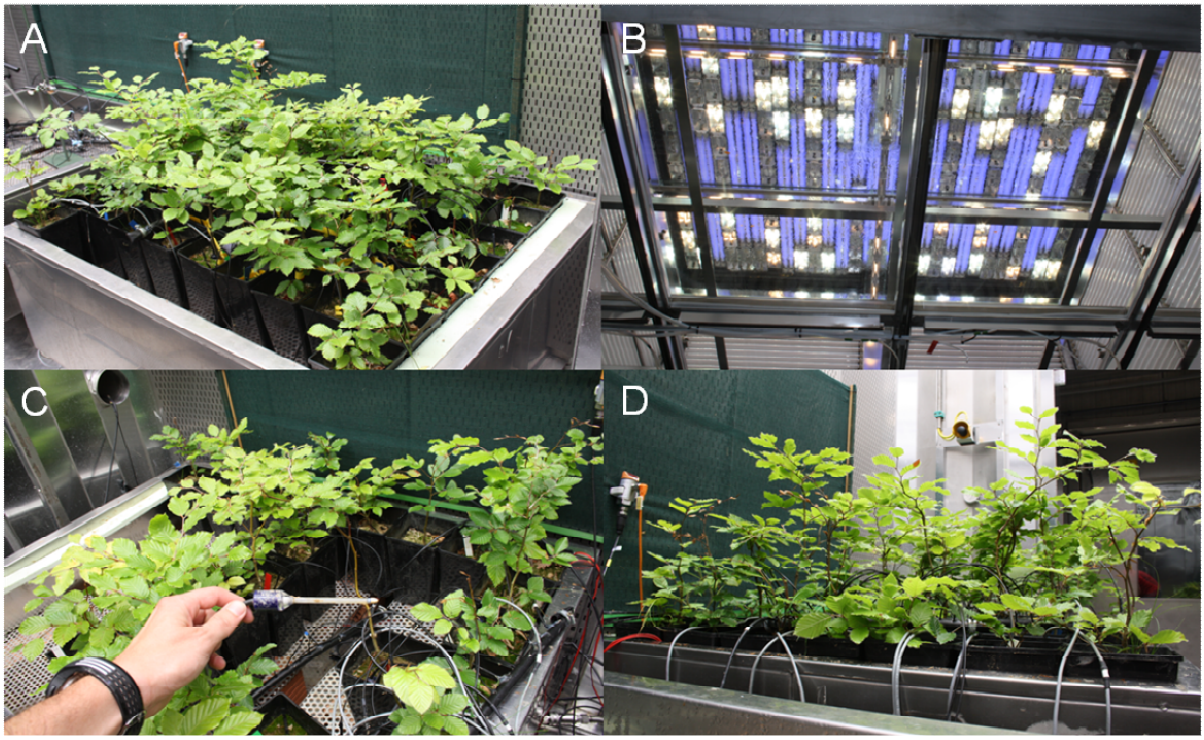

Figure 3: Detailing of the growth chambers. A: detail of a tank inside the chamber containing plants from all treatments. The tanks are able to control root temperature. B: Light system of the growth chambers mimicking real day-light conditions and luminosity. C: Detail of a soil water-content sensor. The sensors avoid water stresses in the soil, automatically triggering irrigation when needed. D: Detail of the plants, sensors and irrigation tubes.

Stable isotope labeling

Before inoculation those plants used for the first labeling campaign were transferred to a separate identical phytotron for the $^{12}CO_2/^{13}CO_2$ labeling. The remaining plants stayed in the original phytotron but were treated in the same way as those in the labeling system except of the labeling itself. The first labeling started immediately after inoculation by changing from ambient to CO_2 with an isotopic signature of $\delta^{13}C$=-10 ‰ to labeling CO_2 with $\delta^{13}C$=-48 ‰ and by casting ^{15}N-ammonium-nitrate solution to the pots (Fig. 3). $^{12}CO_2/^{13}CO_2$ labeling was maintained for a period of 10 day (Fig. 4). Due to incomplete removal of the ambient CO_2 from the labeling chamber the label was

diluted to $\delta^{13}C$ = -27,6 ‰ during the first labeling and $\delta^{13}C$ = -26,0 ‰ during the second labeling. Plant samples were taken before the start of labeling (t = 0 d) for the determination of initial $\delta^{13}C$ and $\delta^{15}N$ of plants, as well as after ten days of labeling (t = 10 d) (Fig. 3). At this second harvest the same number of plants was sampled from the non-labeled phytotrn chamber as from the labeling chamber, to estimate possible changes in ^{13}C discrimination due to the root infection treatments by comparing $\delta^{13}C$ values from the first and second harvest without labeling. This design allowed studying carbon allocation within beech plants irrespective of possible changes in ^{13}C-discrimination. The difference in $\delta^{13}C$ of carbon pools between the unlabeled and the label regimes was 17.6 ‰ (-10 ‰ - -27.6 ‰) and 16‰, respectively, in case that these pools are completely formed by labeled carbon independently of their actual $\delta^{13}C$. A 50 % labeling accordingly results in a difference of 8,8 ‰ (8.0 ‰) between the two labels. The comparison of unlabeled plants at t=0d and t=10d allowed to identify changes in discrimination of ^{13}C during the labeling period. However, no changes in discrimination of ^{13}C were observed. The labeling experiment was repeated two month after inoculation in the same way as described above with those plants remained in the phytotron. The design results in two independent growing repetitions with 8 seedlings per treatment and one labeling repetitions (n=8), respectively, for each labeling period.

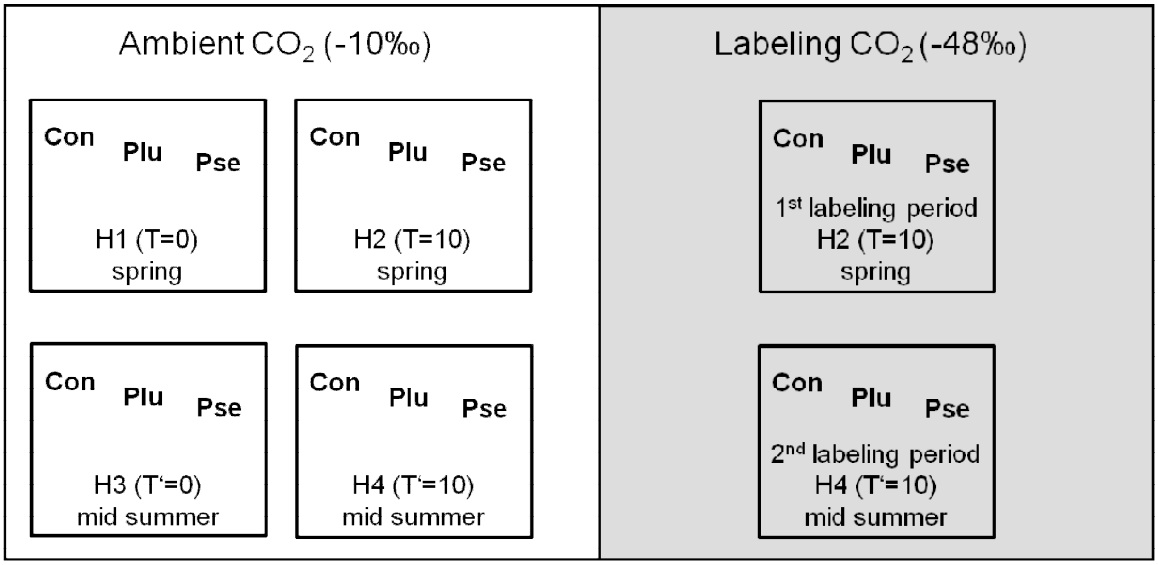

Figure 4: Schematic representation of the chambers arrangement. Con= control plants. Plu= plants infected with *P. plurivora*. Pse= plants infected with *P. pseudosyringae*. H1, H2, H3 and H4, harvests 1,2,3and 4 respectively. T=time point. Plants from the second labeling period were previously maintained at the ambient CO_2 chamber until the mid summer, when they were placed in the labeling CO_2 chamber.

Labeling with ^{15}N was achieved by adding 250 ml of a 98 at% solution of ^{15}N-ammonium-^{15}N-nitrate to each pot. The amount of ammonium-nitrate was calculated so that the actual plant available nitrogen will be enriched to about 3 atom% ^{15}N. With this procedure excessive nitrogen fertilization could be avoided, as only about 3 % of plant available nitrogen will be added.

Stable isotope analysis

All stable isotope analyses was performed on an isotope ratio mass spectrometer (GV Instruments) equipped with an elemental analyzer (EuroVector). For the analysis of $\delta^{13}C$ and $\delta^{15}N$ dried and milled biomass aliquots of 2 mg was necessary.

During the two labeling periods gas samples were taken daily from both phytotron chambers and $\delta^{13}C$ was analyzed within three days at the mass spectrometer to monitor the actually applied label. Calculations for relative specific allocation (RSA) and partitioning were done according to Dyckmans *et al*., (2000a):

RSA carbon= $(\delta_p - \delta_c) / (\delta_l - \delta_c)$

Where δ_p is the $\delta^{13}C$ value of the plant sample, δ_l is the $\delta^{13}C$ value of the labeled carbon in the plant and δ_c is the $\delta^{13}C$ value of the unlabelled carbon in the plant.

RSA nitrogen= $(^{15}N\%_p - {}^{15}N\%_c) / (^{15}N\%_l - {}^{15}N\%_c)$

Where $^{15}N\%_p$ is the isotopic signal of the plant sample, $^{15}N\%_c$ is the isotopic signal of the unlabelled control plants and $^{15}N\%_l$ is the isotopic signal of the labeled nutrient solution.

Partitioning carbon= $\%P_c = (C_{organ} \cdot RSA_{organ}) / (C_{plant} \cdot RSA_{plant})$

Partitioning nitrogen= $\%P_n = (N_{organ} \cdot RSA_{organ}) / (N_{plant} \cdot RSA_{plant})$

Where C_{organ} and N_{organ} are the amount of carbon and nitrogen in the specific plant organ and C_{plant} and N_{plant} are the amount of carbon and nitrogen in the whole plant.

Allocation describes the fraction of new C (N) within a sample, while partitioning describes the amount of new C (N) in an organ relative to the amount of new C (N) on whole plant level.

Physiology and Biomass analysis

Gas exchange measurements were conducted using a CO_2/H_2O diffusion porometer equipped with a broad-leaf LED cuvette (GFS-3000 Portable photosynthesis system, Walz Germany).

The maximum efficiency of photosystem II was measured using a MiniPAM yield analyser (Walz, Effeltrich, Germany) under ambient light conditions.

For plant sampling, the root systems of each tree were excavated and carefully washed. Roots were scanned for analysis with the WinRhizo-Software. Afterwards,

plants was segmented into leaves, twigs, stem, coarse roots (diameter > 1 mm) and fine roots (diameter < 1 mm). Biomass of each fraction was determined to get detailed information on the impact of the pathogens on growth.

qPCR analysis

Genomic DNA was extracted from 20 mg freeze-dried and milled root material using the DNeasy plant mini kit (Qiagen, Hilden, Germany) and was further purified using the Wizard® DNA clean up system (Promega, Mannheim, Germany) according to the manufacturer's protocols. The DNA was diluted 1:10 in H_2O to prevent the inhibition of the PCR reaction. The amount of *P. plurivora* and *P. pseudosyringae* DNA of root extract was determined by TaqMan quantitative PCR using an SDS7700 sequence detection system (Applied Biosystems, Germany). For the *P. plurivora* and *P. pseudosyringae quantification* the primer sets and the fluorogenic probes of Böhm *et al*., (1999) and Tooley *et al*., (2006), respectively, were used. Dilution series of genomic DNA extracted of pure *P. plurivora* or *P. pseudosyringae* mycelium grown in M1-liquide culture was used to calculate a linear standard curve of log [*Phytophthora* DNA] vs. CT-value. The standard curve concentrations ranged from 1 pg DNA mL^{-1} to 10 ng DNA mL^{-1} in five steps.

6.3 Results

In order to confirm the infection of *P. plurivora* and *P. pseudosyringae* in roots of beech, a qPCR analysis was performed. DNA from both pathogens was detected in the root material (Fig. 5). *P. pseudosyringae* DNA was found to be higher than *P. plurivora's* in H2 and H4, but these differences were not statistically significant. The DNA content of both species increased along time. *P. plurivora* DNA content was

found to be about 5 times bigger in H4 in comparison with H2, whereas *P. pseudosyringae* increase was about 2.5 times bigger. This faster growth rate of *P. plurivora* diminished the ratio *P. pseudosyringae* / *P. plurivora* DNA from 3.6 in H2 to 1.7 in H4.

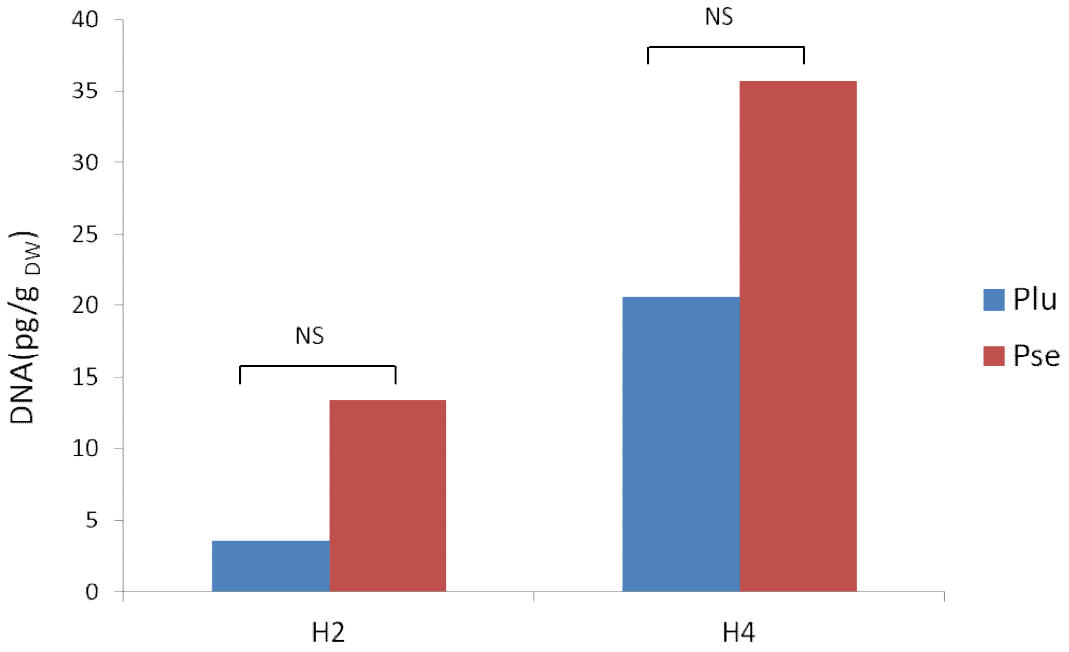

Figure 5: qPCR analysis of *P. plurivora* and *P. pseudosyringae* DNA of three year old beech saplings along harvest 2 (H2) and harvest 4 (H4). Blue bars: Plants infected with *P. plurivora*; red bars: Plants infected with *P. pseudosyringae*. n=8 plants per treatment. NS= statistically not-significant.

