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MYCORRHIZA-INDUCED RESISTANCE AGAINST *THIELAVIOPSIS BASICOLA* IN THE ORNAMENTAL CROP *PETUNIA HYBRIDA*

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Table of contents

Abstract

Petunia hybrida is an ornamental crop of high economic interest but diverse root pathogens can cause high losses, especially in soilless greenhouse production systems, and their control by conventional methods implies an excessive use of pesticides. A more sustainable horticulture requires alternative methods to counter these chemical inputs. The introduction of arbuscular mycorrhiza (AM), known to reduce a number of root diseases in other plant species, into the production itinerary could form an integral part of an appropriate strategy. However, mycorrhizal effects against soil-borne pathogens are not always predictable and mechanisms behind the protective effects of mycorrhiza are largely unknown. In this context, mycorrhiza-induced resistance (MIR) was studied in *P. hybrida* in an inert soilless substrate, and the underlying mechanisms were investigated.

After testing different soil-borne pathogenic fungi causing disease in petunia nursery production, *Thielaviopsis basicola* was selected as a model pathosystem. Three AM fungal species were evaluated for their ability to protect petunia against *T. basicola*; only *Glomus mosseae* BEG 12 turned out to reduce disease symptoms and pathogen spread in roots. Split root experiments showed that this protective effect was systemic and could be induced in non-mycorrhizal parts of mycorrhizal root systems, in agreement with previous studies in other plant pathosystems. The AM fungus, moreover, reduced the amount of phosphate fertiliser input fivefold, and provides tolerance against high salt concentrations in the horticultural substrate.

In order to gain insight into molecular mechanisms involved in the MIR to *T. basicola* in petunia roots, hypotheses were tested by analysing the expression patterns of plant genes which are involved in various pathways of known plant defence responses. Nine genes related to the jasmonic acid pathway of induced systemic resistance (ISR) by plant growth promoting bacteria and three genes activated by salicylic acid, a key molecule in systemic acquired resistance (SAR), were selected. Expression profiles of these genes indicated that local MIR to *T. basicola* in petunia roots does not primarily involve either pathway, whilst systemic MIR in this pathosystem could include elements of both SAR and ISR.

The activation of seven AM-related genes was unaffected by *T. basicola* infection of mycorrhizal petunia roots showing that the pathogen does not affect symbiotic functionality. Results suggest that the part of the symbiotic cell programme covering AM-regulated plant defence genes may constitutively contribute to the expression of local MIR; the role of such genes in this phenomenon merits further attention and analyses.

Résumé

Petunia hybrida est une plante ornementale d'intérêt économique élevé, mais diverses agents pathogènes racinaires peuvent causer des pertes dramatiques en serres, surtout chez les plantes produites dans les substrats artificiels. Leur contrôle par des méthodes conventionnelles implique un usage excessif de pesticides. Une horticulture plus durable exige des méthodes alternatives pour réduire ces intrants chimiques. L'introduction des mycorhizes à arbuscules (MA), connue pour réduire certaines maladies racinaires chez d'autres espèces végétales, dans l'itinéraire de production pourrait constituer une partie intégrante d'une stratégie appropriée. Cependant, les effets mycorhiziens contre les pathogènes racinaires ne sont pas toujours prévisibles et les mécanismes qui régulent les effets protecteurs des mycorhizes sont largement inconnues. Dans ce contexte, la résistance induite par la mycorhize (RIM) a été étudiée chez *P. hybrida* dans un substrat horticole artificiel, et les mécanismes impliqués ont été recherchés.

Après avoir testé différents champignons racinaires provoquant des maladies lors des productions de pétunia en pépinière, *Thielaviopsis basicola* a été sélectionné pour le pathosystème modèle. Trois espèces fongiques MA ont été évaluées pour leur capacité à protéger le pétunia contre *T. basicola*; seul *Glomus mosseae* BEG 12 a réduit la propagation du pathogène dans les racines, ainsi que les symptômes de maladie. Des expériences basées sur un système « split-root » ont montré que cet effet protecteur est systémique et peut être induite dans les parties non-mycorhiziennes de systèmes racinaires mycorhizés, en accord avec des études d'autres pathosystèmes végétaux. Par ailleurs, l'activité du champignon MA réduit de cinq fois l'apport nécessaire en engrais phosphaté, mais améliore pas la tolérance du pétunia aux concentrations élevées du sel dans le substrat horticole.

Afin de mieux comprendre les mécanismes moléculaires à la base de la RIM vis-à-vis *T. basicola* chez le pétunia, diverses hypothèses ont été testées en analysant l'expression de gènes impliqués dans différentes voies de défense des plantes. Neuf gènes liés à la voie de signalisation de l'acide jasmonique, impliquée dans la résistance systémique induite (RSI) par des bactéries favorisant la croissance végétale, et trois gènes activés par l'acide salicylique, une molécule clé dans la résistance systémique acquise (RSA), ont été sélectionnés. Le profil d'expression de ces gènes indique que ces deux voies ne sont pas principalement impliquées dans la RIM locale contre le pathogène, tandis que la RIM systémique pourrait inclure des éléments de la RSA et de la RSI.

L'infection par *T. basicola* des racines mycorhizées de pétunia n'affecte pas l'activation de sept gènes liés à la MA, ce qui montre que l'agent pathogène n'influence pas la fonctionnalité symbiotique. Les résultats suggèrent que la partie du programme cellulaire symbiotique englobant les gènes de défense végétaux régulés par la MA pourraient constitutivement contribuer à l'expression de la RIM locale ; leur rôle dans ce phénomène mérite des études plus approfondies.

Zusammenfassung

Wurzelpathogene zeigen bedeutenden Einfluss auf die Produktion von Zierpflanzen. Vor Allem in erdelosen Produktionssystemen unter Glas verursachen sie erhebliche Verluste und ihre Bekämpfung mit konventionellen Mitteln beinhaltet normalerweise ein hoher Einsatz an Pestiziden. Ein mehr nachhaltiger Gartenbau braucht alternative Methoden, um den Eintrag dieser Chemikalien zu vermeiden. Die Einführung arbuskulärer Mykorrhizapilze (AM Pilze) in das Produktionssystem könnte ein integraler Bestandteil einer entsprechenden Strategie sein. Mykorrhizierte Pflanzen zeigen generell eine erhöhte Resistenz gegenüber bodenbürtigen Pathogenen und Nematoden. Der Erfolg einer solchen Strategie ist allerdings nicht immer vorhersagbar und die Mechanismen hinter den schützenden Effekten der Mykorrhiza sind weitgehend unbekannt.

Die Zierpflanze *Petunia hybrida*, die von verschiedenen Wurzelpathogenen befallen wird, wurde als Modell eingesetzt, um die Mykorrhiza-induzierte Resistenz (MIR) in erdelosen Substraten zu untersuchen. Nach der Überprüfung unterschiedlicher bodenbürtiger pathogener Pilze, die Schäden in der Anzucht verursachen, wurde *Thielaviopsis basicola* als Pathosystem ausgewählt. Drei AM Pilzisolate wurden bezüglich ihrer Fähigkeit untersucht, Petunien gegen *T. basicola* zu schützen. Nur das Isolat *Glomus mosseae* BEG 12 konnte sowohl Krankheitssymptome, wie auch die Ausbreitung des Pathogens in der Wurzel reduzieren. Experimente mit geteilten Wurzeln zeigten in Einklang mit früheren Ergebnissen einen systemisch schützenden Effekt, der auch in den nicht-mykorrhizierten Anteilen eines ansonsten mykorrhizierten Wurzelsystems induziert werden konnte. Der AM Pilz reduzierte darüber hinaus den Bedarf an Phosphatdüngung um das Fünffache. Eine erhöhte Toleranz gegenüber hohen Salzkonzentrationen im Substrat konnte allerdings nicht erreicht werden.

Um Erkenntnisse über die molekularen Mechanismen der MIR gegenüber *T. basicola* in Petunienwurzeln zu gewinnen, wurden durch Analyse von Expressionsmuster der bekannten Pflanzenverteidigung unterschiedliche Hypothesen überprüft. Neun Gene aus dem Jasmonatweg der durch pflanzenwachstumsfördernde Bakterien induzierten systemischen Resistenz (ISR) und drei durch Salizylsäure induzierte Gene der systemisch erworbenen Resistenz (SAR) wurden ausgewählt. Die Expressionsprofile dieser Gene deuteten darauf hin, dass die lokale MIR keinen der beiden Signalwege mit einbezieht, während die systemische MIR sowohl Elemente der ISR wie auch der SAR einbindet.

Die Aktivierung von sieben AM-regulierter Gene war von der *T. basicola* Infektion der Petunienwurzeln nicht betroffen, das Pathogen beeinträchtigt also nicht die symbiontischen Funktionen. Die Ergebnisse deuten außerdem darauf hin, dass der Teil des Symbioseprogramms, der AM-regulierte Verteidigungsgene betrifft, zur MIR beiträgt. Die Rolle dieser Gene bei dem Phänomen bedarf weiterer Untersuchungen.

Abbreviations

AM	Arbuscular mycorrhiza
ET	Ethylene
ETI	Effector-triggered immunity
hai	Hours after inoculation
HR	Hypersensitive response
ISR	Induced systemic resistance
JA	Jasmonic acid
K	Potassium
MAMPs	Microbes associated molecular patters
MIR	Mycorrhiza-induced resistance
N	Nitrogen
P	Phosphate
PAM	Peri-arbuscular membrane
PAMPs	Pathogen associated molecular patterns
PGPF	Plant growth promoting fungi
PGPR	Plant growth promoting rhizobacteria
PTI	PAMP-triggered immunity
PR	Pathogenesis-related proteins
PRRs	Patterns recognition receptors
SA	Salicylic acid
SAR	Systemic acquired resistance
wai	Week after inoculation

1 -

General Introduction

1.1- Introduction

Soil is not only composed of abiotic solid, liquid and gaseous phases but is also characterized by biotic components interacting with each other and forming diverse soil communities. These biotic components are bacteria and fungi, viruses and animal species e.g. worms, protozoas and nematodes. The part of soil directly under the influence of plants, the so-called rhizosphere, represents an important area of interactions among biotic components, and between biotic and abiotic factors.

During evolution, plants have formed different beneficial interactions with a number of soil microorganisms living in the rhizosphere. Such microorganisms support particular needs of plants concerning the uptake of nutrients, the adaptation to harsh abiotic conditions and the protection against pathogenic biotic factors. Beside casual interactions (protocooperation) with some plant growth promoting rhizobacteria and endophytic fungi (Jumpponen and Trappe, 1998; Vessey, 2003), two mutualistic associations with mycorrhizal fungi or nodulating rhizobial bacteria are particularly important for plant nutrition and health (Hayat et al., 2010; Smith and Read, 2008).

Fossil data and molecular phylogeny suggest the presence of arbuscular mycorrhizal fungi in roots since at least 460 million years (Rémy et al., 1994, Redecker et al. 2000), while root nodulation evolved approximately 100 million years ago to meet special nitrogen needs of legumes (Gianinazzi-Pearson and Denarié, 1997) (Fig. 1).

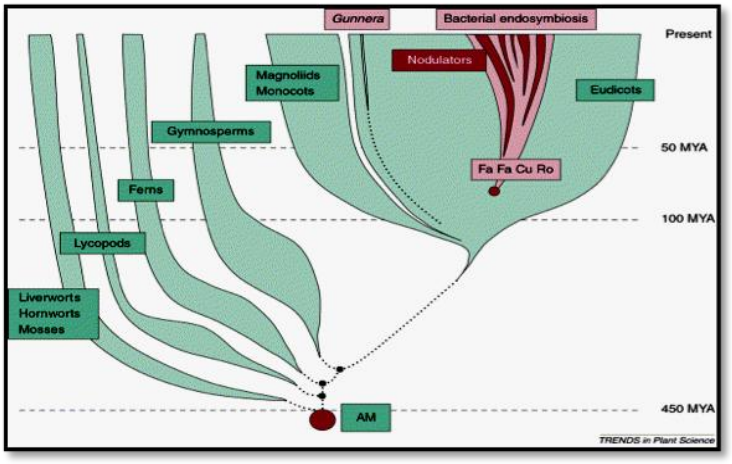


Figure 1. Evolution of plant root mutualistic interactions (from Kistner and Parniske, 2002).