P. plurivora infection in roots did affect the net CO_2 assimilation rate of the saplings at H2 and H4 (Fig. 6). *P. pseudosyringae* infection decreased the CO_2 assimilation only at H2; at H4, it is statistically not significant when compared with control saplings. All the other physiological parameters such as stomatal conductance, ci and PSII quantum yield were not affected by the infection (Fig. 6). Likewise, activity of rubisco (Vcmax) and maximum rate of electron transport (Jmax) as calculated from A/Ci curves of infected plants did not differ from the controls at harvest 4 (Table 1).

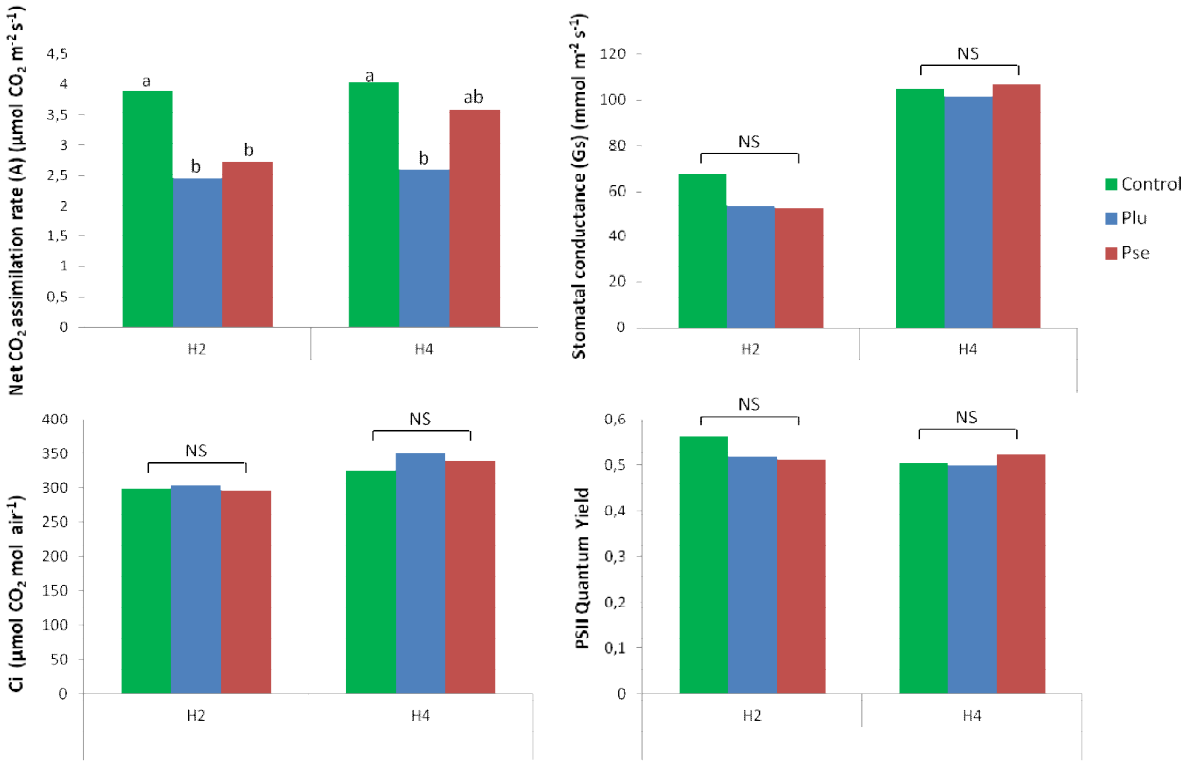

Figure 6: Net CO2 assimilation rate (A) (µmol CO2 m-2 s-1), Stomatal conductance (Gs) (mmol, m-2 s-1), Ci (µmol air-1) and PSII Quantum yield of 3 year old saplings infected or not with *P. plurivora* (blue bars) and *P. pseudosyringae* (red bars) (green bars shows control plants), at harvest 2 (H2) and harvest 4 (H4). Values shown in the bars are the average of three independent measurements done before H2 and H4 respectively. n=8 plants per treatment. Same letters represent no statistically difference, Duncan test (P≤0,05). NS= statistically not-significant.

Table 1: Rubisco activity (Vcmax) and maximum rate of electron transport (Jmax) data fitted from A/ci curves of *Fagus sylvatica* saplings at harvest 4 (n=6).

	A/ci curves fitted parameters		
Treatment	V_{cmax} [µmol CO_2 $m^{-2}s^{-1}$]	J_{max} [µmol e^- $m^{-2}s^{-1}$]	Ratio V_{cmax}/ J_{max}
Control	23,5 a*	40,8 a	1,82 a
Plu	21,6 a	36,8 a	1,77 a
Pse	19,3 a	33,7 a	1,80 a

*Same letters at same numbers represent no statistically difference, Duncan test (P≤0,05).

Figure 7 shows dry weight of leaves, twigs, stem, coarse and fine roots, as well as the total biomass of the beech saplings at harvests 2 and 4. Regardless of the growing phase, or infection in roots, or even lower net CO_2 assimilation rate in infected plants, the biomass of the saplings did not significantly change over the experiment. No statistically differences were found neither to organs nor to total plant mass when comparing all treatments at the two harvests. Furthermore, besides no differences in biomass, no symptoms or mortality were recorded over the experiment.

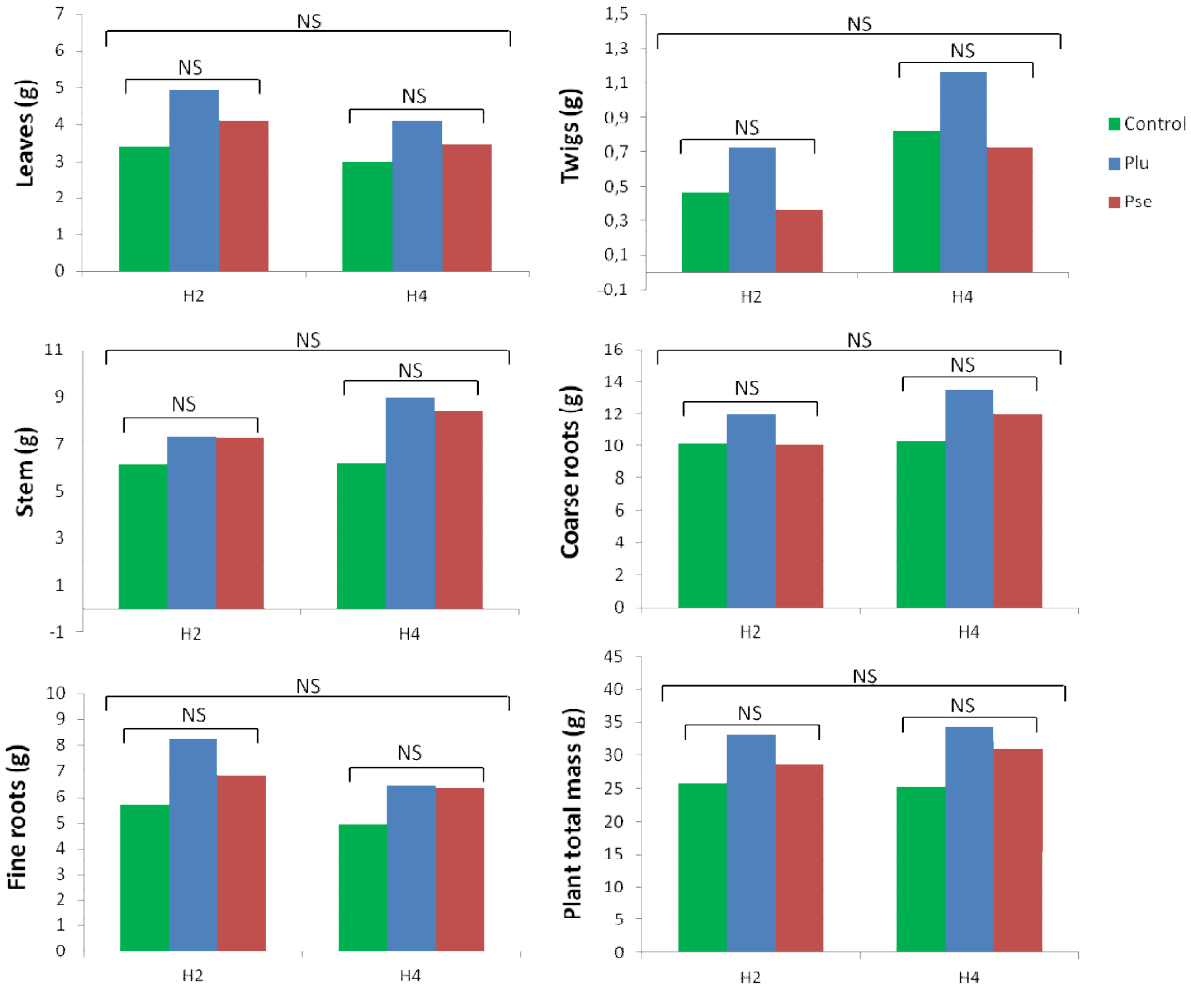

Figure 7: Dry weight (g) of leaves, twigs, stem, coarse and fine roots, as well as the plants total mass along harvest 2 (H2) and harvest 4 (H4). Green bars: control plants. Blue bars: Plants infected with *P. plurivora*; red bars: Plants infected with *P. pseudosyringae*. n=8 plants per treatment. NS= statistically not-significant.

The relative specific allocation (RSA) of both carbon and nitrogen was determined to check if there is any difference in the allocation pattern of new C and N when comparing healthy and root-infected plants. Figure 8 and 9 shows the relative specific allocation of carbon and nitrogen, respectively, in all plant organs at H2 and H4. The saplings new carbon and nitrogen allocation-pattern was not altered under root infection with either *P. plurivora* or *P. pseudosyringae* at any of the harvests (Figs. 8 and 9).

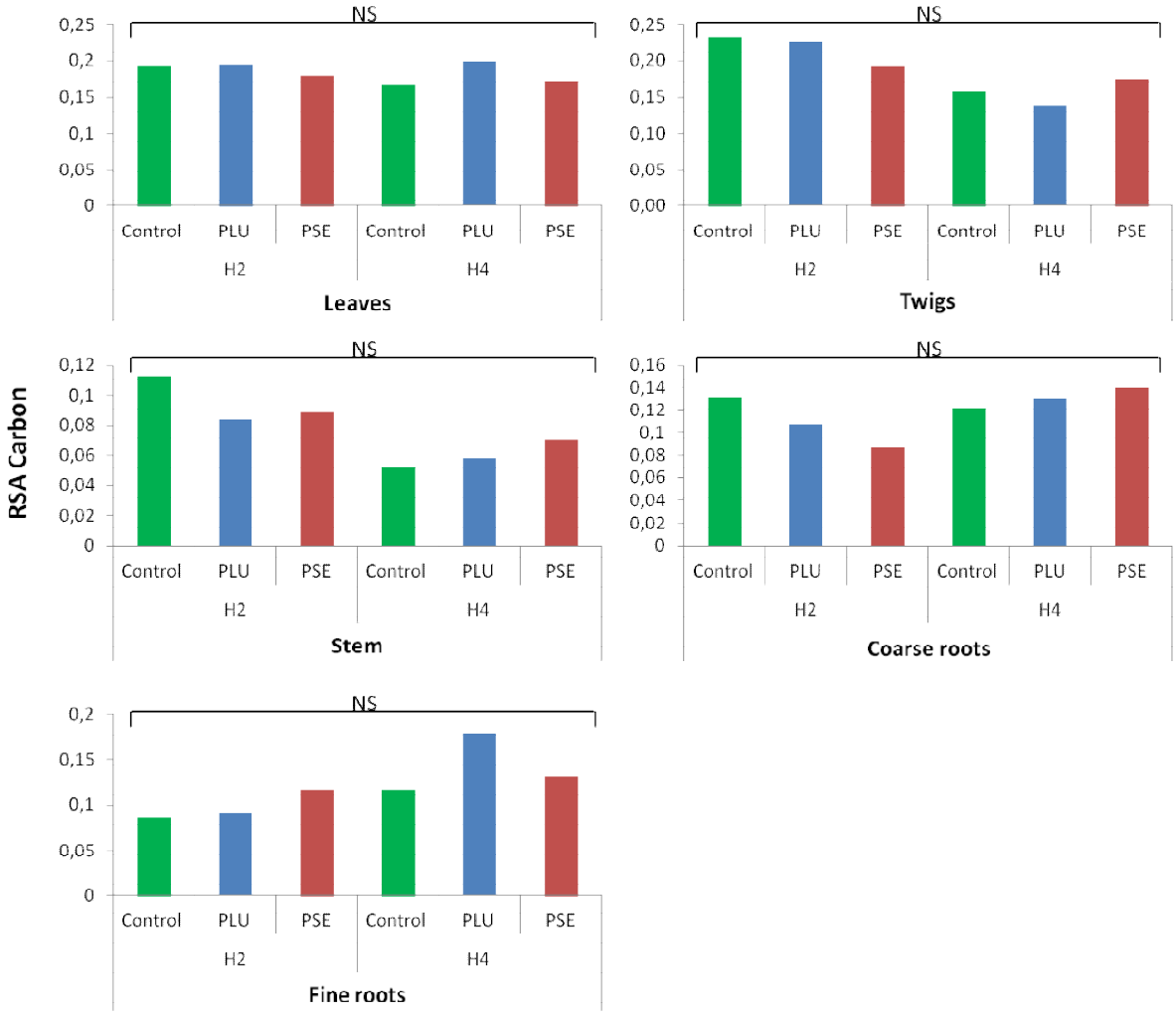

Figure 8: Relative specific allocation of Carbon (RSA Carbon (%)) in leaves, twigs, stem, coarse and fine roots, along harvest 2 (H2) and harvest 4 (H4). Green bars: control plants. Blue bars: Plants infected with *P. plurivora*; red bars: Plants infected with *P. pseudosyringae*. n=8 plants per treatment. NS= statistically not-significant.