Unfortunately, numerous past and present human activities, especially during the 'Green Revolution', have affected the balance of these beneficial interactions by permanently changing soil conditions such as pH, concentrations of essential or toxic elements, water and mineral nutrient capacity, or contamination with organic compounds (Dudal et al., 2002; http://www.fao.org/ag/agl/agll/wrb/doc/0093.pdf). For this and other reasons, world concern about excessive use of chemical fertilizers and pesticides on crops is greatly increasing. Public opinion and political representatives have recognized that side effects are harmful and represent real risks for soil biodiversity, and in turn for future availability of food and feed (Gianinazzi et al., 2010). Searches for alternative methods representing a promising way to counter these chemical inputs could lead to a more sustainable production of crops. An integral part of such methods will be the management of beneficial interactions of plants with microorganisms. Due to their ubiquity, mycorrhizas are of particular interest for use in sustainable plant production systems.

More than 90% of all known terrestrial plant families form mycorrhizas (Wang and Qui, 2006), a term first used by A.B. Frank in 1885 and originating from the Greek words *mükes*, meaning 'fungus', and *rhiza*, meaning 'root'. Mycorrhizas are mutualistic symbioses where the plant provides carbohydrates to the fungus, and in return is protected by the presence of the fungus against biotic and abiotic stresses (Smith and Read, 2008). Different types of mycorrhiza are distinguished: ectendomycorrhiza, orchid, ericoid, arbutoid, monotropoid and the two main types, arbuscular mycorrhiza (AM) and ectomycorrhiza. While most types are more or less restricted to particular groups of plants, AM are found associated with most crops, many tree species, and numerous vegetable and ornamental plants (Newmann and Reddell, 1987). The AM symbiosis is therefore of high interest for agriculture and horticulture.

The AM symbiosis is mainly characterized by the delivery of mineral nutrients, and in particular phosphate, by the fungus to the plant. In addition to this nutritional benefit, a bioprotective effect accompanying the establishment of the symbiosis has been reported since over 30 years (Dehne and Schönbeck 1975; Rosendahl, 1985). The interest of plant producers for the potential role that AM fungi could play in the control of plant diseases has increased over the last years (Whipps,

2004). However, the mechanisms involved in mycorrhizal protection against plant pathogens are still poorly understood (Pozo and Azcòn-Aguilar, 2007). Investigations of this phenomenon are made complex because of different parameters: i) the model system is a combination of interactions between three different partners (plant, AM fungus, and pathogen), ii) the growth conditions should be favourable for each partner, and iii) the partners' identity defines the specificity of the system. Therefore, understanding each interaction (plant/pathogen, root/ mycorrhizal fungus) independently is a prerequisite for improving knowledge in this research area and for identifying the processes behind the bioprotective effects of the mycorrhizal symbiosis.

1.2- Plant/pathogen interactions

In nature, most higher plants are fixed by their roots in soil and they are not able to escape any biotic or abiotic stress conditions that may occur. Plant-microbe interactions cover not only beneficial but also pathogenic associations where the microorganisms involved can be fungi, bacteria or viruses, able to attack shoot or root parts of the plant. Plant pathogens are broadly divided into biotrophic (require living host tissues to complete their life cycle) and necrotrophic (kill the host and feed on released compounds). Model plants such as *Arabidopsis thaliana* have been used in particular to formulate hypotheses concerning contrasting mechanisms of defence against biotrophic and necrotrophic pathogens (Oliver and Ipcho, 2004; Glazebrook, 2005). A class of hemibiotrophic pathogens has also been defined by Perfect and Green (2001) which are characterized by an initial biotrophic period followed by necrotrophy.

In addition to preformed barriers contributing to constitutive resistance, plants have evolved sophisticated mechanisms of induced resistance to translate the recognition of pathogens into an adaptive defence response (Dangl and Jones, 2001).

1.2.1- Plant defence

Despite their presence in aggressive surroundings, plants are in general resistant to most species of potential microbial invaders due to preformed physical or chemical barriers (Walters, 2011). This kind of immunity is able to completely

prevent any pathogen penetration (Thordal-Christensen, 2003). However, when a pathogen is able to overcome such passive resistance and propagate in plant tissues, either disease develops (susceptibility) or plant responses (cell wall appositions, phytoalexins, antifungal proteins…) are induced to reduce pathogen proliferation and disease symptom development (Nümberger et al., 2004). The latter phenomenon, based on non-self recognition by the host plant, requires pathogen signals/molecules that can trigger plant defencse responses (Walters, 2011).

1.2.2- Pathogen recognition: general elicitors

The signalling molecules produced by pathogens which plants are able to recognize and respond to are known as "elicitors". Elicitors rapidly activate a range of plant defence responses that can be either sufficient to stop pathogen spread (incompatible interaction) or insufficient leading to disease (compatible interaction) (Nümberger et al., 2004). Identification of elicitors has unveiled similarities in the molecular basis of immunity in plants with that known for insects and animals (Paré et al., 2005). The first characterized microbial elicitors were predominantly oligosaccharides but later many other compounds were identified such as flagellin or cold-shock protein produced by bacteria, and necrosis-inducing proteins, transglutaminase, elicitins or β-glucans produced by fungi. Altogether, they were called PAMPs for "pathogen associated molecular patterns" (see Nümberger et al., 2004). However, not only pathogenic microorganisms possess these patterns and therefore a broader term was introduced, MAMPs, that substitutes the word pathogen by microbe (Ausubel, 2005). In addition to these exogenous elicitors produced by microbes, plant endogeneous elicitors of defence responses that are generated as a result of physical and/or chemical cleavage of the plant cell wall have also been identified since a long while (Hahn et al., 1981).

1.2.3- Defence responses in shoots and roots

In leaves, PAMP recognition via pattern recognition receptors (PRRs) activates a basal resistance, called PAMP-triggered immunity (PTI), which is translated by different plant responses such as oxidative burst and/or nitric oxide production, the biosynthesis of particular phytohormones like salicylic acid, jasmonate or ethylene, as well as a complex cascade of calcium dependent and

mitogen-activated protein kinases that leads to the activation of transcription factors and in turn of defence response genes (Nümberger et al., 2004). Faced with this plant immunity, pathogens have co-evolved a strategy in which they secrete small effector molecules into the host cell to suppress PTI and establish a compatible interaction. In turn, plants have developed another recognition system, based on 'R' proteins, to detect these pathogen effectors and induce a secondary immune response known as effector-triggered immunity (ETI) (Pieterse et al., 2009). ETI activates a signalling pathway which leads to programmed hypersensitive cell death in order to restrict pathogen invasion and therefore prevent intact tissues from further damage (De Wit, 1998).

Although, there have been major advances in the understanding of host shoot-pathogen interactions, relatively little is known about PAMP-mediated responses in roots (Millet et al., 2010). Root pathogens play an important economical role; monetary losses annually in the US due to soil-borne pathogens of vegetables, fruits or field crops have been estimated at 4 billion US $ (Lumbsden et al., 1995).

Pathogens do not necessarily discriminate between different plant organs, and shoots as well as roots can be targets of the same pathogenic strain. It has been suggested that root pathogens induce no or only weak responses in order to reduce plant fitness costs. Studies focussing on *A. thaliana* to compare leaf and root responses to different PAMPS or MAMPs have pointed to the presence of orchestrated and tissue-specific plant, as well as potential pathogen-encoded, mechanisms to block elicited signalling pathways in roots (Millet et al., 2010). However, further studies are needed to better understand plant defence in roots against biotrophic or necrotrophic pathogens and how MAMP and/or effector signalling pathways are involved in compatible interactions with beneficial microbes.

1.2.4- Phytohormones and induced resistance in plants

Phytohormones are plant chemical messengers that play an important role in growth and development processes and all are known to be also involved in plant responses against biotic stresses (Bari and Jones, 2009). Those considered to play major roles in defence responses include salicylic acid (SA), ethylene (ET) and jasmonic acid (JA). Attack by diverse pathogens results in changes in the level of these phytohormones and thereafter in the expression of defence related genes (Adie et al., 2007; Robert–Seilaniantz et al., 2007). The types of phytohormones implicated in signalling pathways leading to defence gene regulation appear however to depend on whether the pathogen concerned is biotrophic or necrotrophic (Bari and Jones, 2009) (Fig. 2).

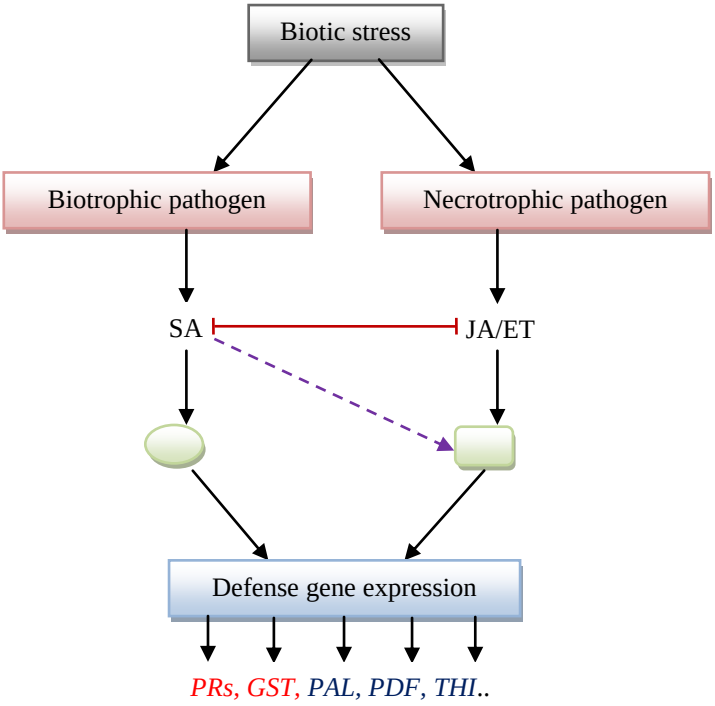


Figure 2. Defence gene responses following biotrophic or necrotrophic pathogen attack and the main hormonal pathways of salicylic acid (SA) (in red), jasmonic acid (JA) or ethylene (ET) (in blue) involved in signaling leading to the expression of genes encoding proteins like PRs (pathogen-related proteins), GST (glutathione-S-transferase), PAL (phenylalanine ammonia lyase), PDF (plant defensin) or THI (thionine).

In an incompatible interaction between a plant and a leaf pathogen, infection triggers rapid and localized responses in and around infected host cells (Walters and Boyle, 2001). These responses include e.g. an oxidative burst, cell wall reinforcements, papilla formation and phytoalexin synthesis. One important process is the so-called hypersensitive response (HR), which results in local resistance and stops further pathogen development. In addition to this localized resistance, defence responses to further pathogen attack can develop systemically in neighbouring non-infected tissues and in distal parts of the plant, leading to a phenomenon known as systemic acquired resistance (SAR) (Heil and Bostok, 2002) (Fig. 3).

SAR, which was initially described by Ray (1901) and Beauvene (1901) working on *Botrytis*, can be induced by fungal, bacterial or viral pathogens (Agrios, 1997). The establishment of SAR is accompanied by an increase in endogenous levels of the phytohormone SA, which may act as a mobile signal locally and systemically (Durrant and Dong, 2004), and by the accumulation of SA-induced pathogenesis-related proteins (PRs) (van Loon and van Kammen, 1970; Gianinazzi et al., 1970, van Loon et al., 2006) and activation of many defence-related genes (Cameron et al., 1999; Kohlr et al., 2002), although the role of other phytohormones such as JA or ET cannot be excluded (Truman et al., 2007; Verberne et al., 2003).

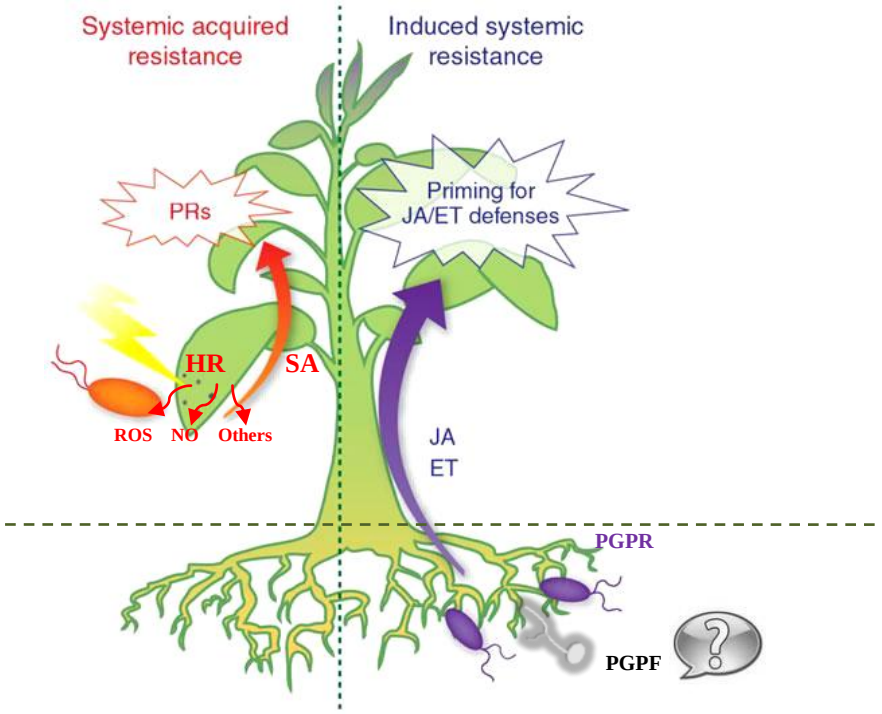


Figure 3. Comparison between two different types of induced resistance in plants. Systemic acquired resistance (SAR) activated by a leaf pathogen induces a local hypersensitive response (HR) in infected leaves. This is followed by a mobile signal, related to a salicylic acid (SA) pathway that travels through the vascular system to enhance pathogenesis-related protein (PRs) gene expression in distal tissues. Induced systemic resistance (ISR) is activated by root colonization with plant growth promoting rhizobacteria or fungi (PGPR and PGPF). This induced resistance is effective locally (in roots) and systemically via a mobile signal dependent on two phytohormones, jasmonic acid (JA) and/or ethylene (ET), that may be transported through the plant to activate defence genes in above-ground plant parts. ISR is not characterized by PR production like in SAR (adapted from Pieterse et al., 2009).

In *A. thaliana*, two proteins have been identified to play a role in the induction of SAR: NPR1 (non-expressor of PR1 gene), a SA-mediated protein regulator of defence gene expression, and EDR (Enhanced disease resistance 1), a putative mitogen-activated protein kinase (MAPK) that functions as a negative regulator of SAR induction (Conrath et al., 2006).

Non-pathogenic organisms also have the potential to activate resistance mechanisms in plants. Infection of aerial tissues by some avirulent fungal or viral strains, for example, can provoke HR and SAR whilst root colonization by non-pathogenic growth promoting bacteria or fungi (PGPR, PGPF) can lead to an analogous protective phenomenon known as induced systemic resistance (ISR) (Wei et al., 1991; van Loon, 2007). ISR is a widespread phenomenon that has been reported

to reduce disease in several plant/pathogen systems (Leeman et al., 1995; Benhamou et al., 1998; Maurhofer et al., 1998). It has been intensively investigated for its potential use in plant protection particularly against foliar diseases (van Loon et al., 1998). Contrary to SAR, ISR is SA independent and is considered to depend on the phytohormones JA and ET (Kloepper et al., 2004) (Fig. 2). Most studies investigating ISR have found that the mobilization of a signal from bacteria colonized roots toward leaves is not mediated by an SA pathway and is not associated with PR protein expression (Paré et al., 2005). However, the identity of the mobile signal involved is still vague.

The first evidence of plant defence responses during ISR was a faster rise in phytoalexin levels in ISR-expressing carnation with reduced susceptibility to *Fusarium* wilt (Peer, 1991), followed by studies reporting the accumulation of other defence compounds such as callose and phenolics. Later, a transcriptome analysis of leaves of PGPR-inoculated *A. thaliana* plants revealed the enhanced expression of 81 genes predicted to be regulated by either JA or ET or by both phytohormones (van Wees et al., 1999; Hase et al., 2003; Verhagen et al., 2004). Also, plants mutated for signalling pathways related to JA or ET pointed to their role in activating ISR (Kloepper et al., 2004). However, no alteration in levels of either phytohormone has been observed during ISR, suggesting an enhancement in the plant sensitivity to them rather that an increase in their production (Pieterse et al., 2000).

Interestingly, the regulator molecule in SAR, NPR1, was also found necessary for a successful establishment of ISR (Pieterse et al., 1998), suggesting that NPR1 could be modulated by both SA or JA/ET signalling pathways where the functionality may vary from inducing PR gene expression in SAR to modulating different defence compound gene expression in ISR (Pieterse and van Loon, 2004; Dong, 2004). Although PGPR-mediated ISR via phytohormones is a common feature, it was demonstrated that some non-pathogenic bacteria induce production of volatiles such as C4 carbon compounds that can also trigger plant defence responses (Ryu et al., 2004; Paré et al., 2005).

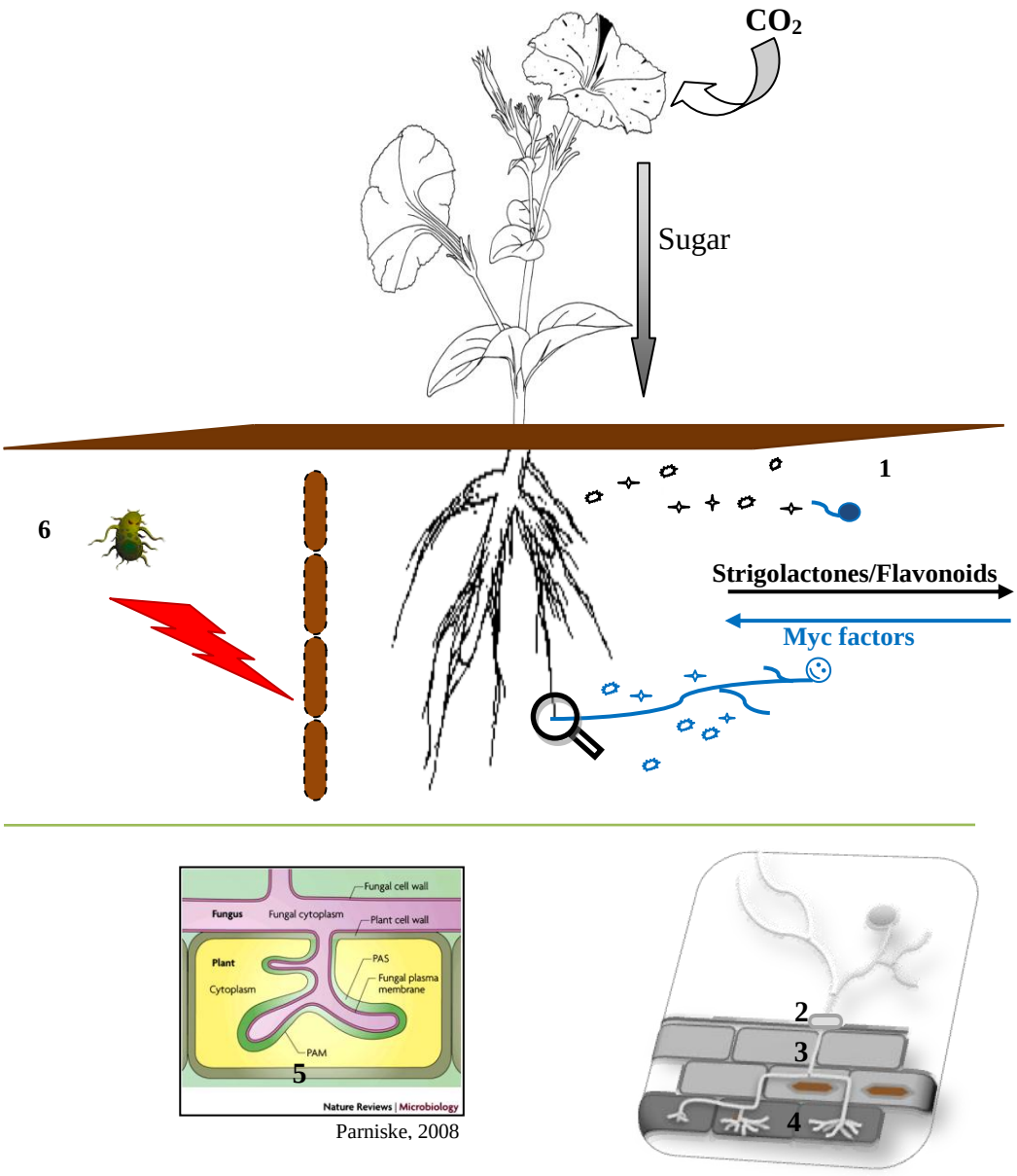
1.3– Arbuscular mycorrhiza

At least 80% of terrestrial plant families form AM symbioses (Newman and Reddell, 1987; Wang and Qui, 2006). AM fungi make up the phylum Glomeromycota where they were originally subdivided into four orders (*Glomerales*, *Archaeosporales*, *Paraglomerales* and *Diversisporales*), and between 150 and 200 species are described (Schüssler et al., 2001). More recently, a new restructuration of species within these orders was performed by Schüssler and Walker (2010). They are obligate biotrophs that can persist in soil as spores. After germination their germ tubes exhibit only limited growth and they must colonize root tissues of a host plant for reproduction and long-term survival (Sekhara Reddy et al., 2009).

1.3.1– AM development

1.3.1.1- Presymbiosis

The dialogue between an AM fungus and plant roots begins before any physical contact. After spore germination (asymbiotic stage), which does not need any plant factor, the fungus responds to the presence of host plant roots by an intense branching of hyphae (presymbiotic phase). This phenomenon is not observed in the presence of non-host roots, which suggests that the AM fungus perceives a signal released by a host plant (Giovannetti et al., 1993). Plant signals in root exudates activate fungal gene expression and respiration (Tamasloukht et al., 2003, 2007). Plant-derived flavonoids seem to play a role (Gianinazzi-Pearson et al., 1989), but recently a new plant hormone, strigolactone, was identified in the plant root exudates, which is suspected to be a component of signalling to the fungus to induce hyphal respiration and branching (Akiyama et al., 2005; Besserer et al., 2006) (Fig. 4.1).



Parniske, 2008

Figure 4. Developmental stages of AM. The presence of AM fungi near host plant roots leads to an exchange of signals between the two symbionts. This is the presymbiotic phase (1). In contact with the epidermal surface, the fungal hyphae form a structure similar to an appressorium called "hyphopodium" (2). The next step is inter-or intracellular penetration (3) of root tissues until they reach the inner cortical cell layer. Here, intracellular fungal hyphae branch and form "the arbuscule" structure in the host cell by invaginating the cell membrane (4). This structure constitutes the active site for nutrient and probably also carbohydrate exchange between plant and fungus across the periarbuscular space (PAS) formed between the hyphal membrane and the plant periarbuscular membrane (PAM) (5). In addition to nutrient uptake, root colonization by AM fungi can lead to plant protection against a wide range of root pathogens (6).

In turn, the fungus releases signal molecules (collectively called Myc factors) that induce symbiosis-specific responses in the host root with transcriptional activation of symbiosis-related genes prior to contact (Kosuta et al., 2003; Weidmann et al., 2004). Apart the fact that Myc factors are diffusible compounds that elicit plant symbiotic responses, there was no clear idea about their structure until recently when the structure of a *Glomus intraradices* diffusible signal was identified. The AM fungus secretes a mixture of sulphated and non-sulphated lipochitooligosaccharides (LCOs) that have structural similarities with rhizobial Nod factors (Maillet et al., 2011). However, it is suggested that more Myc factors should exist because LCOs do not induce all expected early plant responses to AM fungi (Bonfante and Requena, 2011).

1.3.1.2- Symbiotic phase

After contact of a fungal hypha with the host root surface, the first step in the symbiotic interaction is the formation of a hyphopodium which is considered as the entry point structure for AM fungal hyphae into the root (Fig. 4.2). During hyphopodium formation, but preceding the first signs of root penetration, the underlying epidermal cell responds with a striking program of cellular reorganization to form the prepenetration apparatus (Genre et al., 2005). This depends on a number of plant genes, which have first been recognised by the analysis of pea mutants defective in the development of the nodule symbiosis with nitrogen-fixing rhizobia (Gianinazzi-Pearson and Denarié, 1997). Corresponding genes have later been identified in the two model legumes *Medicago truncatula* and *Lotus japonicum*.

A SYM pathway for AM and rhizobial nodule symbiosis (RNS) has been described which involves at least 7 genetically defined steps in *L. japonicus*, of which 3 are common to *M. truncatula*: i) a receptor kinase (SYMRK/DMI2), ii) a gene encoding an ion channel (POLLUX/DMI1), and iii) a calcium and calmodulin dependent protein kinase (CCaMK/DMI3) (Parniske, 2004). In addition to these 3 genes, 2 nuclear porins (NUP85, NUP133) are also required for Ca^{2+} spiking which is an early response of root hairs to Nod factor application or to AM fungi approaching roots (Kosuta et al., 2008) (Fig. 5).