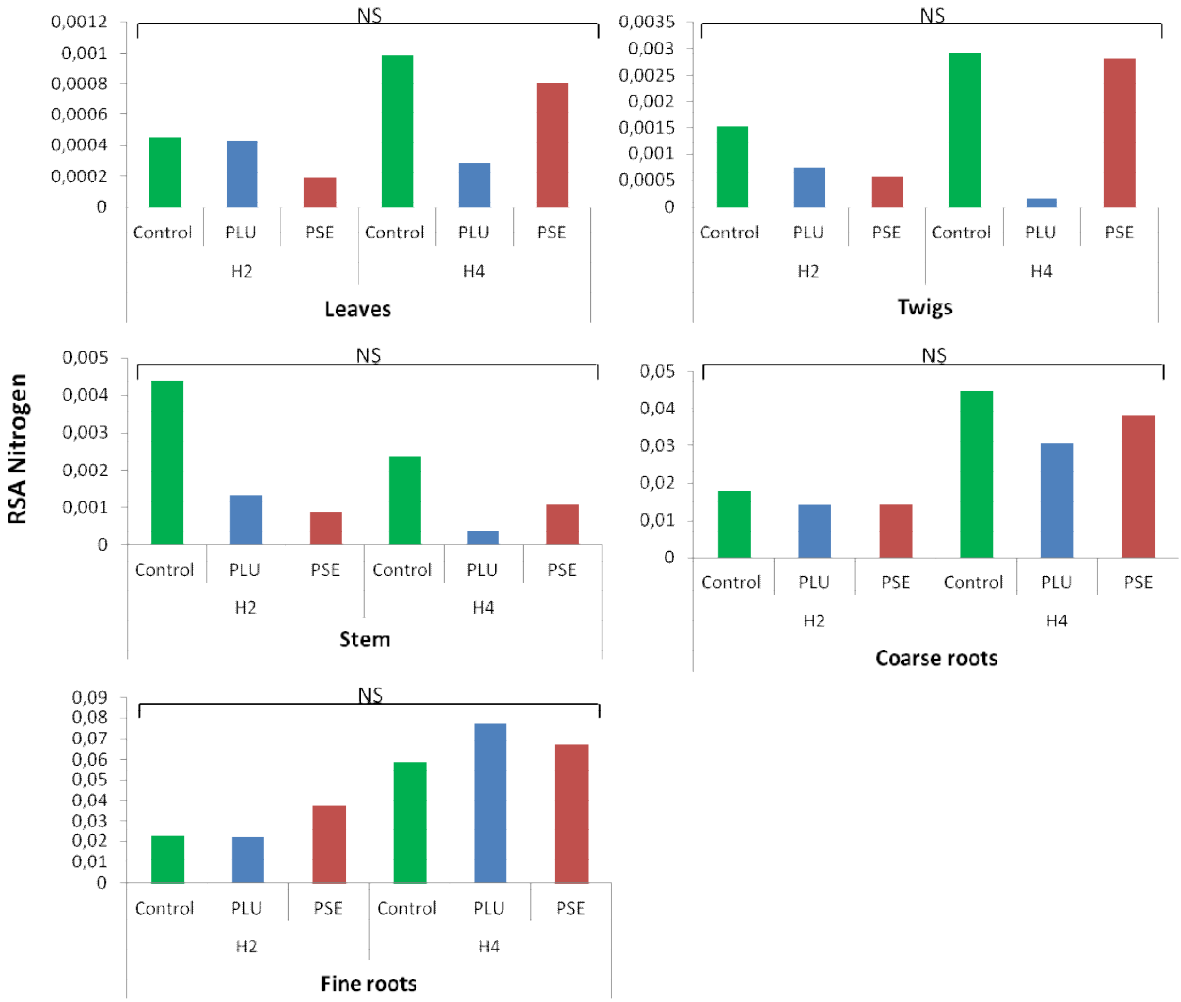

Figure 9: Relative specific allocation of Nitrogen (RSA Nitrogen (%)) in leaves, twigs, stem, coarse and fine roots, along harvest 2 (H2) and harvest 4 (H4). Green bars: control plants. Blue bars: Plants infected with *P. plurivora*; red bars: Plants infected with *P. pseudosyringae*. n=8 plants per treatment. NS= statistically not-significant.

Although no difference was found regarding RSA of new carbon and nitrogen in infected saplings at any point of the experiment, it was found that *P. plurivora* and *P. pseudosyringae* did alter partitioning of carbon at H2 and H4 (Fig. 10). Plants under infection with *P. pseudosyringae* at H2 partitioned significantly less carbon in the coarse roots when compared with control plants. Saplings infected with *P. plurivora* showed a tendency of less partitioning of carbon in coarse roots at H2 (likewise in plants infected with *P. pseudosyringae*), though these differences were not statistically different from control plants. A clear tendency of less carbon allocation can be also seen in leaves of infected saplings at harvest 2; however these differences are also not

significant. At H4, *P. plurivora* and *P. pseudosyringae* partitioned significantly less carbon at twigs. The partitioning in other plant-organs did not differ from control plants.

Overall, there is a tendency in harvest 2 that the Phytophthora infection increased the partitioning of carbon in above ground organs. This pattern was accentuated in the *P. pseudosyringae* infected plants. The partitioning of carbon in below ground organs was increased in H4 in comparison to H2, regardless of the treatment (Fig. 10).

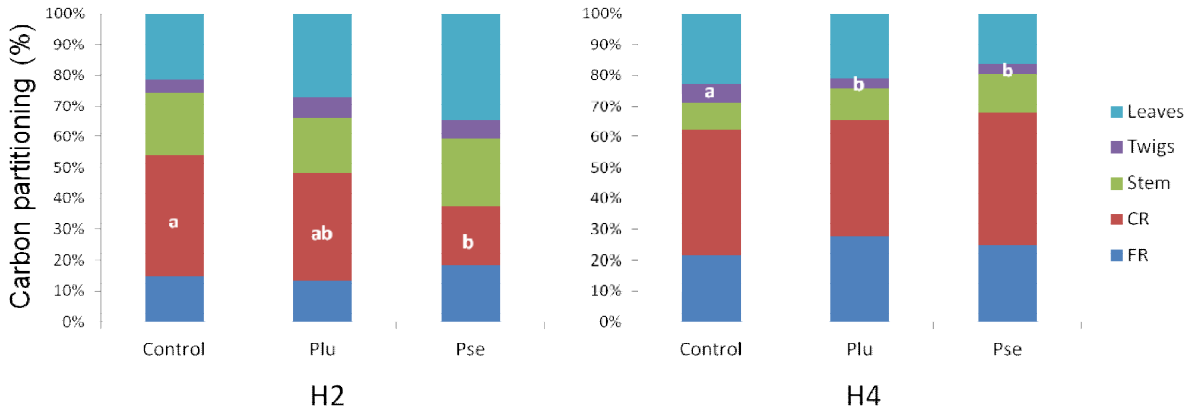

Figure 10: Partitioning of Carbon (%) in leaves, twigs, stem, coarse and fine roots (CR and FR respectively), along harvest 2 (H2) and harvest 4 (H4). Treatments: non-infected plants (Control), plants infected with *P. plurivora* (Plu), plants infected with *P. pseudosyringae* (Pse). n=8 plants per treatment. Same letters represent no statistically difference, Duncan test (P≤0,05).

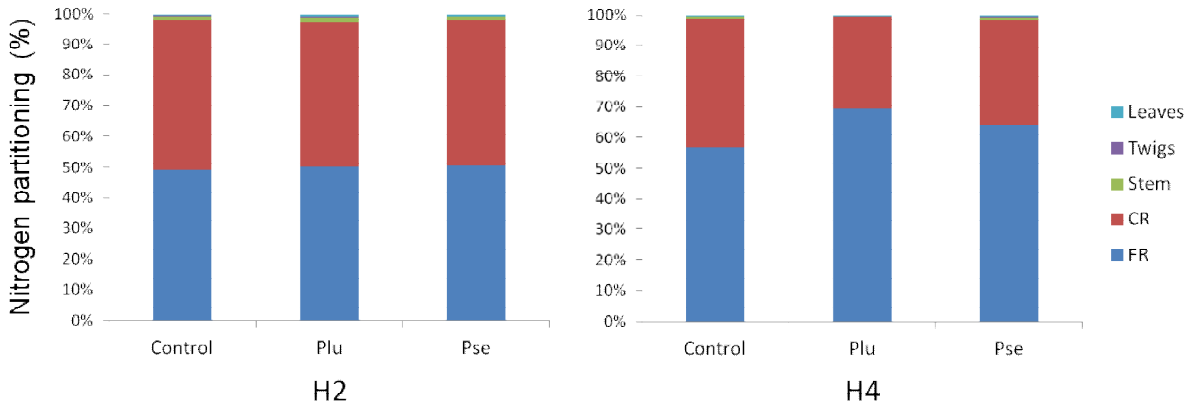

Figure 11: Partitioning of Carbon (%) in leaves, twigs, stem, coarse and fine roots (CR and FR respectively), along harvest 2 (H2) and harvest 4 (H4). Treatments: non-infected plants (Control), plants infected with *P. plurivora* (Plu), plants infected with *P. pseudosyringae* (Pse). n=8 plants per treatment. Same letters represent no statistically difference, Duncan test (P≤0,05).

Regarding partitioning of nitrogen, no statistically differences were found in the allocation pattern when comparing data from different organs in healthy and infected plants at any of the harvests (Fig. 11). In addition, no clear tendency is visible.

Interestingly, the overall tendency in H2 and H4 is that the majority of the nitrogen (more than 98%) stays at the below ground organs, regardless of the treatment (Fig. 11).

6.4 Discussion

Within this study, the patosystems *P. plurivora* / *F. sylvatica* (susceptible interaction) and *P. pseudosyringae* / *F. sylvatica* (tolerant interaction) were set and analysed during two distinct growth phases: spring and mid summer. The responses of 3 year-old beech saplings were examined in terms of physiology and allocation as well as partitioning of carbon and nitrogen.

In general, the DNA content of both pathogens can be considered low in this study when compared with previous publication of Fleischmann *et al*., 2005, and the figures 6 (chapter 2), 4 (chapter 3) and figure 3 (chapter 5) in which the DNA content of *P. plurivora* and *P. pseudosyringae* are substantially bigger than the one's found in the current analysis. Nevertheless, it has to be pointed out that all these previous results were originated from a different inoculation method. In former studies, the plants, that were place inside falcon tubes filled with water, were inoculated via zoospores; in this study, plants were inoculated through an inoculum. Clearly, the inoculation with zoospores in falcon tubes seems to be largely more effective. Another important difference to explain the low DNA content in this study when compared with previous

ones is the age of plants. In this study, plants were 3 years-old, while in the former reports the seedlings were 3 to 4 months old.

qPCR analysis confirmed the infection with both pathogens. It was expected a bigger DNA content of *P. plurivora* in beech-roots, as it was shown to have a significant faster growth rate at same temperatures when compared with *P. pseudosyringae* (Fig. 2, chapter 5). An opposite tendency was found in which DNA content of *P. pseudosyringae* was bigger, however the differences are not significant. Nevertheless, the growth rate of the pathogens when comparing harvest 2 and 4 were in agreement with previously reports (Fig. 2, chapter 5; Jung *et al*., 2003; Jung and Burgess, 2009), it was found that *P. plurivora* grows twice faster than *P. pseudosyringae* at the same temperature range.

Despite of the low pathogen DNA found in the roots, plants infected with both *P. plurivora* and *P. pseudosyringae* showed significant changes in net CO_2 assimilation. The decrease in net CO_2 assimilation found in plants infected with *P. plurivora* is in accordance with previous reports of Fleischmann *et al*., 2002 and 2004, and figure 3 (chapter 2), figure 2 (chapter 3) and table 1 (chapter 5). However, the decrease in net CO_2 assimilation found in plants infected with *P. pseudosyringae* is in opposite of the results found in Fleischmann *et al*., 2002 and 2004 and table 1 (chapter 5), in which plants infected with *P. pseudosyringae* performed as good or even better as healthy control trees.

The decrease in net CO_2 assimilation found in infected plants during the experiment did not alter the biomass of the plants, neither to *P. plurivora* nor to *P. pseudosyringae* infected plants. In previous pathogenicity tests with young saplings, both species had quite contrasting effects on beech. Both pathogens caused root rot and

strongly decreased the length of fine roots as well as the number of root tips. However, *P. plurivora* caused stronger visible damage on coarse roots than did *P. pseudosyringae*. Most importantly, *P. plurivora* caused high mortality of beech, while all saplings survived an infection with *P. pseudosyringae* (Fleischmann et al. 2004; Fleischmann et al. 2002; Jung et al. 2003). In the current study no symptoms or mortality was recorded in any treatment at any time point.

Investigations using stable isotope analysis of carbon and nitrogen allocation and partitioning in infected plants are scarce in the literature. Most of the work remains to healthy plants under the scope of ecology and not plant-pathology. Fleischmann and Oßwald, (unpublished data) in labeling experiments using stable isotope analysis and infected beech saplings found that the allocation of C and N-metabolites within beech plants was strongly affected during the early stages of a *P. plurivora* root infection. In consequence, the partitioning of newly assimilated carbon increased in leaves at the expense of roots due to *P. plurivora* infection. The opposite was found for nitrogen (Fleischmann and Oßwald, unpublished data).