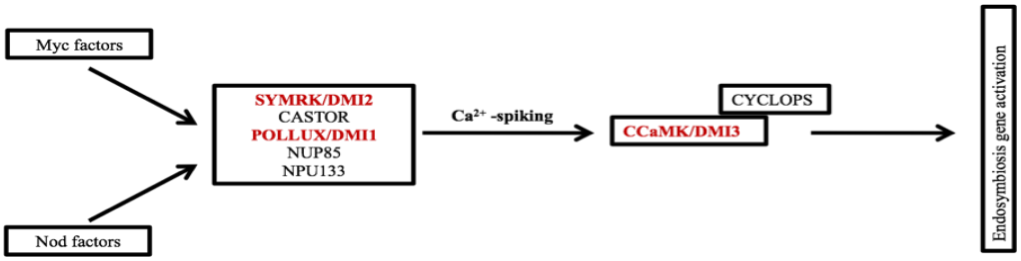


Figure 5. The common SYM pathway between mycorrhiza and nodulation symbioses. Microbial factor recognition by a plant receptor kinase (SYMRK/DMI2) leads to ion channel activation (CASTOR, POLLUX/DMI1) and Ca^{2+} spiking that regulates symbiosis gene expression via a calmodulin calcium dependent protein kinase (CCaMK/DMI3) associated to a CYCLOPS protein. DMI (Does not make Infection): nomenclature used in *Medicago truncatula* to describe the three proteins common with *Lotus japonicus*.

Following the colonization of an epidermal cell, the AM fungus traverses the epidermal layer and the outer cortex intercellularly as in *Arum*-type, or intracellularly, as in *Paris*-type mycorrhiza (Smith and Smith, 1997). Within a few days after initial penetration of the root, the fungus forms the first arbuscules (*Arum*-type) or hyphal coils (*Paris*-type) in cortical cells. Here, entering of the apoplast is also accompanied by the formation of a prepenetration apparatus (Genre et al., 2008), and arbuscule formation by a tremendous structural reorganisation in the surrounding plant cell (Bonfante and Perotto, 1995; Gianinazzi-Pearson et al., 1996).

Signals exchanged in the mycorrhizosphere lead to specific gene expression patterns in the fungus and the plant. The activation of three main classes of plant genes has been consistently reported during the colonization process. These are related to: i) membrane processes and cell wall turnover, ii) metabolic functioning and iii) plant defence reactions (Gianinazzi-Pearson et al., 1996; Blee and Anderson, 2000; Franken et al., 2000; Gallou et al., 2011a). Many defence-related genes or proteins have been reported to be induced in AM tissues, and nearly all the studied genes are up-regulated in arbuscule-containing cells (Gianinazzi-Pearson et al., 1996; Dumas-Gaudot et al., 2000, Breuillin et al., 2010). Plant defence activation remains lower than in plant/pathogen interactions and this seems to be a key element in the establishment of compatibility between the mycorrhizal partners (Gianinazzi-Pearson et al., 1996). In natural and man-made ecosystems, however, these controlled plant defence reactions are confronted with numerous other external factors like nutrient and water availability, plant pathogens or soil pollution.

On the fungal side, little is known about signalling molecules or genes that could be involved in AM development during different first steps of root interactions. To date, Tollot et al. (2009) has described the transcription factor STE12 from *G. intraradices* with a potential role in AM fungal invasion into roots, and Kloppholz et al. (2011) described a fungal effector which interacts with the plant transcription factor ERF19 and suppresses in this way plant defence responses during the whole colonization process.

Parallel to intraradical growth, AM fungi form a network of extraradical hyphae which explores far into the soil and gives the root system a much greater excess to mineral nutrients by taking them up and transferring them to the plant (Neumann and George, 2005). The extraradical hyphae also contribute to stabilization of soil aggregates and improve soil quality concerning, for example, water availability to the plant (Miller and Jastrow, 1990; Kabir and Koide, 2002). Another important aspect is the establishment of common mycorrhizal networks which connect plants of the same or of different species (Selosse et al., 2006), and via which plants can exchange not only mineral elements (Meding and Zasoski, 2008) but also communicate with each other (Song et al., 2010). The AM fungal life cycle is completed as the extraradical mycelium produces a new generation of spores which are major survival organs and able to tolerate adverse soil conditions for many years (Neumann and George, 2005).

1.3.2– Arbuscular mycorrhiza functions

1.3.2.1- Exchange of nutrients

Arbuscules represent a checkpoint between the two mycorrhizal symbionts where a high transporting activity occurs not only from plant to fungus, but also in the direction fungus to plant, via the symbiotic interface made up of the plant periarbuscular membrane and fungal plasma membrane separated by an apoplastic zone (Hause and Fester, 2005).

In AM plants, there is a net increase in photosynthesis which results in a photoassimilate increase in AM roots, estimated to be up to 20% (Bago et al., 2000). Carbohydrates from “source leaves” are transferred as sucrose via the phloem to the

"root sink" and converted into glucose plus fructose (Blee and Anderson, 1998). Glucose seems to be transferred to the fungal symbiont (Solaiman and Saito, 1997; Boldt et al., 2011). However, a monosaccharide transporter recently isolated from *G. intraradices* did not only transport glucose, but also xylose indicating plant cell wall sugars as alternative carbon source for AM fungi (Helber et al., 2011). Localisation of its expression, moreover, suggested that the transfer of carbohydrates does not solely occur at the arbuscules but also at other intraradical hyphae.

In mycorrhizal plants, the pathway of direct uptake of inorganic phosphate (Pi) from the soil at the root surface is suppressed and replaced by the mycorrhizal pathway that involve import of Pi into fungal hyphae via Pi transporters, translocation of Pi to the arbuscule interface, and release to root cells where plant Pi transporters transfer the Pi into cortical cells (Bucher, 2007; Smith et al., 2011). Many plant Pi transporters have been characterized and classified into high or low affinity transporters, of which some are AM specific.

Although improved nutrient assimilation by AM associations concerns mainly Pi, the fungal partner can also provide the host plant with N (Hawkins et al., 2000). The current model predicts that nitrate and ammonium are taken up by the extraradical mycelium, arginine is transported in the fungal hyphae and ammonium is finally transferred towards the plant (Govindarajulu et al., 2005; Chalot et al., 2006; Guether et al., 2009). Fungal transport capacities for N and P are in a similar range (Smith and Read, 2008), but the plant needs ten times more N than P, so that the fungal-mediated transfer of N is probably of less importance for mycorrhizal effects on plant growth.

1.3.2.2- Bioprotection against environmental stress

Abiotic stress

In addition to influencing plant nutrition, AM fungi improve the performance of their hosts on polluted soils (Aloui et al., 2009; Rivera-Becerril et al., 2002; Gonzalez-Chavez et al., 2002), under drought stress (Augé, 2001) or at high salt concentrations (Ruiz–Lozano et al., 1996). Consequently, AM contributions have

been investigated in different fields like landscape regeneration, alleviation of desertification or bioremediation of contaminated soils (Jeffries et al., 2003).

The mechanisms contributing to such tolerance against abiotic stresses in AM plants are not fully understood (Schützenduebel and Polle, 2002). Pathways of heavy metal chelation do not appear to operate in such AM-enhanced tolerance (Rivera-Becerril et al., 2005) and recent investigations have indicated the implication of anti-oxidative activities through, in particular, reactive oxygen species (ROS) accumulation (Aloui et al., 2009). In fact, several observations have shown that AM induced tolerance against different abiotic stresses (heavy metals, salt or drought) may be ROS-dependent (Ruiz-Lozano et al., 1996, 2001; Bowler and Fluhr, 2000; Huang et al., 2010). In parallel to enhanced plant anti-oxidant activities, it was shown that on the fungal side accumulation of six glutathione-S-transferases was up-regulated in extraradical hyphae of *G. intraradices* growing in a heavy metal contaminated soil (Waschke et al., 2006).

Biotic stress

Interactions between AM and pathogens has received attention since first studies showed that the symbiosis can reduce both the incidence and the severity of diseases. These effects have been consistently reported against different pathogens (Dehne and Schönbeck, 1979; Dehne, 1982; Cordier et al., 1998; Benhamou et al., 1994; Yao et al., 2003; Li et al., 2006). The effect of AM symbiosis on leaf pathogens is variable and appears to depend on the pathogen lifestyle. For example, AM plants have been reported to be more susceptible to leaf biotrophic pathogens such as powdery mildew and rust fungi, but more resistant to phytoplasma or necrotrophic fungal pathogens (Gernns et al., 2001; Lingua et al., 2002; Fritz et al., 2006; de la Noval et al., 2007; Gallou et al., 2011b). In contrast, the development of AM consistently reduces disease caused in roots by a number of soil-borne pathogens. The most frequently reported effects relate to reduction in:

* incidence and/or severity of root rot or wilting caused by fungi (*Rhizoctonia*, *Fusarium* or *Verticillium*)

* root rot caused by oomycetes (*Phytophthora, Pythium* or *Aphanomyces*)

* deleterious effects caused by parasitic nematodes (*Pratylenchus* or *Meloidogyne*) (for a full list, see table 1 in Whipps, 2004).

Bioprotection of roots against such pathogens generally depends on a fully established mycorrhizal symbiosis (Bärtschi et al., 1981; Rosendahl, 1985; Slezack et al., 2000), although there are reports suggesting pre-symbiotic effects of AM fungi (Caron et al., 1986; Krishna and Bagyaraj 1983; St-Arnaud et al. 1997; Gallou, 2011b). However, in contrast to investigations of the influence of AM on abiotic stress, the effect of different AM fungal isolates on biotic stress tolerance to pathogens has been rarely compared (Franken and George, 2006). In the only study, by Pozo et al. (1999), *G. mosseae* was shown to reduce the disease index of tomato roots infected with *P. parasitica,* while *G. intraradices* did not.

Although AM bioprotection against plant pathogens has been often confirmed, the mechanisms underlying it remain unclear. Several hypotheses have been proposed to explain this phenomenon based on the fact that establishment of an AM symbiosis causes physiological and developmental changes in the host plant. These include: i) plant nutrition improvement, ii) competition for photosynthates and root colonization sites between an AM fungus and a pathogen, iii) modification in root biomass and architecture, and iv) changes in rhizosphere microbial populations. These changes could play a role in AM-induced bioprotection by compensating root damage caused by the pathogen, or by stimulating components of rhizosphere microbiota with antagonistic activity towards certain root pathogens (Azcòn-Aguilar and Barea, 1997, Barea et al., 2005). However, results from several studies exclude the hypothesis of improved nutrition (Shaul et al., 1999; Fritz et al., 2006). Another proposed hypothesis is that colonization of roots by AM fungi primes defence mechanisms leading to mycorrhiza–induced resistance (MIR) (Cordier et al., 1998; Pozo et al., 2002; Pozo and Azcòn-Aguilar, 2007).

1.4– Mycorrhiza–induced resistance (MIR)

As for pathogens and PGPR, the presence of mycorrhizal fungi in host tissues can induce enhanced defence responses against pathogen attacks; this raises the notion of mycorrhiza–induced resistance (MIR). This notion is not recent; in fact, early work already showed that AM bioprotection is associated with a stimulation of

defence mechanisms (Baltruschat and Schönbeck, 1972; Dehne and Schönbeck, 1979).

Based on the fact that an AM fungus is a biotrophic microorganism, a hypothetical signalling pathway comparable to PGPR and biotrophic pathogen signalling may exist. In addition to the above-mentioned Myc factors, symbiotic AM fungi seem to possess MAMPs as plants respond upon colonization also with the expression of defence–related genes. That this expression is only slight and transient (Gianinazzi-Pearson et al., 1996) seems to depend on a fungal activity; in this context, a small effector molecule (SP7) was recently identified in *G. intraradices* that contributes to the biotrophic status of the AM fungus in roots by counteracting the plant immune system (Kloppholz et al., 2011).

Altered endogenous levels of phytohormones have also been observed during AM interactions indicating a role in the communication between AM fungi and host plants (Ludwig-Müller, 2000; Hause et al., 2007; Herrera-Medina et al., 2007). The three phytohormones SA, JA and ET, which are involved in the signalling pathways of defence gene expression, are likewise regulated. This has led to the hypothesis that direct or indirect triggering of signalling pathways regulated by these phytohormones could be important for AM fungal protection against biotic stress (Garcia–Garrido and Ocampo, 2002). However, the specific role of the three phytohormones in MIR is not well understood, and it may be a matter of interplay especially between JA and SA since they are known to have antagonistic effects in biotic interactions (Kunkel and Brooks, 2002).

MIR appears to be both a localized and a systemic phenomenon. A detailed study on tomatoes showed that arbuscule-containing cells were immune against *P. parasitica* due to cell wall reinforcement associated with phenolics and callose deposition (Cordier et al., 1998). Activation of defence related genes by arbuscule development is a well-described event in AM and is considered to prime plant cells to such immunity (Dumas-Gaudot et al., 2000). Split root systems have shown that pathogen development is also limited in non-mycorrhizal parts of mycorrhizal root systems (Davis and Menge, 1980; Rosendahl, 1985; Cordier et al., 1998; Elsen et al., 2003). However, there are only a very few studies concerning possible molecular

mechanisms underlying MIR although there is some evidence for the involvement of callose, PR-1a, β-1,3 glucanases and phenolic compounds (Cordier et al., 1998; Pozo et al., 2002).