In this study, infected plants showed no statistic differences regarding allocation (RSA) when compared with control plants at any time point. However, infection with *P. plurivora* and *P. pseudosyringae* did alter the pattern of partitioning of newly assimilated carbon. It was found in H2 less partitioning of newly assimilated carbon in coarse roots in *P. pseudosyringae* infected plants. Fleischmann and Oßwald (unpublished data) found decrease in partitioning in fine roots of infected plants rather than in coarse roots as found in this study for *P. pseudosyringae* infected plants. The partitioning of newly assimilated carbon at harvest 4 was also altered. *P. plurivora* and *P. pseudosyringae* infected-plants were found to have less new carbon at twigs in

comparison with control plants. This plant response can be interpreted as an attempt to counter-attack or contain the infection in the root system, so that the newly assimilated carbon is invested in the infected roots instead of growth of above ground biomass. The allocation and partitioning of nitrogen did not differ at any time of the experiment regardless of the treatment. Horst *et al*., 2010 found out that *Ustilago maydis* strongly alters the nitrogen allocation in maize leaves. It was demonstrated that *U. maydis*-induced tumors reduced assimilation of soil-derived ^{15}Nitrate in leaves. However, the authors did not show evidence of differences in assimilation or partitioning of other plant-organs.

Dyckmans *et al*., (2000a) have investigated the carbon and nitrogen allocation and partitioning of healthy three-year-old beech saplings under ambient and elevated CO_2. The growing conditions were similar to the actual study, however the labeling period was longer and labeling started already with bud break. Different patterns of allocation and partitioning were found in comparison with the results showed in the current experiment. The authors demonstrated that the partitioning of new carbon in beech six weeks after bud break are higher in leaves (almost 60%) and stem (about 25%). It means that during leaves flush, most of the Carbon from actual assimilation is incorporated in the biomass of developing leaves. The higher values of partitioning in this study were found for coarse roots (39%) and leaves (21%), although stem showed values of 20%; these results are consistent with short-term labeling experiments using other tree species showing that carbon assimilates are first used in leaves and translocation to below ground organs just gains relevance after completion of leaf expansion (Burke *et al*., 2002; Dickson *et al*., 1990; Mordacq *et al*., 1986).

Regarding nitrogen, Dyckmans *et al*., (2000a) found out the higher levels in leaves and fine roots (38 and 37% respectively). In other publication Dyckmans *et al*., (2000b) found out that more than 60% of nitrogen were partitioned to fine roots after 12 weeks from bud break. Here it is shown that the majority of the nitrogen stays at below ground organs (coarse roots 48%, and fine roots 49%). Only about 3% of the nitrogen goes to the above ground organs. Dyckmans *et al*., (2000a) suggested that the tree internal N may limit incorporation of newly assimilated nitrogen to developing organs like leaves and twigs. Consistently with the results shown here, Dyckmans and Flessa (2001) also found out more partitioning of newly incorporated nitrogen into fine and coarse roots of nitrogen-deprived plants, which would indicate the formation of nitrogen stores.

Overall the low significance of results found in this work can be explained either by the low amount of pathogen DNA in roots (which means a weak infection) or by the older age of the plants (3 years-old), which results in more resistance to attack of pathogens. Further experiments and repetitions should be done to draw more accurate conclusion.

Chapter 7

Final discussion and Outlook

This thesis, entitled: “Deciphering mechanisms of pathogenicity and resistance induction in the interaction between *Phytophthora* spp. and European beech (*Fagus sylvatica* L.)” was aimed to investigate in detail the interaction between *Phytophthora* species and Beech, providing useful information for future management strategies to combat *Phytophthora* diseases. In this context, fundamental questions such as: What are the molecular mechanisms for pathogenicity; which are the main plant defense strategies against *Phytophthora* diseases and how to activate or manipulate them, were addressed.

Chapter 2: The acidic elicitin of *Phytophthora plurivora* is essential for the susceptible interaction with *Fagus sylvatica* (European beech)

In chapter 2, the significance of elicitins for *P. plurivora*-beech interaction was deciphered. It was demonstrated that *P. plurivora* lost its virulence towards *F. sylvatica*, when the acidic elicitin Plurivorin was specifically inactivated by the use of Plurivorin antibodies. Since elicitins are putatively produced in virtually all *Phytophthora* species (Takemoto *et al*., 2005), it is very likely that the same functions are found for other susceptible Phytophthora-plant interactions. In consequence, elicitins could be a target for protecting plants against *Phytophthora* infections. Very likely applying antibodies in order to block *Phytophthora* elicitins will not work under natural conditions, because these peptides would be degraded very soon. However, feasible techniques, such as the use of protein inhibitors or even genetic engineering, could be developed for targeting elicitins in *Phytophthora* diseases.

Chapter 3: Phosphite protects *Fagus sylvatica* seedlings towards *Phytophthora plurivora* via local toxicity, priming and facilitation of pathogen recognition

In chapter 3, it was demonstrated that phosphite leaf treatment of beech plants enhanced plant defense responses and finally caused resistance against *P. plurivora*. A

remarkable outcome of treating plants with phosphite is that the highly susceptible interaction between *F. sylvatica* and *P. plurivora* was converted into a resistant one by the activation of plant own defense mechanisms.

Phosphite was presented as a powerful weapon to manage *Phytophthora* diseases. It is environmental friendly, relatively cheap and easy to manipulate. Furthermore it is toxic to *Phytophthora* species and remarkably effective in inducing plant defence. One very interesting question still remains unanswered: to what extent phosphite primes or facilitates plant recognition during infection? According to Frost *et al*., (2008), the priming phenomenon in woody plants is even more beneficial than in herbaceous plants because it endures over several growing seasons, often called "delayed induced resistance". It does not impose any energy cost to the plants in the absence of pathogens, but implements a fast and strong defence reaction under pathogen attack. Thus, priming woody plants can be an extremely powerful strategy to manage diseases.

Chapter 4: Zoospore attraction by root exudates - development of a new trap to quantify attraction.

Very little attention has been given to the early steps of interaction between soil borne pathogens and their hosts in the rhizosphere. This initial interaction is fundamental and can determine the fate of infection. This communication process is strongly influenced by root exudates before the pathogen starts attacking the host. Pathogens can sense and move towards plants through root exudates. Some plants produce more attractant root exudates than others, what might be crucial for the host-pathogen interaction. Selecting hosts that produce less-attractant root exudates would be a very useful management strategy, and could be done by using the zoospore trap

presented in chapter 4. However, information on the composition of root exudates is scarce in literature. Once the structure of the different components is elucidated they also can be tested using the zoospore trap described above.

Chapter 5: Controlling root colonization of *Fagus sylvatica* seedlings by *Phytophthora plurivora* and *P. pseudosyringae* using specially designed thermo boxes

In chapter 5, it was demonstrated that the high virulence of *P. plurivora* towards *F. sylvatica*, as compared to *P. pseudosyringae* could not be traced back to its faster growth rate. It would be of interest to compare the effector arsenal of both pathogens and to investigate the corresponding plant responses. The box device controlling root temperatures presented in chapter 5 could also be used to get more detailed information how below ground temperature influences soilborn plant-pathogen interactions.

Chapter 6: Physiological investigations and allocation of C- and N-metabolites in European beech (*Fagus sylvatica* L.) infected with *Phytophthora plurivora* or *Phytophthora pseudosyringae*

The study of carbon and nitrogen allocation in plants is extensively used in plant-ecology but very scarce in plant-pathology. Differences in the pattern of Carbon and Nitrogen allocation during infection can provide very useful information on how plants defend themselves. In chapter 6, carbon and nitrogen allocation of *F. sylvatica* saplings were investigated by use of stable isotope labeling techniques under infection with *P. plurivora* (susceptible interaction) and *P. pseudosyringae* (tolerant interaction). In general, Phytophthora infection had little impact on the plant internal resource allocation. The most important result of this analysis was that *P. pseudosyringae* infected plants allocated less newly assimilated carbon in twigs possibly as an attempt to recover infection in the root system. In addition, the contrasting effects of both

species in seedlings (susceptibility and tolerance) were not recorded in older plants. It could be explained by low amount of DNA found in roots or interpreted as a general more resistance of older plants. Further experiments should be repeated and the inoculation method should be improved.

Undoubtedly, the intrinsic point that permeates all the findings of the investigations described above is the co-evolution between *Phytophthora* spp. and their host plant. The co-evolution process drives all biological processes in the biosphere and has particularly importance to the field of plant-pathology.

Co-evolution

"Co-evolution between plants and their natural enemies is generally believed to have generated much of the Earth's biological diversity" (Rauscher, 2001).

Co-evolution, under a plant-pathologist view, is a cycle of evolutionary events occurring in the plant-pathogen interactions that increase fitness of pathogens in detriment of plants and vice-versa. In other words, it is the evolution of two interacting species, each adapting to changes in the other. Figure 9 of chapter 1 shows a schematic illustration of co-evolution between plants and pathogens.

In the initial steps of the co-evolution process, selection pressure imposed by pathogens results in evolution of a new plant character providing resistance. Consequently, the selection pressure turns into the pathogen side, as the resistance plant-character reduces virulence and therefore pathogen-survival. A further step in this process is initiated with the evolution of a new pathogen character, increasing its fitness and circumventing the newly evolved plant resistance, what is called counter-resistance. These steps are repeated tending to infinite (Rauscher, 2001; Janzen, 1980; Gould,

1980). As a result, on one hand, plants exhibit an extraordinary variety of chemicals and morphological diversity related to defense, coming from adaptation in different sets of co-evolution. On the other hand, changes in behavior, physiology and metabolism are found in pathogens as an attempt to counter-attack resistance evolved in plants.

Plants and pathogens co-exist in the same environment for millions of years. A high number of evolutionary events should have happened during all this time. It would then explain the high number of effectors (such as RxRL) and receptors (such as R-proteins) or defense molecules recorded in recent sequencing analysis of pathogens and plant DNA (Kamoun, 2006). Moreover, it creates a very intricate, complex, fine-tuned system, in which every single disturbance can promote advantage to one of the sides involved.

It opens a new horizon to the plant-pathology field, since one can disturb this complex system between plants and pathogens, giving advantage to the plants. Most of the plants can defend themselves against pathogens; however successful pathogens secrete effectors to mock plant-recognition of infection, including *P. plurivora* (Chapter 2, fig. 10, Table 1; Schlink, 2010). Any disturbance of the mode of action of effectors could activate plant-defense responses. The results of chapter 2 and 3 are evidence for this conclusion. In chapter 2, a very punctual disturbance of the system, such as blocking the acidic elicitin α-plurivorin of *P. plurivora* among hundreads of other effectors, resulted in loss of virulence and simultaneously activation of plant defence. On the other hand, in chapter 3, it was hypothesized that lysis of cell walls by phosphite would release several pathogen cytoplasmic-molecules (effectors), activating plant defence. Scientists also figured out that silencing of one single effector can compromise pathogenicity. One recent example is given by Yu et al. (2012), who proved that silencing the RxLR effector Avh241, among about 627 RxRL in total (Tyler *et al*. 2006)

resulted in loss of virulence of *P. sojae* to soybeans. Kale (2001) stated that "effector blocking technologies could be developed and utilized in a variety of important crop species against a broad spectrum of plant pathogens".

Recognition

Recognition of environmental signals either by plants or by pathogens is fundamental for their interaction. In each plant-pathogen interaction, recognition is the main factor driving resistance or susceptibility. If a plant lacks recognition of infection, invariably, there will be susceptibility and even mortality. In the case of recognition, plants can activate their defense responses counter-attacking infection. In the majority of cases, plants are able to defend themselves, resisting or tolerating the effects of an invader.

On the other hand, pathogens are also able to recognize plants or even specific hosts in their environment (Tyler, 2006). This recognition was shown in chapter 4 where *Phytophthora* species identified their host plant by recognizing specific host plant root exudates. It was also demonstrated that the zoospores attraction can vary when comparing root exudates from different plants and that a susceptible cultivar was more attractive to zoospores than a confirmed resistance one.

Altogether, understanding and manipulating the main factors involved in recognition can be a powerful strategy to manage plant diseases, either by facilitating plant recognition of pathogens or by introducing plant varieties that are no or less attractant to pathogens.

Woody plants vs. herbaceous plants

Most of the work analyzing plant defense mechanisms is mainly done nowadays with herbaceous model plants such as *Arabidopsis thaliana*, tobacco, tomato, potato,

barley, maize or rice. All these model plants share same features; they are small, short living and rapid-growing plants. Trees on the other hand are in general much larger and live longer than herbaceous plants, they take longer to reproduce and they have different growth patterns, including secondary growth. In addition, as stated by Eyles et al. (2010) "Trees may be subjected to different patterns of herbivore and pathogen pressure and require different modes of protection".

The co-evolution of trees and herbaceous plants with pathogens also shows dissimilarity. The rate of molecular evolution, which is a process of evolution at a scale of DNA, RNA and proteins, is an important indicator of the evolution tempo for a given species (Smith and Donoghue, 2008); it depends on several factors such as body size, metabolic rate, DNA repair and generation time (Gilloly *et al*., 2005). Smith and Donoghue (2008) concluded that trees have consistently lower rates of molecular evolution when compared to herbaceous plants. The direct implication of these conclusions is that the knowledge originated from extensive studies on plant-pathogen interactions with model herbaceous plants may not always be transferred to woody plants. The same conclusion was drawn by Hammerschmidt (2007), investigating host-parasite interactions of conifers.

A good example supporting the arguments stated above is the contrasting functions of elicitins when comparing *Phytophthora* infections with trees and herbaceous plants. In the *Phytophthora* – tree interaction, elicitins have effectors functions, being directly correlated with pathogenicity (chapter 2; Horta *et al*., 2010; Manter *et al*. (2010). However, in herbaceous plants, such as tobacco (Kamoun *et al*., 1994, Keller et al., 1999), radish and turnip (Kamoun *et al*., 1993), elicitins share PAMP characteristics, resulting in resistance. The findings regarding the functions of elicitins

strengthen the point that each plant-pathogen interaction is unique and that results of herbaceous plants cannot be generalized to trees.

The outcome of the five parallel investigations carried out along this thesis supplies useful information to manage *Phytophthora* diseases, especially by taking advantage of co-evolutionary aspects of the plant-pathogen interactions, manipulating recognition and attacking diseases through exploitation of the plant own defenses.

Appendix:

This appendix provides supplemental information for the thesis:

- Figures 1 to 14 provide supplemental Confocal laser scanning images from chapter 2.
- Table 1 provides a list of oligo-nucleotides used in the thesis.

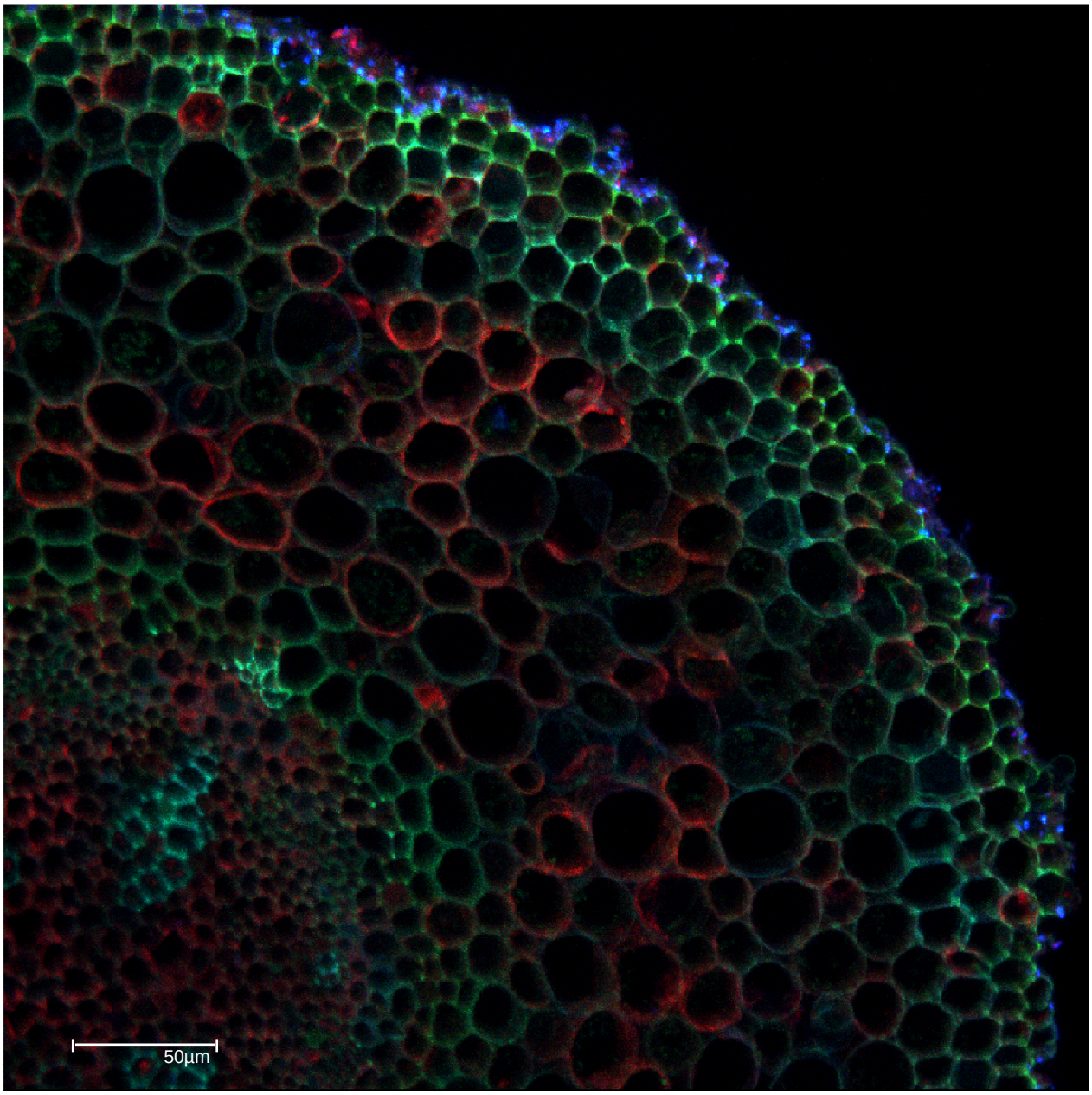

Figure 1: Confocal image of *F. sylvatica* root section, 6 hours post-inoculation with 5 × 10^5 zoospores of *P. plurivora*. Small blue dots, probably germinating zoospores of *P. plurivora* (blue), are mainly visible on the rhizodermis, whereas α-plurivorin (red) can already be observed close to the walls of the cortical cells and in the vascular cylinder at this early time point after infection. Bar represents 50 µm.

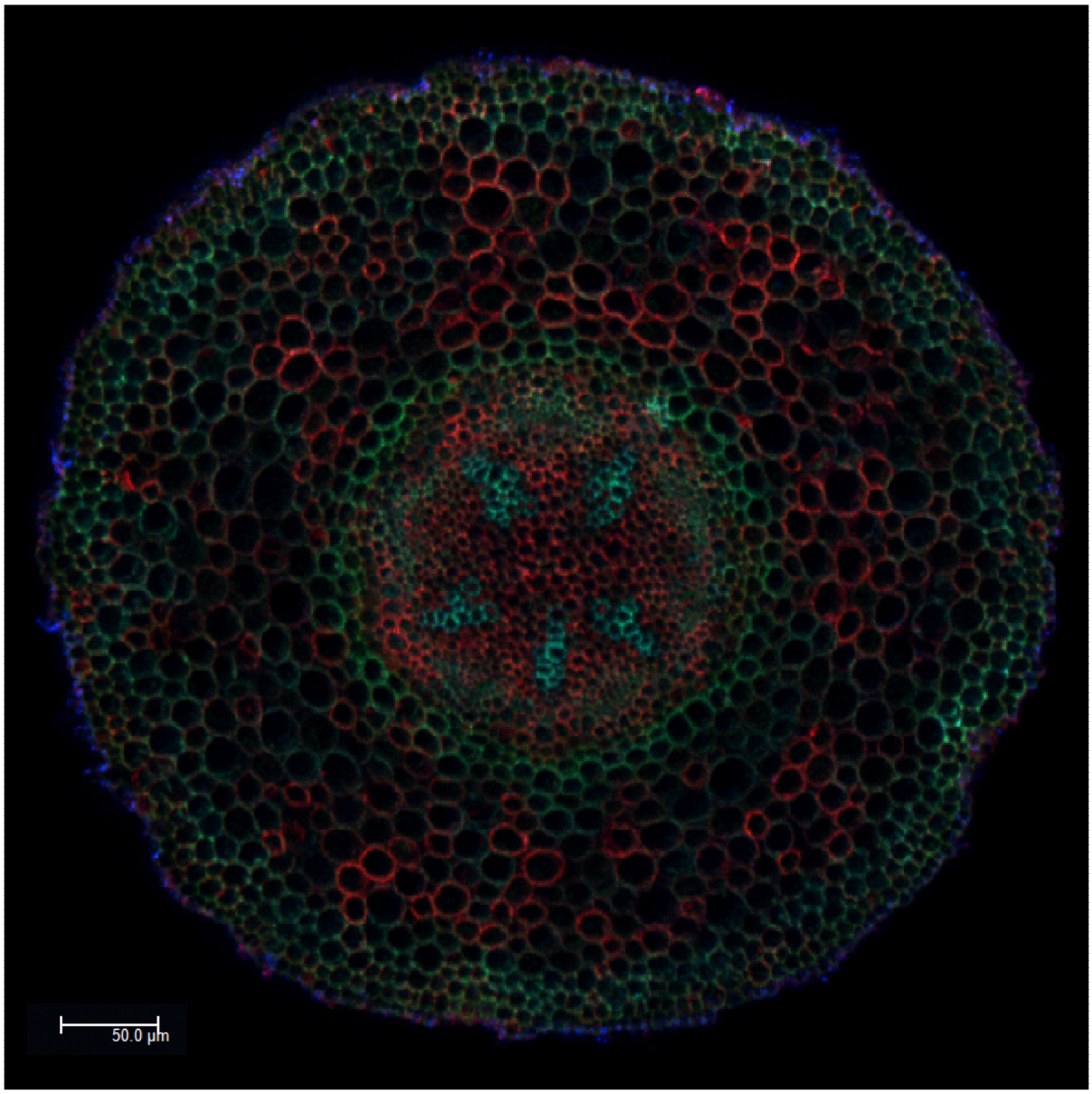

Figure 2: Lower magnification of Figure 1 (above), presenting an integral view of the entire root cross section at 6 hpi. At this early stage of infection, *P. plurivora* was just in the progress of colonising the cells of the rhizodermis (blue). However, large amounts of elicitin α-plurivorin (red) could already be observed distributed throughout the cortex, the vascular cylinder and the pith. Bar represents 50 µm.

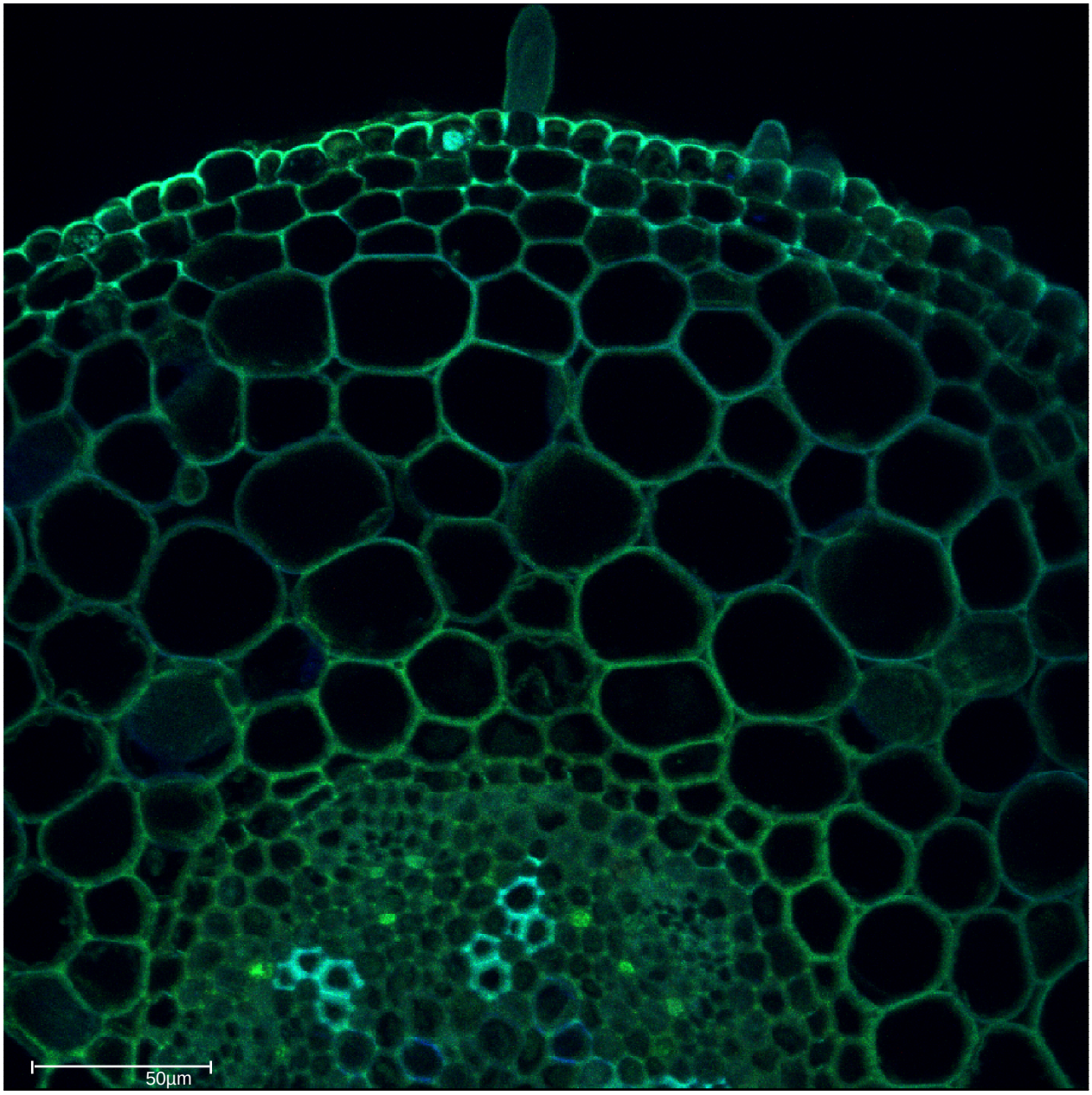

Figure 3: Confocal image of a cross section of an *F. sylvatica* root from a control plant. These seedlings were not infected and not treated with the anti-α-plurivorin antiserum. However, after cross sectioning, the tissue was incubated with the primary and secondary antibodies against *P. plurivora* and α-plurivorin. The image clearly shows that there was no unspecific binding for either of the antibodies. The green colour represents the natural auto-fluorescence of *F. sylvatica* roots. Bar represents 50 µm.