1.5– AM in the Solanaceae

A. thaliana cannot be used as a model plant for investigating symbiotic interactions due to its inability to form mycorrhiza. Therefore *M. truncatula* and *L. japonicus* have become model species for AM research especially after the uncovering of a common SYM pathway that exists in both symbioses. It is suspected that the RNS developmental program evolved from an ancestral AM SYM pathway, and that modifications subsequently occurred specific to RNS (Gianinazzi-Pearson and Dénarié, 1997; Parniske, 2008). A non-legume family could therefore provide a better model to study the AM-specific SYM pathway.

Members of the Solanaceae family, which includes important crops such as tomato, potato, eggplant, tobacco and petunia, are used as model systems in research on many plant biology topics including plant-microbe interactions. Tomato, tobacco and potato, have also become models for understanding mechanisms underlying AM functions. The first mycorrhiza-specific plant phosphate transporter was identified in potato (Rausch et al., 2001) and, later, different AM up-regulated phosphate transporters were characterized from tomato and potato. This transporter characterization uncovered functional redundancy in symbiotic phosphate transport in the Solanaceae (Nagy et al., 2005). In parallel, tomato and tobacco have been used to study AM bioprotection. As mentioned previously, tomato roots are protected by *G. mosseae* against *P. parasitica* infection (Cordier et al., 1998; Pozo et al., 2002) and a proteomic study was carried out to investigate this effect at a molecular level (Dassi et al., 1996). In tobacco, AM was shown to improve tolerance against *Thielavopsis basicola* infection and this was correlated with an increase in proline and arginine contents of the roots (Giovannetti et al., 1991).

1.6- *Petunia hybrida* Mitchell: a model plant

1.6.1- *Petunia* genus: origin and interest

The genus *Petunia* (first established by Jessieu in 1803) assembles commercially important flowering plants originating from South America. The name petunia derives from "Petum" meaning "tobacco" in the language Tupi-Guarani. The geographic distribution of the genus includes temperate and subtropical regions of Argentina, Uruguay, Paraguay, Bolivia, and Brazil, with a centre of high diversity in southern Brazil.

Petunia is an ornamental crop of high economic interest. Many advantages make the culture of petunias favourable for gardeners, such as their easy growth, their versatility and a huge range of colours and flower shapes. One very important quality is their relatively high tolerance to drought, probably related to their origin. For all these reasons, petunias belong to the most sold bedding plants worldwide. For greenhouse growers, petunia is listed as the top genus grown per number of plants sold (Tambascio, 2007).

Plant geneticists' interest in petunia began in the late fifties of the last century. Predicting flower colours on the basis of Mendel's laws enabled the definition of over thirty genes involved in flavonoid biosynthesis (Gerats and Vandenbussche, 2005). Moreover, the finding in petunia of reversible co-suppression of homologous genes (Napoli et al., 1990) had an unexpected outcome in 1998 with the revolutionary discovery of RNA interference (RNAi) (Fire, 1999).

1.6.2- *Petunia hybrida* Mitchell: advantages and qualities

Petunia hybrida, derived from crosses between *Petunia axillaris* (large white flower) and *Petunia integrifolia* (purple flower), is the most widely cultivated of the 30 extant petunia species. *P. hybrida* Mitchell variety is an inbred colchidiploid (2 n = 14) and has a relatively large genome (1200-1500 Mbp) (Mishiba et al., 2000; Bossolini et al., 2011). It is characterised by white flowers that produce a strong fragrance in the evening and at night (Verdonk et al., 2005). The hybrid has been considered as a genetic model plant since the early 1980s (Gerats and Vandenbussche,

2005). In particular, the high mutation rate in the *P. hybrida* line W138 has turned out to be very useful for mutant screens especially after the molecular basis for the mutations was shown to be the non-autonomous transposable element dTph1 (Gerats et al., 1990). Because transformation of petunia is also applicable (Conner et al., 2009), forward and reverse genetic approaches are nowadays possible (Wegmüller et al., 2008). Together with a large EST collection, commercially available microarrays (Breuillin et al., 2010) and the currently on-going genomic sequencing (Franken and Drüge, personal communication), petunia has become an interesting model for studies on the genetics and the molecular physiology of plants (Bossilini et al., 2011).

The fact that reverse genetics can be used as a strategy with petunia has led to the isolation of a petunia mutant, *pam1* (*penetration and arbuscule morphogenesis1)*, which is affected in the development of AM. The corresponding gene has been characterized as a VAPYRIN homologue with 11 ankyrin repeats which could be involved in the transport via the tonoplast of a component with an essential function during intracellular colonization by AM fungi (Sekhara Reddy et al., 2007; Feddermann et al., 2010). In contrast to previously described tomato and maize mutants which are affected at early stages of root colonization or have reduced level of mycorrhization (Barker et al., 1998; David-Schwartz et al., 2001, 2003; Paskowski et al., 2006), the *pam1* mutant is defect in intracellular accommodation, arbuscular development and morphogenesis of the fungal endosymbiont (Sekhara Reddy et al., 2007) and can contribute to our understanding of the AM-specific SYM pathway at later stages of the symbiosis.

1.6.3- Petunia in ornamental crop production

Ornamental crops like petunia are mainly produced as potted plants in artificial substrates, and their marketability is greatly influenced by conditions used during their production, such as substrate quality, drainage, irrigation, water quality and fertilization (Chavez et al., 2008). Soilless culture substrates associated with rich fertilizer regimes are increasingly applied to meet present-day consumer demands for ornamental and nursery plants (Gruda, 2009). Whilst these offer significant advantages for high crop yield and product quality through complete control over water and nutrient supplies (Grillas et al., 2001), the use of substrates with poor or no

ion exchange capacity can lead to mineral nutrient losses or to short-term unintentional exposure of plants to high ion concentrations. This in turn results in short periods of salt stress which reduce vigour and yield and are detrimental, if not lethal, especially for young plants (Rosendahl and Rosendahl, 1990). Moreover, ornamental petunia production is confronted with attack by root pathogens like *Fusarium oxysporum*, *Rhizoctonia solani* or *Thielaviopsis basicola* (Dreistadt, 2001) which cause high losses in greenhouses.

1.7- Thesis objectives

As mentioned above, petunia is particularly sensitive to the accumulation of soluble salts caused by high fertilizer concentrations in the growing media (Kang and van Iersel, 2009), and production in greenhouses is confronted with the threat of root diseases caused by different root rot pathogens (Wright et al., 2004). In this context, the introduction of mycorrhiza into petunia production systems could be a useful strategy in nurseries to reduce fertilization excess and root diseases.

AM can be termed a biological means for plant disease control but knowledge about the mechanisms underlying AM-induced bioprotection is still very fragmentary. In an attempt to fill this gap, the work of my thesis was focused on AM in petunia as a model system to analyze the molecular basis of induced resistance to biotic stress (root fungal pathogen). In order to reach this goal, candidates for each microbial partner (AM fungus, fungal pathogen) were selected and conditions were optimized for their development in petunia. Mycorrhiza-induced bioprotection was then evaluated and plant gene expression analyzed.

In this context,

i. the feasibility of applying an AM fungus to reduce phosphate fertilization for petunia in a soilless substrate was investigated (chapter I)

ii. a pathosystem causing root rot and damping off of petunia was optimized (chapter II)

iii. an experimental system for AM-induced bioprotection in petunia was established (chapter III)

iv. plant gene expression responses were monitored during AM-induced bioprotection in order to gain insight into possible mechanisms involved (chapter IV)

v. the existence of systemic bioprotection in root systems was examined (chapter V)

2 -

Materials and Methods

2.1- Biological materials

Petunia hybrida Mitchell W138

The *P. hybrida* Mitchell variety line W138 was selected as it is a model plant in different research fields (Gerats et al., 1990). For this work *P. hybrida* Mitchell W138 seeds or cuttings were provided by Dr. Uwe Drüge (IGZ, Erfurt, Germany).

AM fungi

Three different AM fungi that belong to two Glomeromycota orders (Glomerales and Diversisporales) were used in this work: *Glomus mosseae* (Nicolson & Gerd.) Gerd. & Trappe BEG12, *Glomus intraradices* Schenck & Smith (Agrauxine), and *Gigaspora rosea* Nicolson & Schenck BEG9, as a vermiculite-based inoculum produced by Agrauxine (Quimper, France) or a soil-based inoculum produced by The International Bank of Glomeromycota (IBG, Dijon, France). The Glomeromycota phylum has recently been revised and some fungi separated into new taxon based on their generic analysis; for example, *G. mosseae* is defined as *Funneliformis mosseae* and *G. intraradices* as *Rhizophagus intraradices* (Schüssler and Walker, 2010). However for the current work, the previous fungal names commonly used in the literature are adopted.

Root fungal pathogens

Four pathogenic fungi were tested for disease development in petunia roots: *Pythium aphanidermatum* (Edson) Fitzp. isolated from cucumber, *Fusarium oxysporum* Schlecht. isolated from infected petunia plants, *Rhizoctonia solani* Kühn AG3 isolated from a diseased potato plant and *Thielaviopsis basicola* (Berk. and Broome) Ferraris (syn. *Chalara elegans*) received from the German Resource Centre for Biological Material.

P. aphanidermatum was grown for 10 days on carrot agar medium (annexe 2) at 25°C and inoculum produced by agitating 20 plugs of infected medium for 12 hours in 1 L of sterile water. Inoculum of *F. oxysporum* was produced for 10 days at 26°C on PDA agar medium. *R. solani* was produced 10 days at 22-25°C on either PDA agar or barley seeds according to Schneider et al. (1997). *T. basicola* was grown

on V-8 juice (Gemüsesaft, Penny, Germany) agar medium (annexe 2) for one week at 22°C with a 16/8h photoperiod under cool-white fluorescent light (4 18W/865 New generation lamps, Philips, Hamburg, Germany).

2.2- Petunia propagation

From seeds

P. hybrida Mitchell (W138) seeds were disinfected for 10 min with sodium hypochlorite (NaOCl), washed three times with sterile water and germinated 4 weeks in autoclaved vermiculite at 22/24°C with 16/8 h photoperiod under cool-white fluorescent light (as indicated above).

From cuttings

P. hybrida cuttings from IGZ-Erfurt (Klopotek et al., 2010) were treated as follows on the same day of cutting:

- Cuttings were rooted in boxes filled with Perlite ('Perligran A', particle size 0–6 mm; Knauf Perlite GmbH, Dortmund, Germany) for 19 days in growth chambers (Vötsch, Balingen-Frommern, Germany; day/night 22/20°C and 16/8 h, light intensity 200 μmol m^{-2} s^{-1}, 60% relative humidity) and watered with tap water.
- Cuttings with similar root growth were selected for transplantation into pots.

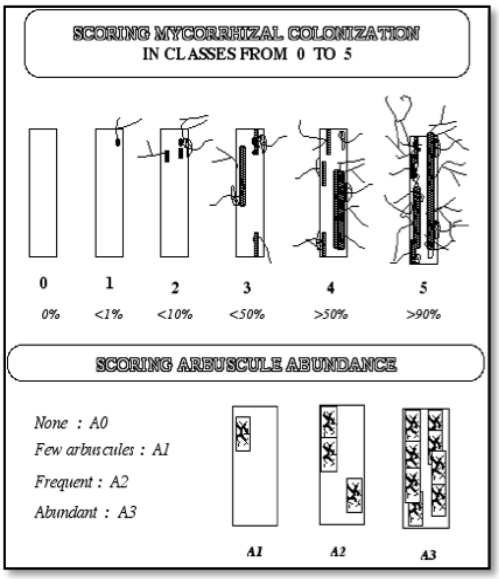
2.3- Petunia mycorrhization

Seedlings or rooted cuttings were transplanted individually into 400 cm^3 pots filled with a mixture of vermiculite (Vermex M, Efisol, France) and sand (Quartz3, Botanic, France) at a 1:1 ratio (v/v) and inoculated (mycorrhizal, M) or not (non-mycorrhizal, NM) with inoculum from one of the three AM fungi *G. mosseae*, *Gig. rosea* or *G. intraradices*. Non-mycorrhizal plants were watered with an inoculum filtrate (two times filtration, Whatmann n°1 filter paper, Schleicher and Schuell, Dassel, Germany) to introduce associated microorganisms in the case of vermiculite-based inoculum (Chapters I, III-2.1), and autoclaved inoculum was also added for

soil-based inoculum controls (Chapters III-2.2/2.3, IV, V). Plants were cultivated in growth chambers (day/night 22/20°C and 16/8 h, light intensity 200 µmol m^{-2} s^{-1}, 60% relative humidity). Pots were arranged in a randomised block design and plants were fertilized twice a week with a modified Hoagland nutrient solution (Oyarzun et al., 1993) or ferty8 solution (annexe 1) containing 10% phosphate (0.1 mM KH_2PO_4) (15 ml/pot).