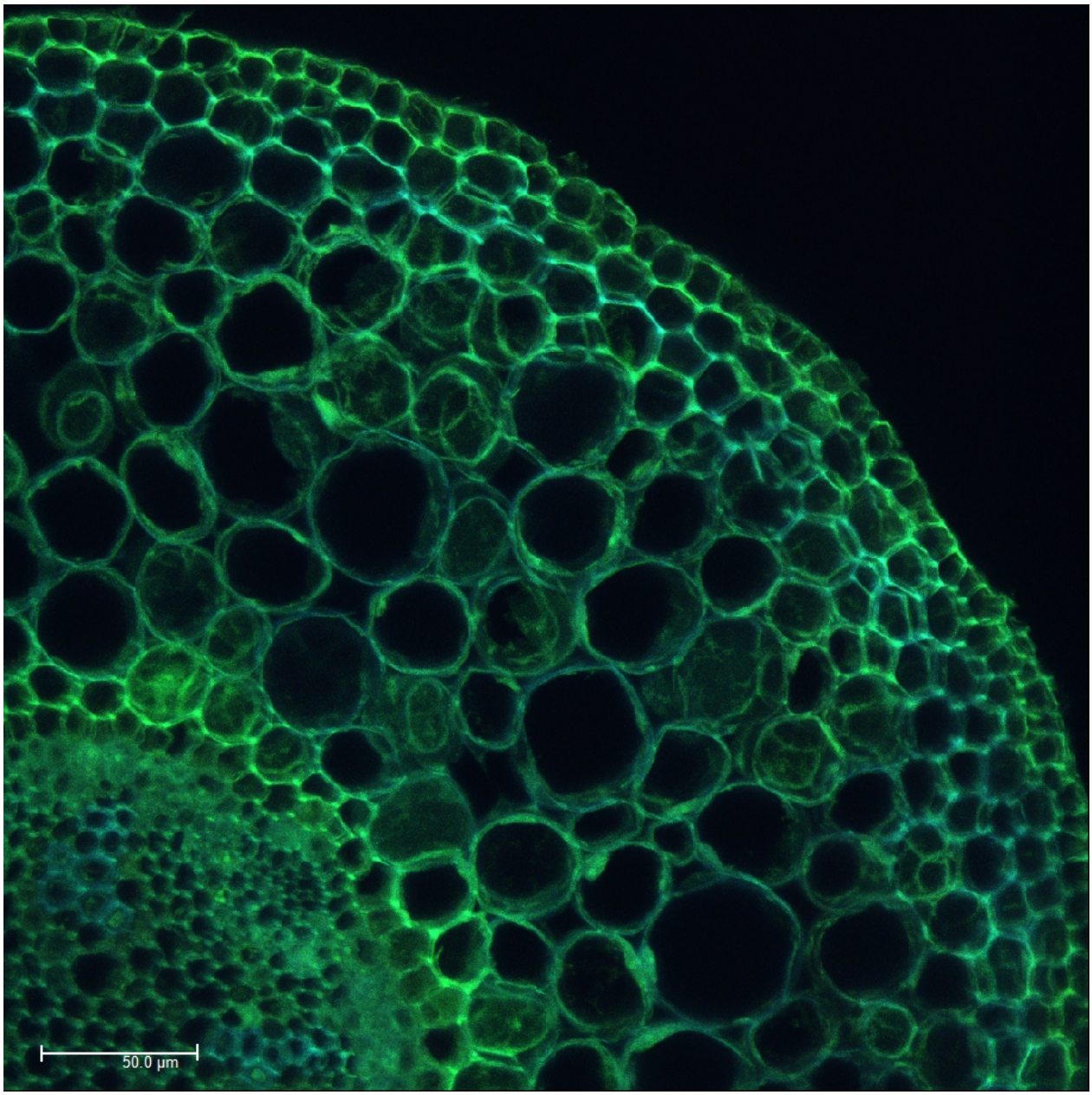

Figure 4: Confocal image of a cross section of an *F. sylvatica* root from a control plant. These seedlings were not infected but treated with the α-plurivorin antiserum. After the cross sectioning, the tissue was incubated with the primary and secondary antibodies against *P. plurivora* and α-plurivorin. The image clearly shows that there was no unspecific binding for the antibodies. Bar represents 50 µm.

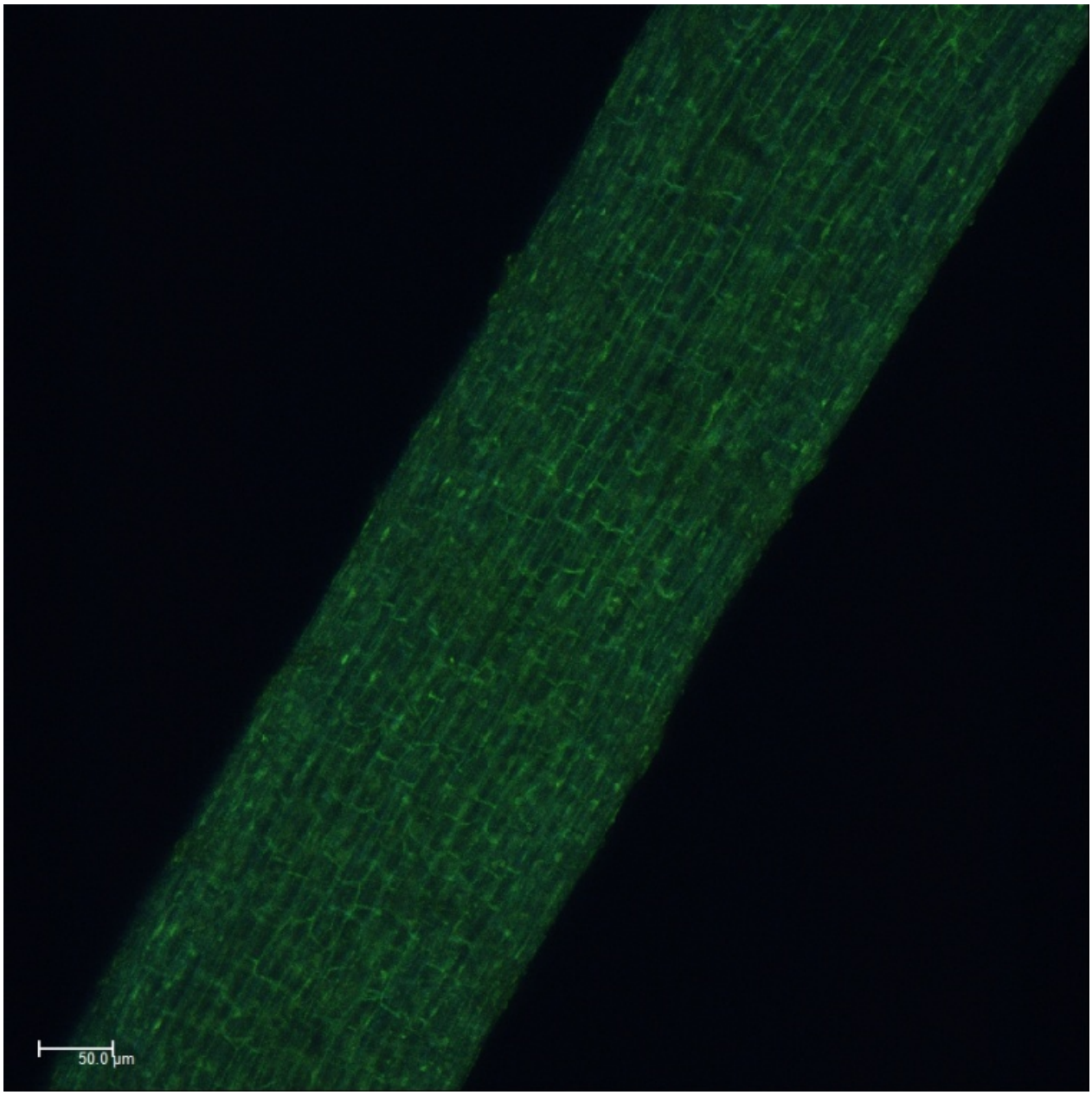

Figure 5: Confocal image of a root surface of beech seedling control plants. These seedlings were not infected or treated with the α-plurivorin antiserum. However, after harvesting, the tissue was incubated with the primary and secondary antibodies against *P. plurivora* and α-plurivorin. The image clearly shows that there was no unspecific binding for either of the antibodies. The green colour represents the natural auto-fluorescence of *F. sylvatica* roots. Bar represents 50 µm.

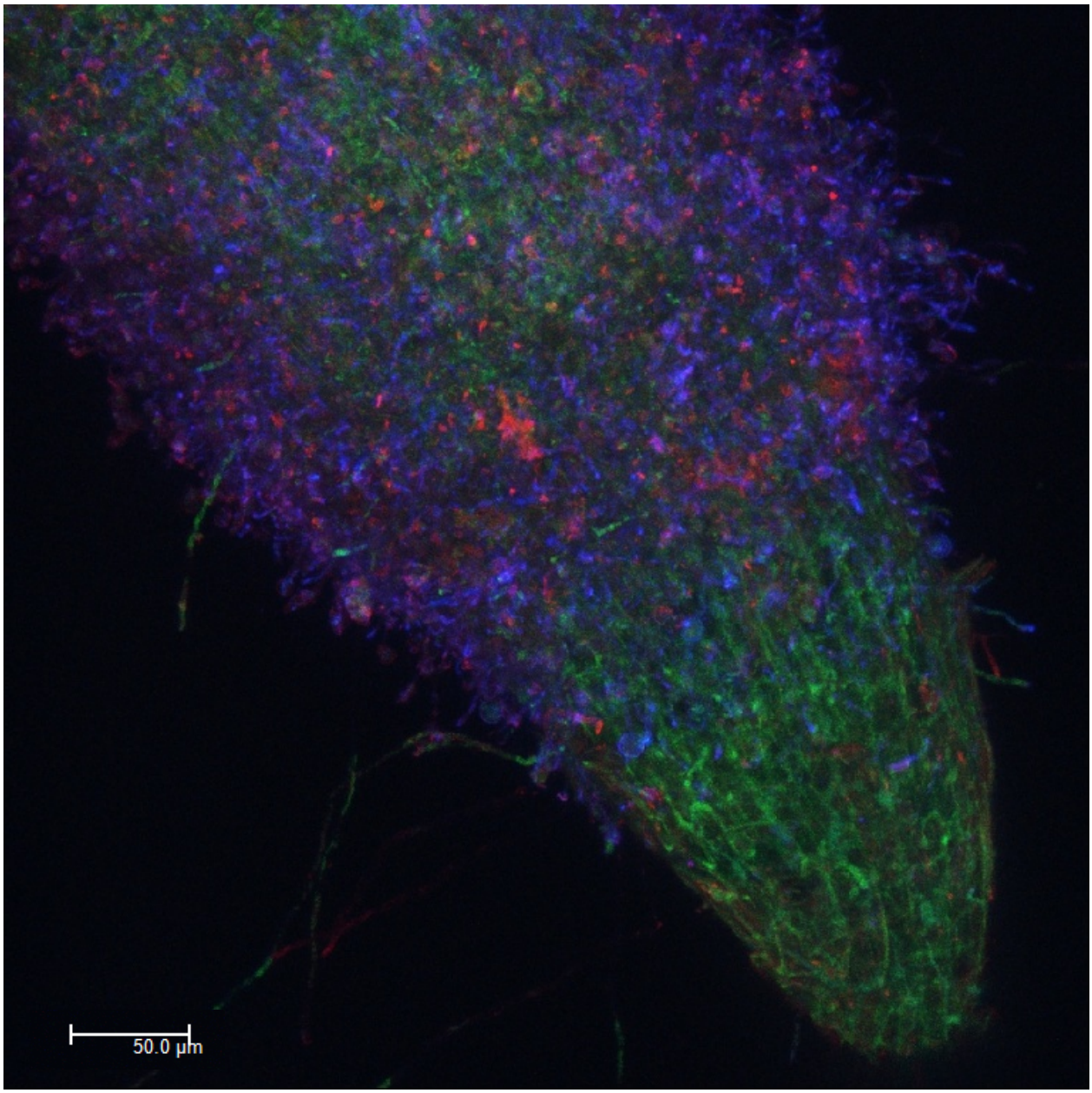

Figure 6: Confocal image of an *F. sylvatica* root tip from an infected seedling not treated with anti-α-plurivorin antiserum at 96 hpi. Massive mycelial growth (blue) was visible at the root tip close to the elongation zone in parallel with α-plurivorin production (red). Bar represents 50 µm.

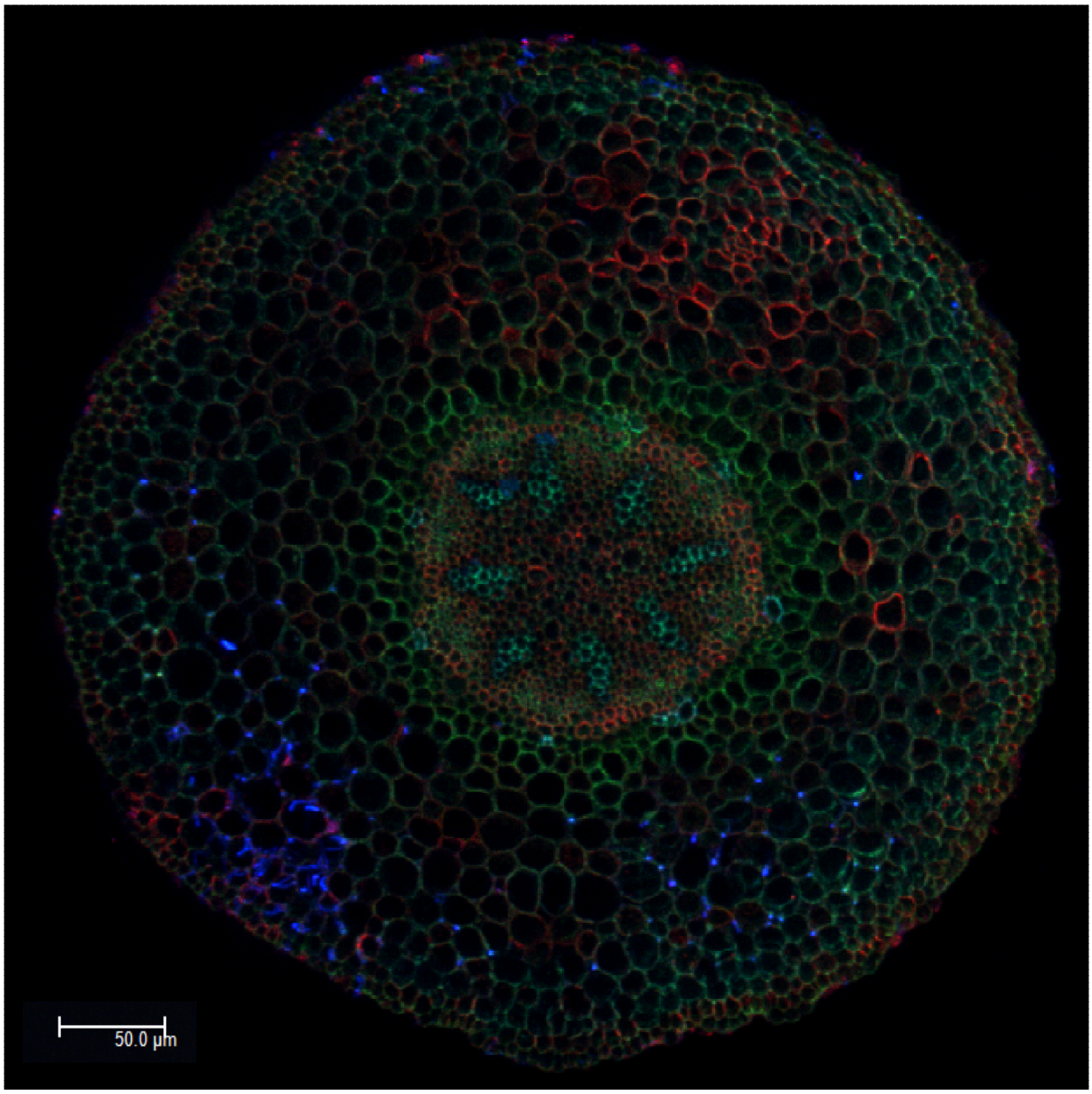

Figure 7: Confocal image of a cross section of an *F. sylvatica* root from an infected seedling not treated with the anti-α-plurivorin antiserum at 12 hpi. At this early stage of infection, *P. plurivora* (blue) was colonising the cortex tissue; however, α-plurivorin (red) could already be detected within the vascular cylinder. Bar represents 50 µm.