In order to compare effects of mycorrhization to those of phosphate fertilization, non-mycorrhizal plants were divided into four sets and fertilized with a Hoagland nutrient solution containing final concentrations of 0.1 mM, 0.26 mM, 0.51 mM or 1 mM KH_2PO_4. All plants were grown under the same conditions as described above.

At harvest, roots were washed under running tap water, cleared in 10% KOH and stained with 0.05% trypan blue in glycerol (Phillips and Hayman, 1970) for AM quantification. Root systems were cut into 1 cm pieces and mycorrhizal colonization was quantified microscopically based on 30 root fragments/plant according to Trouvelot et al. (1986) (Fig. 6). Mycorrhizal parameters were calculated using the "Mycocalc" programme (http://www2.dijon.inra.fr/mychintec/Mycocalc-prg/download.html).



Mycorrhizal parameters calculated

* Frequency of mycorrhiza in the root system

F% = (nb of fragments myco/total nb)*100

* Intensity of the mycorrhizal colonisation in the root system

M% = (95n5+70n4+30n3+5n2+n1)/(nb total)

* Arbuscule abundance in mycorrhizal parts of root fragments

A% = (100mA3+50mA2+10mA1)/100

* Intensity of the mycorrhizal colonisation in the root fragments

m% = M*(nb total)/(nb myco)

* Arbuscule abundance in the root system

a% = A*(M/100)

Figure 6. Determination of mycorrhization by scoring, for each root fragment, mycorrhizal colonization in class as from 0 to 5 and arbuscule abundance in 4 categories (A0, A1, A2 and A3).

2.4- Determination of shoot biomass, water content and phosphorus concentration

To determine dry matter and water content, shoots were weighed fresh, subsequently dried for 3 days at 80°C and then weighed again. 500 mg of dried shoot material was dissolved in 5 ml of 65% nitric acid (HNO_3) and ashed for 20 min at 200°C. Phosphorus was colorimetrically analysed by the Service Laboratory, IGZ, Grossbeeren using the ammonium-molybdate-vanadate method (Gericke and Kurmies 1952) and a spectrophotometer at 436 nm wavelength.

2.5- Salt stress treatment

Five weeks after *G. mosseae* colonization, petunia plants (M or NM) were challenged with salt stress by providing them twice a week with a modified Hoagland nutrient soluton (0.1 mM KH_2PO_4) containing 250 mM NaCl. Plant growth and phosphorus concentration were determined as described in 2.4.

2.6- Fungal pathogen inoculation

In vivo

Three or five week-old petunia plants were inoculated separately with each pathogen:

* *P. aphanidermatum* was introduced by injecting 20 ml of inoculum into the substrate close to the stem-base after adjusting the concentration with sterile water to 10^6 conidia/ml, determined using a Thoma chamber. Control plants received 20 ml of sterile water.

* *F. oxysporum* was inoculated by adding three infected PDA plugs per plant into the substrate (2 cm below the substrate surface, 1 cm distance from the stem-base); non-infected agar plugs were used for control plants.

* *R. solani* was inoculated by introducing three infected barley seeds into the substrate (2 cm in depth, 1 cm from the stem-base); sterile barley seeds were used for control plants.

* *T. basicola* fungal mycelium and conidia were collected from the V-8 juice agar surface and diluted with sterile water to a concentration of 10^6 conidia/ml, determined using a Thoma chamber. Plants were inoculated by injecting 20 ml of this suspension per plant into the substrate close to the stem-base; control plants received 20 ml of sterile water.

All plants were grown under conditions described in 2.3.

In vitro

Disinfected *P. hybrida* seeds were germinated on M-medium (Bécard and Fortin, 1988) for four weeks; seedlings were subsequently transferred to M-medium without sucrose. *F. oxysporum* and *R. solani* were inoculated by placing a 5 cm agar plug from a fresh culture at half distance between two seedlings per Petri dish. Inoculation with *P. aphanidermatum* and *T. basicola* was done by placing 10 µl of pathogen suspension (at 10^6 conidia/ml) onto root tips. Non-inoculated agar and sterile water, respectively, were used for control plants. Seedlings were two weeks incubated under the same conditions as for seed germination (see 2.2)

2.7- Disease severity (DS) estimation

Disease severity caused by each fungal pathogen was rated into five classes based on the percentage of root length with brown regions: 0, no infection; 1, <10%; 2, 10-50%; 3, 50-80%; 4, >80%. For each biological repetition, 30 root fragments/plant were analysed under the microscope and disease severity was calculated according to Fakhro et al. (2010) by: $\sum$ (n_x times x)/30 (n = number of fragments, x = each category from 0 to 4).

The presence of the four fungi was also analysed in the plant stem base (collar). 1 cm pieces were disinfected 35 s in Ethanol (70%) and 2 min in NaOCl (1%), washed 2 times in sterile osmosed water and dried on sterile filter paper. For each pathogen, disinfected collar pieces were incubated under the conditions specified in 2.1. Mycelium growth was checked daily during one week.

For *T. basicola,* its presence in petunia roots was also monitored by reverse transcriptase-polymerase chain reaction (RT-PCR) detection using a specific reverse

primer designed manually on the *T. basicola* LSU rDNA sequence and checked with Amplify3X program, and the universal forward ribosomal gene primer LR1 for eukaryotes (van Tuinen et al., 1998) (Table 1). To verify primer specificity, DNA was extracted from 50 mg of *T. basicola* fresh mycelium by grinding and incubation in 500 µl CTAB (hexadecyltrimethylammoniumbromide) lysis buffer (Sigma) containing Proteinase K at 65°C for 1 h. After adding an equal volume of phenol and centrifugation 10 min at 10,000 g, DNA was recovered in the aqueous phase, washed with 500 µl chloroform/isoamyl alcohol (24:1/v:v) and re-centrifuged. The supernatant was recovered, DNA precipitated overnight at -20°C, and centrifuged down at 10,000 g for 30 min (4°C). The pellet was washed with 70% cold ethanol and DNA resuspended in 50 µl sterile water. After photometrical control of quality and quantity at wavelengths 260/280 by Nanodrop 2000 (Thermo Scientific, USA), DNA was stored at -20°C. DNA amplified by PCR using the *T. basicola* LSU primer pair and 1 µg genomic DNA as template (protocol as for semiquantitative RT-PCR; see below) was cloned into TOPO vector (Invitrogen, USA), sequenced at MWG (Ebersberg, Germany) and the sequence verified by TBLASTX analyses in public databases.

2.8- RNA extraction from petunia roots and first-strand cDNA synthesis

Total RNA was extracted from petunia roots from different treatments using Qiagen RNeasy Plant Mini Kit (74904) following the manufacturer's instructions (Qiagen, Hilden, Germany). RNA quantity and quality was controlled by 1% agarose gel electrophoresis and photometric analysis (Nanodrop 2000) or on the bioanalyser 2100 (Agilent, France) before storage at -20°C until needed. Total RNA was DNase-treated using RNase-free DNase (Promega kit RQ1) according to the manufacturer's instructions. DNA-free RNA was reverse transcribed into first-strand cDNA and amplified via the protocol described by Weidmann et al. (2004).

2.9- Reverse transcriptase (RT)-PCR

cDNA of transcripts of each selected gene was amplified by reactions carried out in 20 µl PCR mix containing Taq polymerase buffer with 1.5 mM $MgCl_2$ (MP

Biomedical, USA), 0.75 U of Taq polymerase (Invitrogen, place), 1 µM dNTPs, and 0.5 µM of each gene-specific primer pair (Table 1), using cDNA from 1 µg root-extracted RNA, non-diluted for *T. basicola* detection and 1:10 diluted for petunia transcript analyses. Reactions were conducted in a T3000 thermocycler (Biometra, Germany) with the following program: 94°C for 5 min, 25 cycles (93°C for 1 min, 60°C for 1 min, 72°C for 1 min), 10 min at 72°C. PCR products were separated by 1% agarose gel electrophoresis for 25 min at 100 volts, gels were stained 10 min in ethidium bromide and documented under UV light using GelDoc EQ apparatus (BioRad, USA). If PCR products showed the right size, they were cloned into TOPO vector (Invitrogen, USA) according to the manufacture's protocol, sequenced (MWG, Ebersberg, Germany) and the sequence was verified by TBLASTX analyses using public databases.

2.10- Real-time RT-PCR

Real-Time PCR reactions were carried out to quantify selected gene transcripts using the Step One Plus Real-Time PCR System Thermal Cycling Block (Applied Biosystems, USA) and SYBER-green as fluorescent dye. In order to determine primer efficiency (E) in real-time RT-PCR, cDNA amplifications were quantified using 5 cDNA dilutions to produce a linear slope. Primer efficiency was calculated using the formula: $E=10^{(-1/slope)}-1$ (Invitrogen guide for important parameters of quantitative PCR analysis). Each reaction (15 µl total volume) contained 7.5 µl SYB green mix (ABsoluteTM QPCR® SYBR Green ROX Mix 2x; Thermo Scientific, UK), 0.5 µM of primer pair for each gene (Table 1) and 2 µl of 1:10 diluted cDNA from 1 µg root-extracted RNA. The amplification program was performed as follows: 95°C for 15 min, 40 cycles (95°C for 15 s, 60°C for 1 min). A melting curve (95°C for 15 s, 60°C for 1 min, 95°C for 15 s) was recorded at the end of every run to exclude primers generating non-specific PCR products (Ririe et al., 1997). Three biological repetitions, each with two technical repetitions, were analyzed for each treatment. Baseline range was adjusted to 0.2 to minimize the effect of non-specific amplification at low values and Ct values were automatically calculated using the Step One software.

2.11- Relative gene expression (R)

T. basicola abundance

To quantify *T. basicola*, the amount of pathogen LSU rRNA in 1 µg root-extracted RNA was estimated. The real-time RT-PCR threshold cycle for the pathogen LSU rRNA from roots of inoculated plants (Ct_{sample}) was subtracted from the threshold cycle obtained from roots of non-inoculated plants, considered as reference ($Ct_{reference}$). The presence of the pathogen gene in inoculated roots was calculated as $R=2^{-\Delta Ct}$.

Petunia genes

Three housekeeping genes were considered for normalization of transcript quantification of targeted petunia genes: actin, glyceraldehyde phosphate dehydrogenase (*GAPDH*) and ubiquitin (*UBQ*). In preliminary RT-qPCR analyses of gene expression, no significant differences in Ct values were found for each of the three candidate genes in petunia roots across different treatments (20±0.7, 21±0.5, 17±0.1, respectively), and *UBQ* was selected as reference gene. The relative expression ratio of each target gene in treated versus control roots was computed according to the formula of Pfaffl (2001) using the *UBQ* reference gene:

$$\text{ratio}= \frac{(E_{target})^{\Delta Ct\ target\ (control\text{-}treated\ sample)}}{(E_{ref})^{\Delta Ct\ ref\ (control\text{-}treated\ sample)}}$$

E_{target}: real-time PCR efficiency of target gene transcript

E_{ref}: real-time PCR efficiency of a reference gene transcript

Ct: threshold cycle

2.12- Statistical analysis

Statistical analyses of data were carried out using one-way or two-way analysis of variance (ANOVA) provided by the program package Statistica (version 7.0; StatSoft Inc., Tulsa, OK, USA). Means were thereafter separated by Fisher-test procedure at P=0.05. T-test was used for comparing means of treatments where two-way ANOVA detected an interaction at P=0.05. Percentage values were ARCSIN transformed before t-test and/or ANOVA.

Table 1. Petunia and *T. basicola* genes selected for expression profiling: putative function, primers and amplicon size. Primers were designed manually using EST sequences from different sources (last column); primer pairs of each gene were tested using Amplify3X program (Engels, 1993) before ordering from Eurofins MWG Operon (Ebersberg, Germany). Primer specificity was checked by cloning (TOPO vector, Propmega) and sequencing (MWG, Ebersberg, Germany) the PCR products. Annealing temperature was 60°C for each primer pair.

Genes	Cell function	Primer sequence (5'-3')	Amplicon size (bp)	References
Reference housekeeping genes				
Ubiquitin (*UBQ*)	Miscellaneous	TGGAGGATGGAAGGACTTTGG AACACACATAACAAAAGCGATGCCA	233	Franken, personal communication
Actin	Cytoskeleton	ATCTATGATTGGGATGGAAGC CTCTCTGGGGGAGCAACAACC	214	Franken, personal communication
Glyceraldehyde phosphate dehydrogenase (*GAPDH*)	Primary metabolism	GGAATCAACGGTTTTGGAAGAATTGGGCG GGCCGTGGACACTGTCATACTTGAACA	135	Franken, personal communication
AM-related genes				
Phosphate transporter *PT3:* AM-upregulated	Phosphate membrane transfer	ATCCCAAAAAGGTTGATGCTGG ATCATAGTATACATATACCACTACG	248	Breuillin et al., 2010
Phosphate transporter *PT4:* AM-specific	Phosphate membrane transfer	CAAATATGGTTGGATTTTGTTGC ATGATAAACTTGCCAATGTAATATCC	184	Breuillin et al., 2010
Potassium transporter *KT:* AM-upregulated	Potassium membrane transfer	CTAGAAAATTACATTCCTGAAGC CTTGTTCCTGCAGCTCTTCATCC	124	Franken, personal communication
Chitinase class III (PR8) *Chit 3* : AM-specific	Hydrolytic enzyme	CAAAATGGCAATGAAGGGACG CAGAACTCACGTTAACGCATCC	166	Franken, personal communication
Glutathone-S-transferase *GST:* AM-specific	Oxidative damage protection	TCCTTGTCACCCATTGCCCCCTC CCGATCTCGTGCACGTTTCTGGG	156	Breuillin et al., 2010
Pathogen related protein 10a *PR10a:* AM-upregulated	RNase activity	GGATGAGAATTCATGCATGG AGTTGAAATAGTCAACAGAAGC	131	Breuillin et al., 2010
JA defense-related genes				
Lipoxygenase (*LOX*)	JA biosynthesis	AACGGTGCTGGAATTGTGC TCTGTCTTGCTTCACATGC	167	Franken, personal communication
Allene oxidase cyclase (*AOC*)	JA biosynthesis	CGGGGATTACGGTCACATCGCTG GTGATGGCTCCACCGTAGGCG	233	Ahkami et al., 2009
Chitinase class I (PR3) *Chit1a*	Anti-fungal enzyme	ATCACCGGCCGATGGACGCC AATTGTCTCCAGGGGCCACGTTC	153	Breuillin et al., 2010
Chitinase class I (PR3) *Chit1b*	Anti-fungal enzyme	GGCAGAACCTCCTCCAACACTGTC TCCTCTTCTGCATCACCCACGAA	193	Linhorst et al., 1990
Phenylalanine amonia lyase (*PAL1*)	Phenylpropanoid biosynthesis	GTCGAGCCACACCCTGCCAC TGGCTTTGGAGTTGGGCCTGC	236	Verdonk et al., 2005
Chalcone synthase *CHS*	Phytoalexin production	GAGCAGAAGGGCCAGCCACAA TAGTCCGCCCCTGGCATGTCA	391	Breuillin et al., 2010
Enhanced disease resistance *EDR1*	Defense response	GTGCTATAACTCGGCCACC TGTATGTAAGCAATCCATGC	158	Breuillin et al., 2010
Callose *CAL*	Cell wall reinforcement	TGCGCGTTGCTTATGTTGAGGAG GCGGACCTGGAAGCTTTACGCG	132	Breuillin et al., 2010
NADPH:cytochrome P450 reductase *P450*	Anti-oxidant defense	CACCGGCGCACTTATCTTCTCCA TGTGTAGGCGGGGAGCAACG	352	Breuillin et al., 2010
SA defense-related genes				
endo-1,3-beta-Glucanase *PR2*	Anti-fungal protein	CAATTGGTGACGCTGGTCTGG AATGTTAACGAGCAAAGGTGC	176	Breuillin et al., 2010
Thaumatine-like *PR5*	Anti-fungal protein	CCGGTGATTGTGGTGGGGTCCTA CCCTGCACTAGGCTTAGTTGGGG	167	Breuillin et al., 2010
Proteinase inhibitor (PI) *PR6*	Anti-fungal protein	RYTTTCTTKCTTCTTGCATC CAAAAAGACGAACWCGATTAC	292	Zahn et al., 2005
***Thielaviopsis basicola* LSU rDNA**				
T. basicola_2	LSU rDNA	GAAAGAGCCACATTCCCTAAG	500	
LR1	eukaryote LSU rDNA	GGTTGGTTTCTTTTCCT		van Tuinen et al., 1998

3 -Results

Chapter I: Mycorrhization of *Petunia hybrida* Mitchell in a soilless system

Chapter II: Pathosystem establishment in *Petunia hybrida* Mitchell

Chapter III: Mycorrhiza-induced bioprotection of *Petunia hybrida* Mitchell against *Thielaviopsis basicola*

Chapter IV: Molecular investigations of bioprotection against *Thielaviopsis basicola* in *Petunia hybrida*

Chapter V: Investigation of systemic bioprotection by *Glomus mosseae* against *Thielaviopsis basicola*

Chapter I:

Mycorrhization of *Petunia hybrida* Mitchell in a soilless system

I.1- Petunia mycorrhization studies

There have been few studies focusing on AM in petunia, and interest in this ornamental for both applied and fundamental research is recent. In an earlier investigation using *P. hybrida* cv. Blue bird, it was shown that the inoculation of phosphate-deficient soil with AM fungi had significant positive effects on petunia biomass, flowering time and uptake of phosphate (Pi) and potassium (Gaur et al., 2000). In comparison to chemicals, the reduction in costs for Pi fertilizers as a result of mycorrhization was estimated at 30%. In a later study, inoculation of *P. hybrida* cv. Mix with *G. mosseae* or *G. intraradices* significantly stimulated plant biomass, flower number and nutrient uptake. In addition, the AM fungi were able to mitigate the adverse effects of drought. All mycorrhizal effects were, however, reduced by high Pi fertilization (Shamshiri et al., 2011). The inhibitory effect of inorganic phosphate on AM, an ubiquitous phenomenon, has recently been investigated in *P. hybrida* at the molecular level. Using a petunia microarray, analyses showed that phosphate supply does not induce genes which may inhibit AM development, but rather reduces root colonization by down-regulation of genes related to AM functioning like genes encoding enzymes involved in carotenoid biosynthesis (Breuillin et al., 2010).

Soilless substrates (like vermiculite, perlite, sand, peat…) are more and more widely used in modern horticulture. They have become the basis of intensive greenhouse production methods for vegetable as well as ornamental crops including petunia (Gruda et al., 2008; Chavez et al., 2008). However, as already pointed out in the general introduction, soluble salt accumulation may occur and bedding plants like petunia can be particularly affected by such stress (Kang and van Iersel, 2009). Earlier reports have highlighted the interest of AM fungal inoculation as a strategy for the production of petunia, particularly in nutrient-deficient soils (Gaur and Adholeya, 2005). Therefore, the ability of AM to reduce phosphate fertilization and stress growth conditions of soilless growing media is of potential interest for horticultural production. For this reason and in order to establish a controlled system for further investigations, experiments were carried out to determine the feasibility of using vermiculite/sand as an inorganic soilless substrate for the production of mycorrhizal petunia plants.

I.2- Results

Petunia plants (*P. hybrida* Mitchell W138) were grown from seeds and subjected to the treatments summarized in Table I-1 in three independent experiments.

Table I-1. Experimental conditions used to compare between *G. mosseae-P. hybrida* interactions in a vermiculite/sand substrate.

	Exp1	**Exp2**	**Exp3**
Control plants			
Fertilization: Hoagland	0.1 mM KH_2PO_4	0.1 mM, 0.26 mM, 0.51 mM and 1 mM KH_2PO_4	0.1 mM KH_2PO_4
Mycorrhizal plants			
AM fungus	*G. mosseae*	*G. mosseae*	*G. mosseae*
Fertilization: Hoagland	0.1 mM KH_2PO_4	0.1 mM KH_2PO_4	0.1 mM KH_2PO_4
Treatments			
after 5 weeks	Harvest	Harvest	Salt stress (250 mM)
after 7 weeks	–	–	Harvest

I.2.1- Mycorrhiza development, plant growth and phosphate nutrition

Petunia seedlings were inoculated with *G. mosseae* at planting and grown under low phosphate (0.1 mM KH_2PO_4) conditions (Exp. 1). Five weeks after inoculation, the intensity of mycorrhizal colonization of the root system (M%) was 9.7±0.3 and arbuscule abundance in the root system (A%) 2.8±1.6. In spite of these low values, a significant positive effect on shoot dry mass (2 fold), shoot water content (1.7 fold) and phosphorus (P) content (2.5 fold) of petunia plants was observed in mycorrhizal as compared to non-mycorrhizal plants (Fig. I-1).

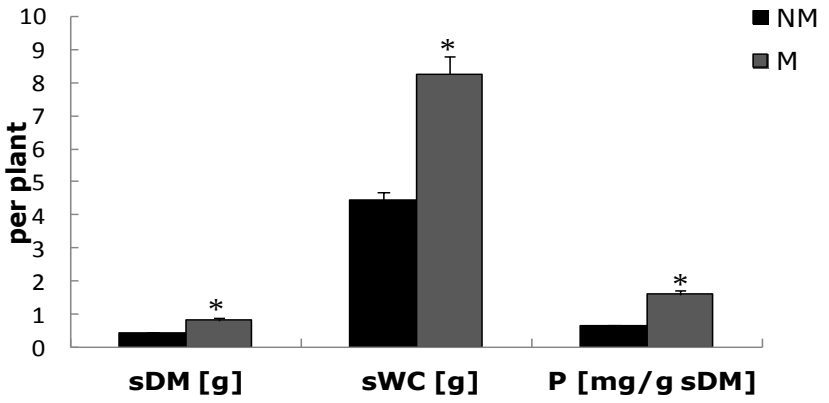


Figure I-1. *Petunia hybrida* shoot dry mass (sDM), water content (sWC) and phosphorus content (P) 5 weeks after inoculation with *G. mosseae* (M), compared to corresponding control plants (NM). Significant differences between treatments (t-test, *P*=0.05, *n*=3) are indicated by asterisks. Bars= standard errors.

The effect of *G. mosseae* was compared to phosphate fertilization (Exp. 2) by fertilizing non-mycorrhizal plants with 0.1 mM, 0.26 mM, 0.51 mM or 1 mM KH_2PO_4. Plants were harvested after 5 weeks. Parameters of root colonization were not significantly different to those in Exp. 1 (M%= 15±3 and A%= 10±5). Shoot dry weight and P contents of control plants increased with increasing levels of Pi in the nutrient solution. T-test Comparisons indicated significant differences (*P*=0.05, n=4) between *G. mosseae*-colonized plants and all NM treatments except for those receiving 0.51 mM KH_2PO_4 (NM2) (Fig. I-2).

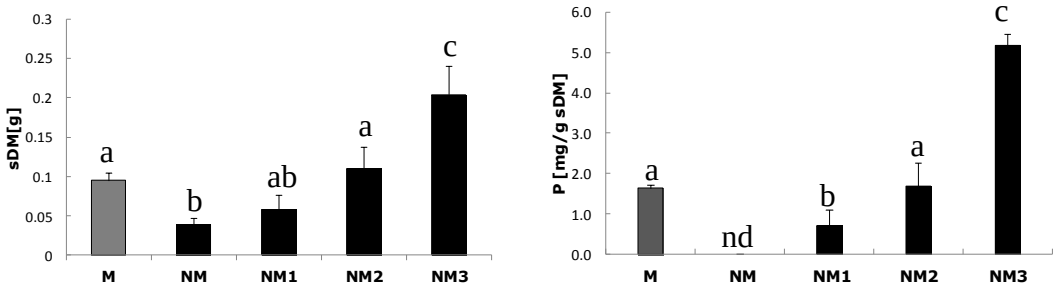


Figure I-2. *Petunia hybrida* shoot dry mass (sDM) and phosphorus content (P) 5 weeks after inoculation with *Glomus mosseae* and fertilized with 0.1 mM KH_2PO_4 (M), compared to control plants fertilized with 0.1 mM (NM), 0.26 mM (NM1), 0.5 mM (NM2) or 1 mM KH_2PO_4 (NM3) (nd: not enough material to determine the phosphorus content). Letters above columns indicate significant differences between the mycorrhizal and the non-mycorrhizal treatments (one-way ANOVA, *P*=0.05, n=4). Bars= standard errors.