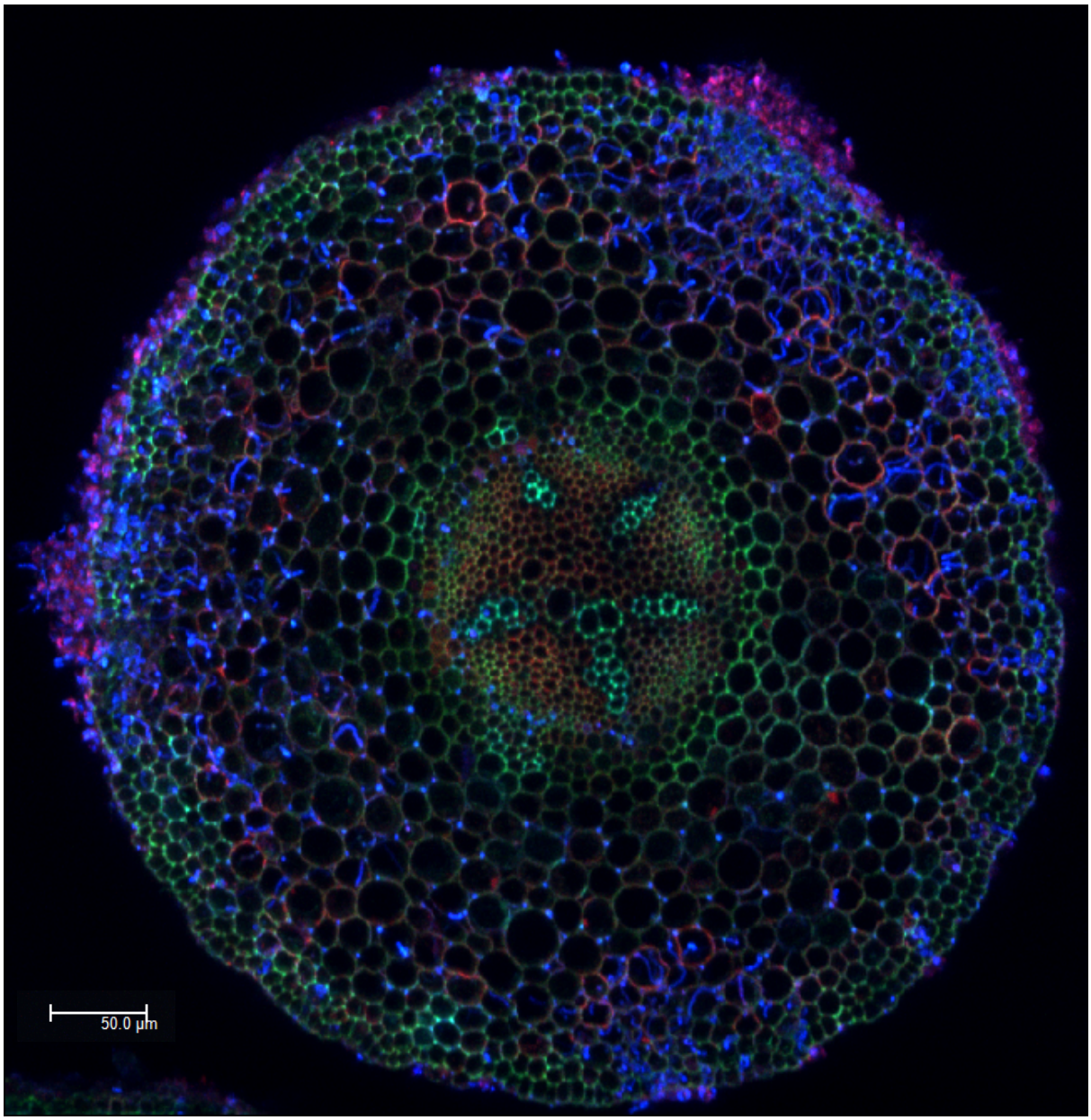

Figure 8: Confocal image of a cross section of an *F. sylvatica* root from an infected seedling not treated with the anti-α-plurivorin antiserum at 24 hpi. At this time point of infection, *P. plurivora* had almost completely colonised the cortex tissue and began to invade the central cylinder. Notably, the elicitin of *P. plurivora* is always adjacent to the mycelia when the pathogen is growing on the root surface. However, the opposite is true when *P. plurivora* penetrated and was growing inside the root tissue: α-plurivorin is mainly located next to the cell wall and is always far from the inter- and intracellular growing mycelia. Bar represents 50 µm.

Figure 9: Confocal image of a cross section of *F. sylvatica* root from the infected treatment and no immunoprecipitation of α-plurivorin at 96 hpi. At this stage of infection *P. plurivora* completely colonized the whole root, cell disrupting and damage in the cortex and central cylinder can be seen together with high amounts of α-plurivorin overall. At this point mortality of plants is already recorded. *P. plurivora* is immunolabelled with a blue fluorescence die. α-plurivorin is immunolabeled with a red fluorescence die. Bar represent 50 µm.

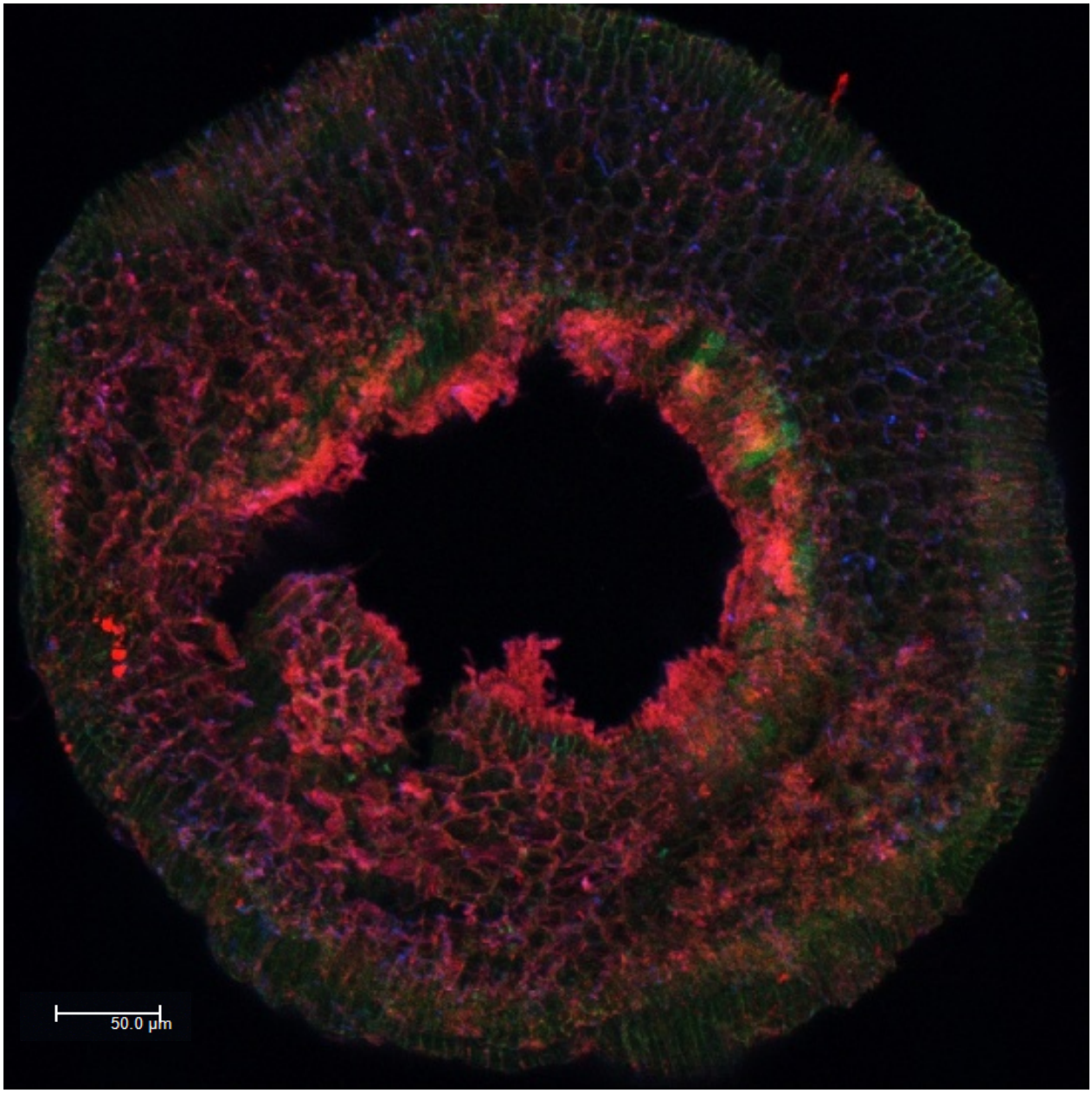

Figure 10: Confocal image of a cross section of an *F. sylvatica* root from an infected seedling not treated with the anti-α-plurivorin antiserum at 192 hpi. At the end of the experiment, *P. plurivora* almost completely destroyed the entire root. Severe damage was visible in the cortex and the central cylinder where high amounts of α-plurivorin (red) accumulated. At this time point, most of the plants had died and showed severe wilting. Bar represents 50 µm.

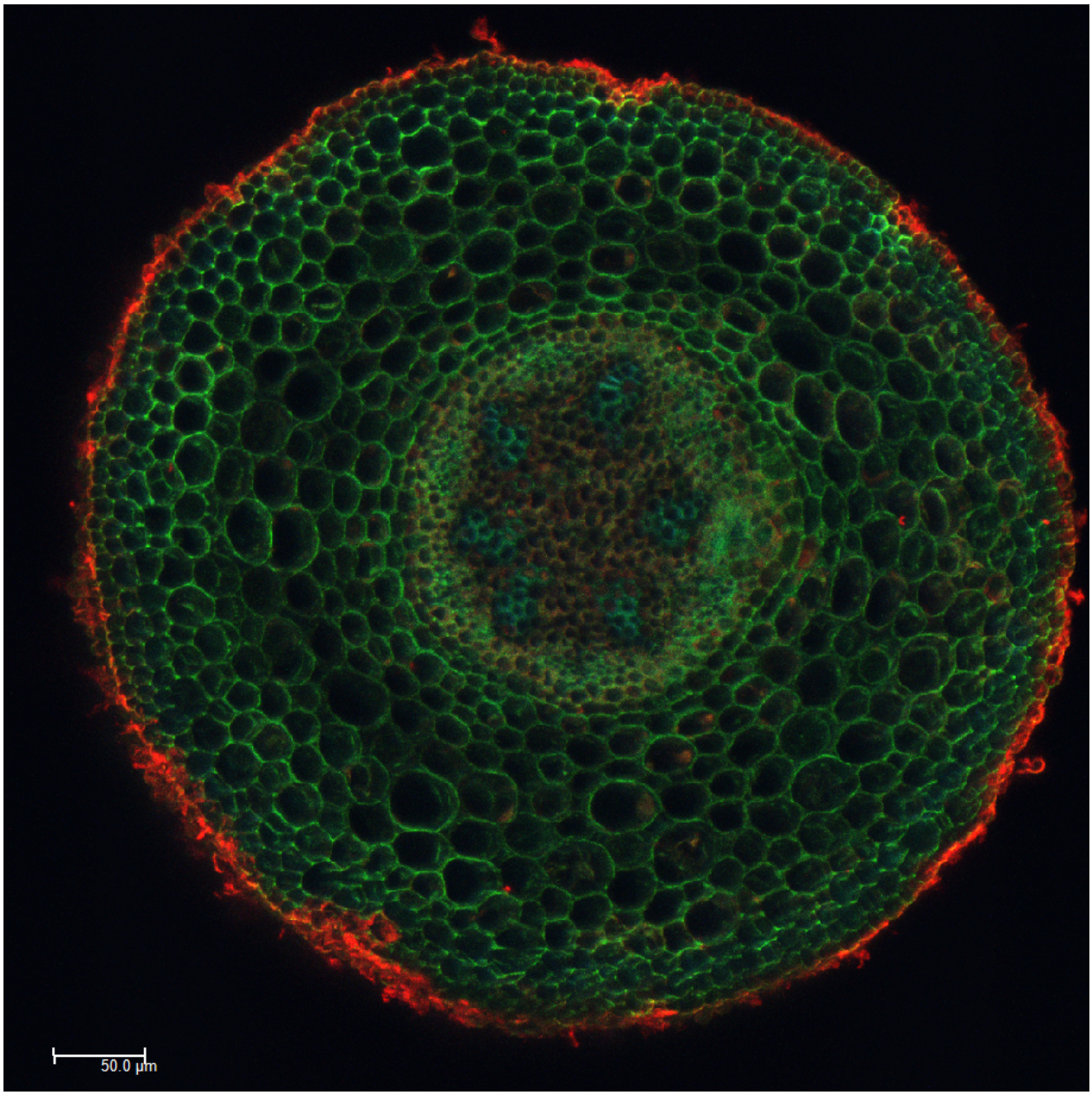

Figure 11: Confocal image of a cross section of an *F. sylvatica* root from an infected seedling treated with the anti-α-plurivorin antiserum at 12 hpi. Due to the immunoprecipitation of α-plurivorin, the effector protein could not enter the root tissue, therefore high amounts of α-plurivorin (red) accumulated mainly at the root surface. Bar represents 50 µm.

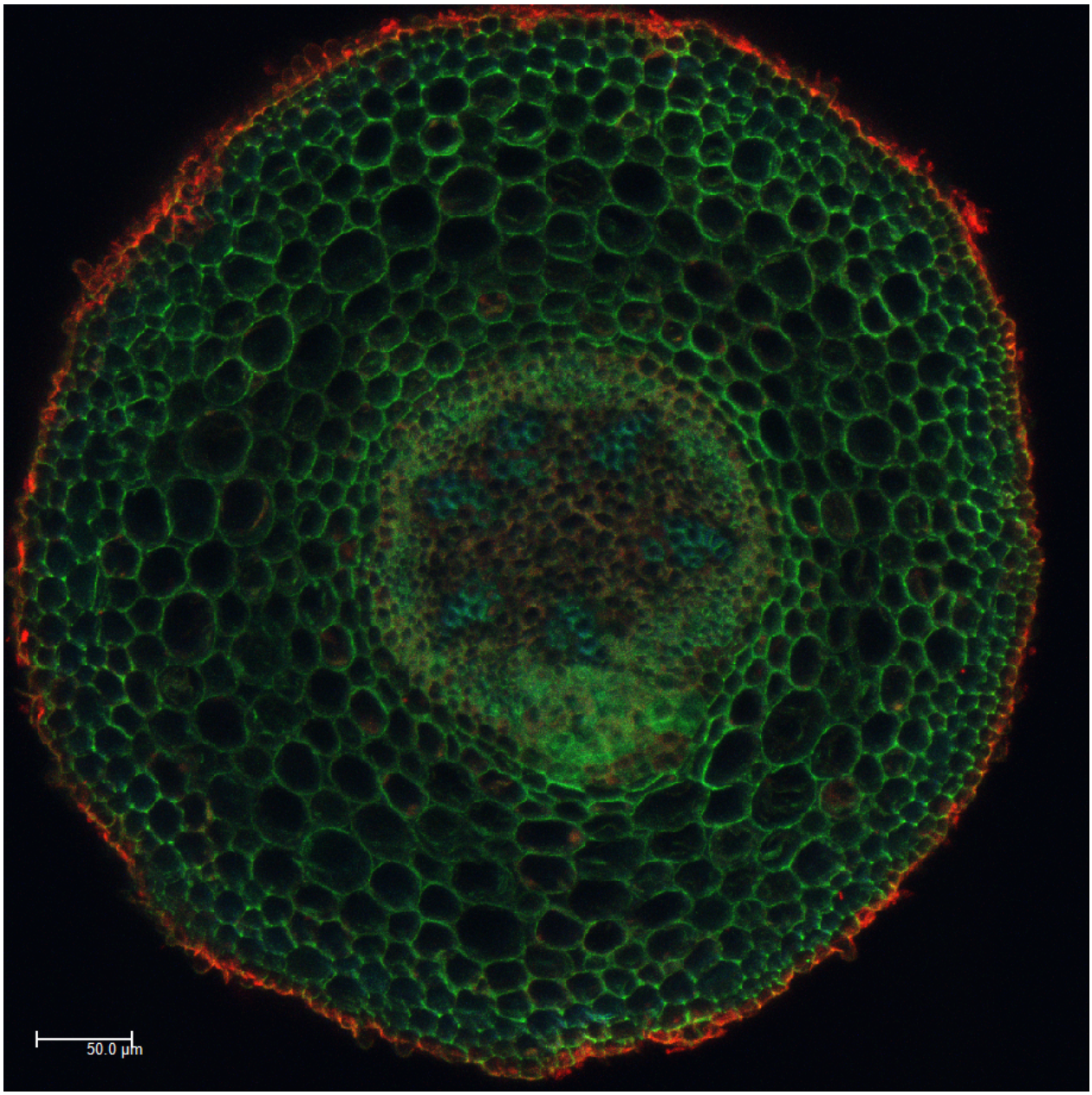

Figure 12: Confocal image of a cross section of an *F. sylvatica* root from an infected seedling treated with the anti-α-plurivorin antiserum at 24 hpi. At this time point, a similar situation was observed as shown in Supplementary Figure 17. Again α-plurivorin (red) accumulated mainly outside the root surface; no *P. plurivora* hyphae were found in the cortex or the vascular cylinder. Bar represents 50 µm.

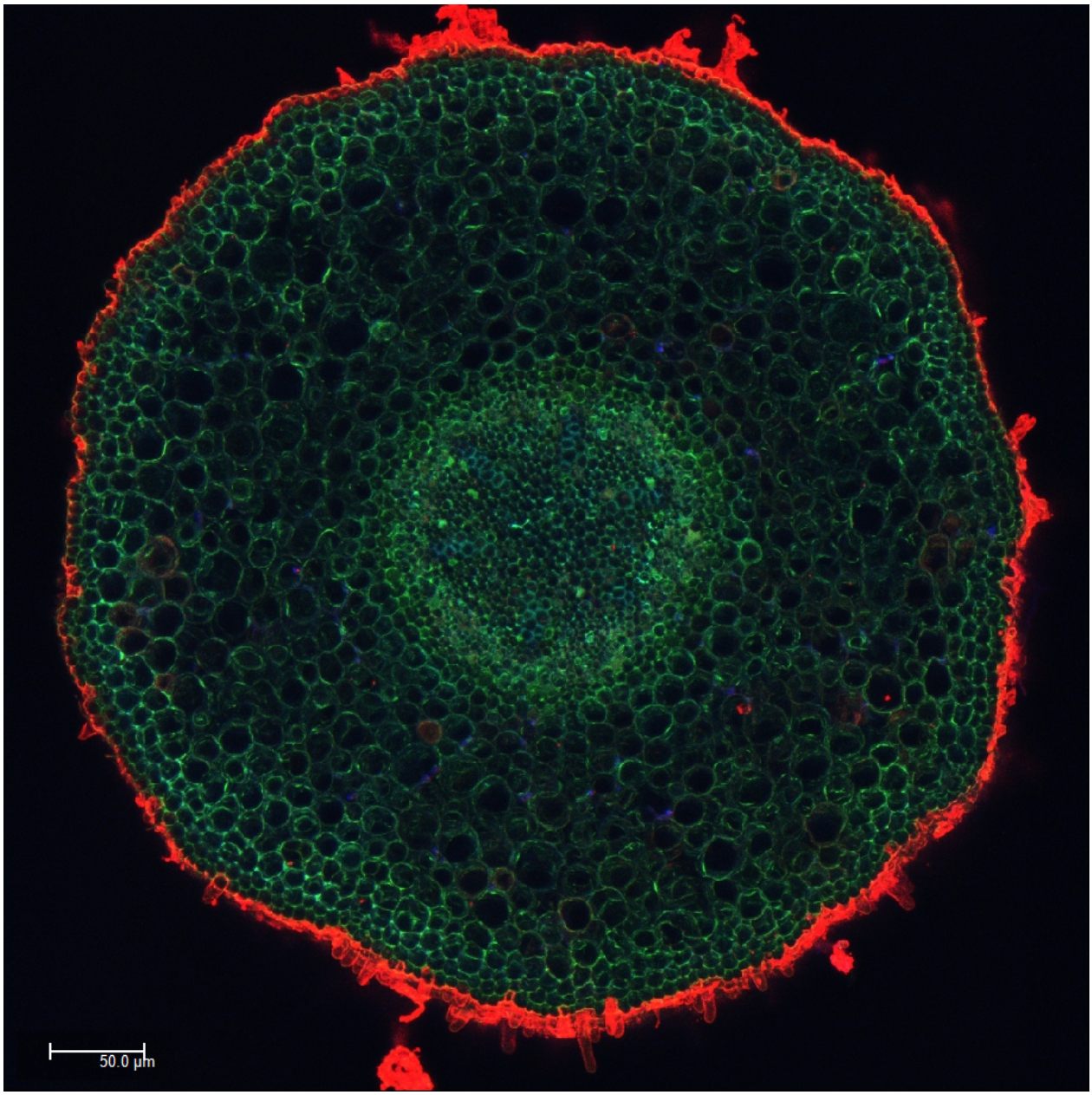

Figure 13: Confocal image of a cross section of an *F. sylvatica* root from an infected seedling treated with the anti-α-plurivorin antiserum at 96 hpi. At this time point, a similar situation was seen as shown in Supplementary Figures 17 and 18. Again, α-plurivorin (red) accumulated mainly on the root surface, and only a few hyphae were visible in the cortex (blue). However, no destruction of root tissue was found. Bar represents 50 µm.

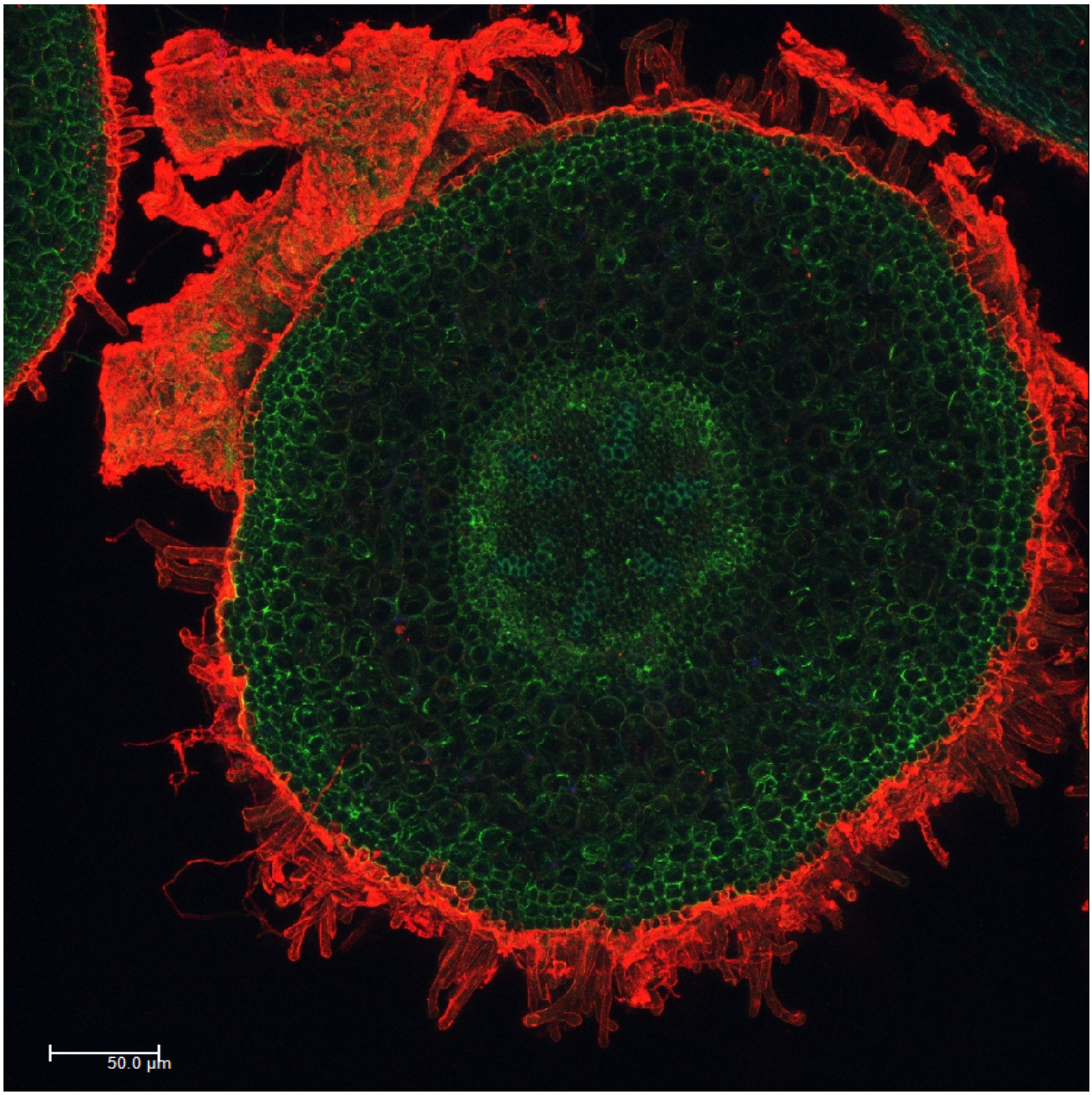

Figure 14: Confocal image of a cross section of an *F. sylvatica* root from an infected seedling treated with the α-plurivorin antiserum at 192 hpi. Again, α-plurivorin (red) accumulated in large amounts mainly on the root surface. No destruction of any root tissue was observed (compared with Supplementary Figure 16). All of these plants survived and did not show any disease symptoms. Bar represents 50 µm.

Table 1: oligonucleotides used within this study.

name	**primer**	**primer sequence 5' – 3'**
P. plurivora ITS1	P5	TCAACCCTTTTAGTTGGGGGTC
P. plurivora ITS1	P6	TTTAAAACAAAAAGCTACTAGCCCAGAC
P. plurivora ITS1	F3	FAM-CTTTTTTTGCGAGCCCTATCATGGCGA-TAMRA
P. pseudosyringae	FMPps1c	AGTTTCATTAGAAGATTATTTAC
	FMPps2c	AAAATTGTTTGATTTTATTAAGTATC
α-plurivorin	uni1	ATGAACTTCCGCGCTCTSYTYGC
	uni2	CGAGAAGCCGTTCGCGTA
Actin	Actin-Fw	AGAGATTCCGTTGCCCAGAA
	Actin-Rv	TGGATTCCAGCAGCTTCCA
PR1	PR1-Fw	CACTGTGATTGAGGGTGATG
	PR1-Rv	GCTCTTCAACACAGATCCTC
PR2	PR2-Fw	TCAAAGGGGGTACACCAAAG
	PR2-Rv	TCARCAGTGACATCCCATAGTC
PR3	PR3-Fw	GGTGGAAGATCGCATTGGGTTC
	PR3-Rv	CACAAGACTACAAGGTCAGGCATCC
PRP	PRP-Fw	GGTTTCAAGAGGAAAAAGTGCCAGT
	PRP-Rv	GCTTGCCATCCAGGTTTGTTC
ACO	ACO-Fw	CTGGTGGGATCATCTTACTC
	ACO-Rv	CAATAGAATGGCGCATAGGG
WRKY	WRKY-Fw	TTTCTCACTGGACACGCTGG
	WRKY-Rv	GATGGCTACCGTTGGAGGAA
Tubulin	Tubulin-Fw	TGAGTTGCTCAGGGTGGAAAA
	Tubulin-Rv	CGAGCCCACTGTCATCGAT
GADPH	GADPH-Fw	GATAGATTTGGAATTGTTGAGG
	GADPH-Rv	AAGCAATTCCAGCCTTGG

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