I.2.2- Salt stress

Five weeks after *G. mosseae* inoculation, petunia plants (M or NM) were challenged with a modified Hoagland nutrient solution (0.1 mM KH_2PO_4) containing 250 mM NaCl (twice a week for two weeks), in order to analyse if *G. mosseae* increases petunia tolerance to salt stress in the vermiculite/sand substrate. While values for mycorrhizal colonization and arbuscule abundance in non-stressed conditions were similar to those in Experiments 1 and 2, mycorrhization parameters significantly increased in salt-stressed plants (Table I-2). Colonisation by *G. mosseae* (M%) and arbuscule abundance (A%) in root systems were 2.5 and 4.8 fold higher, respectively, compared to mycorrhizal plants not grown under salt stress.

Table I-2. Mycorrhizal colonization in *Petunia hybrida* roots 5 weeks after inoculation with *Glomus mosseae* and an additional 2 weeks in the absence (M) or the presence of salt stress (Ms: 250 mM NaCl). Significant differences compared to the M treatment are indicated by asterisks according to one-way ANOVA followed by Tukey-test (P=0.05, n=3). ± means standard error.

	F%	M%	A%	m%	a%
M	96 ±3	10 ±0.5	3 ±2	10 ±2	25 ±5
Ms	99 ±5	25 ±3*	14.5 ±4*	25 ±5*	58 ±7*

Dry mass, water content and phosphorus contents were again clearly enhanced in non-stressed mycorrhizal plants (Fig. I-3). However, all parameters declined with salt stress and this was more pronounced in mycorrhizal than non-mycorrhizal petunia. Under salt stress, only shoot dry mass (1.5 fold) was still enhanced in mycorrhizal plants compared to non-mycorrhizal controls.

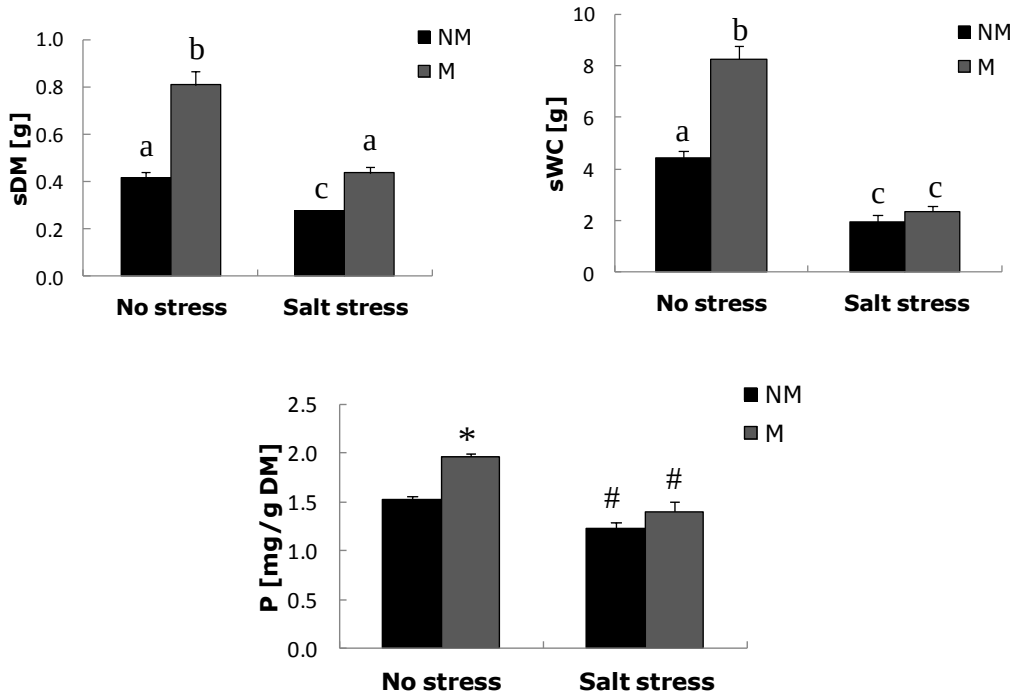


Figure I-3. Interaction between mycorrhiza and salt stress in *Petunia hybrida* 5 weeks after inoculation with *Glomus mosseae* and an additional 2 weeks in the absence or presence of salt stress (250 mM NaCl). Shoot dry mass (sDM), water content (sWC) and phosphorus content in shoots (P) in plants inoculated with *G. mosseae* (M) compared to corresponding control plants (NM). Two-way ANOVA (P=0.05, n=3) revealed significant interaction between the factors *G. mosseae* and abiotic stresses for sDM and sWC parameters. Letters above columns indicate significant differences between treatments using Tukey-test (P=0.05, n=3). Two-way ANOVA (P=0.05, n=3) revealed no significant interaction between the factors *G. mosseae* and abiotic stresses for the parameter P. Significant differences between mycorrhizal plants and controls are indicated by asterisks and significant differences between stressed and corresponding non-stressed colonized or non-colonized plants are indicated by hash icons. Bars= standard errors.

I.3- Discussion

Petunia is an ornamental crop of high economic interest which is particularly sensitive to the accumulation of soluble salts caused by high fertilizer concentrations in the soilless growing media (Kang and van Iersel, 2009). Arbuscular mycorrhizal (AM) fungi are well-known for their positive effects on plant physiology (Smith and Read, 2008) and, as such, represent a potential means to counteract the aforementioned problem in two ways: reduction of mineral fertiliser and increase of the plant's salt tolerance. Moreover, several studies have shown petunia to be

mycorrhiza-responsive in soil-based growth conditions (Gaur et al., 2000; Sekhara-Reddy et al., 2007; Shamshiri et al., 2011).

Results from the present work clearly show that colonization of *P. hybrida* Mitchell W138 by the AM fungus *G. mosseae* positively affected biomass, shoot water content and P content of 5 week-old plantlets in a vermiculite/sand soilless culture system. Such effects in an inert substrate with poor or no ion exchange capacity are probably due to the efficient Pi uptake capacities of extraradical hyphae developing out from the roots (Smith and Read, 2008). The fact that in vermiculite/sand the AM effect at 0.1 mM KH_2PO_4 fertiliser concentration was similar to that of 0.5 mM KH_2PO_4 in non-mycorrhizal plants indicates a five-fold economy of Pi fertilization due to the use of mycorrhiza in the system. This is in agreement, for example, with the previous estimation by Gaur et al. (2000) of a 30% reduction in costs for mineral fertilizers in petunia production when introducing AM fungi. However, caution should be taken in extrapolating from previous studies which have been carried out using different AM fungal strains and conditions. For example, previous reports of a better capacity of *G. mosseae* compared to *G. intraradices* in improving nutrient supply to petunia plants (Gaur et al., 2000; Shamshiri et al., 2011) contrast with the report of increased levels of P content in petunia after inoculation with a different *G. intraradices* strain (Sekhara Reddy et al., 2007).

As expected, petunia turned out to be highly sensitive to salt stress: plant biomass was decreased and uptake of water and phosphate was reduced. Inoculation with *G. mosseae* continued to increase petunia biomass but not shoot water or P content in the presence of a high salt concentration, and no positive interactions were observed between the factors ‘mycorrhiza’ and ‘salt’. Elimination by the salt stress of the mycorrhizal responsiveness of petunia was not due to a negative effect on AM fungal development itself as mycorrhization parameters were enhanced in plants grown in the presence of salt, suggesting that mycorrhizal effectiveness rather than root colonization had been affected. Salt-stress tolerance induced by mycorrhiza has not been analyzed in petunia before, but has in several other plants including the closely related species tomato where results indicated negative to positive interactions (Al-Karaki 2000, 2006; Al-Karaki et al. 2001; Hajiboland et al. 2010; Huang et al.

2010). Screening of other AM fungi and different salt concentrations to better define mycorrhiza-induced salt tolerance in petunia will be useful.

Soilless substrates are increasingly used in horticulture and in particular for annual ornamental plants like petunia which require growing media with adequate water retention and aeration (Erstad and Gislerod, 1994). The results from the present study show for the first time that *G. mosseae* BEG12 has potential as a biological agent for sustainable petunia production in a soilless substrate, so meeting petunia growth requirements and consumers' demands for ecologically-produced ornamental crops.

Chapter II:

Pathosystem establishment in Petunia hybrida Mitchell

II.1- Introduction

Most of the characterized root pathogens are filamentous fungi, oomycetes or filamentous bacteria (Okubara and Paulitz, 2005). In general, they are necrotrophic pathogens with wide host ranges and do not appear to have closely co-evolved with specific hosts as biotrophic pathogens have done. Widespread examples are fungi of the genera *Pythium*, *Fusarium*, *Thielaviopsis*, and *Rhizoctonia* which cause root rot diseases and can be easily spread in greenhouses where only fungicide application is effective against them. The following four fungi, known to cause root rot and damping-off in different Solanaceae plants, were selected in order to establish a pathosystem with petunia for studies of AM-induced bioprotection (chapter IV).

II.1.1- *Pythium aphanidermatum* (Edson) Fitzp.

Pythium species belong to the Oomycota and are serious threats in worldwide plant production (Hendrix and Campbell, 1973; Moorman et al., 2002). They cause economic losses on several important crops and bedding ornamentals, including petunia (Mitchell and Deacon, 1986, Veit et al., 2001; Kessler, 2004). *Pythium aphanidermatum* is a highly aggressive representative of the genus; it reproduces both sexually and asexually and can infect host plants in three different forms: oospores, zoospores and sporangia (Matthews, 1931). *P. aphanidermatum* is referred to as a water mold; the zoospores are able to spread easily via greenhouse irrigations, wet surfaces and substrates with high water retention. Infection by this pathogen causes severe root and crown rot in petunia, which results in wilting and death of plants. Resistant cultivars do not exist and efficient fungicides (i.e., propamocarb, etridiazole and metalaxyl) pose environmental problems (Postma et al., 2008). The strain used in the present study was originally isolated from an infected cucumber plant in the Department of Plant Health at the IGZ (Grossbeeren, Germany).

II.1.2- *Fusarium oxysporum* Schlecht

Fusarium wilt is a major problem for the production of a wide variety of crops (Nelson et al., 1981). *Fusarium oxysporum* is the most abundant species among the genus *Fusarium* (Ascomycota) and was first described from a solanaceous plant (eggplant) suffering from a vascular wilt disease (Matuo and Ishigami, 1958). Pathogenic strains can penetrate roots inducing either root rot or tracheomycosis in the vascular system (Fravel et al., 2003). *F. oxysporum* is characterized by flask-shaped conidiophores which are produced asexually. Growing on culture medium, *F. oxysporum* mycelium turns from white to purple and can be easily discriminated from other fungal colonies. Chlamydospores are able to remain infective in soil for 30 years, and when a host plant breaks their dormancy, they germinate and hyphae subsequently infect the roots of this host. For this reason, it is known as the "silent assassin". *F. oxysporum* used in this work was isolated from infected roots of *P. hybrid*a (Hayek et al., unpublished).

II.1.3- *Rhizoctonia solani* Kühn

Rhizoctonia solani [anamorph; telemorph: *Thanatephorus cucumeris* (Frank) Donk] is a widespread soil-borne pathogen that belongs to the phylum Basidiomycota. It was originally isolated from a Solanaceae plant (potato) and described by Kühn (1858). It is responsible for important damage to many economically important agricultural and horticultural crops including petunia (Adam, 1988; Wright et al., 2004). *R. solani* causes root rot that induces reduction in plant growth and yield, sometimes even leading to plant death (Berta et al., 2005). The fungus survives for many years as sclerotia in soil or as mycelium in organic matter under numerous environmental conditions, and it has an extremely wide host range (Grosch *et al*., 2004).

The form genus *Rhizoctonia* is considered as a heterogeneous assemblage of filamentous fungal taxa that do not produce asexual spores (known as non-sporing imperfect stage) (González García et al., 2006) and share a number of common features in their anamorphic states. Since sexual stages are rare, grouping is evaluated based on hyphal anastomosis reactions between isolates; isolates showing successful hyphal fusions belong to one anastomosis group (AG). Twelve such AGs were

originally described and considered to be genetically isolated (Anderson, 1982; Schneider et al., 1997). Presently, thirteen AGs have been described with different levels of host specificity (Carling et al., 2002). A *R. solani* AG3 isolate was tested for pathogenicity towards *P. hybrida* plants.

II.1.4- *Thielaviopsis basicola* (Berk. and Broome) Ferraris (syn. *Chalara elegans*)

Thielaviopsis basicola is a soil-borne fungus with a worldwide distribution and has been identified as a pathogen of more than 137 plant genera including petunia (Maria et al., 2006; Park et al., 2006; Leahy, 1998). The major disease symptom is black root rot (BRR) caused by dark cortical lesions, which are easily identified microscopically. Colonization of the root often leads to root pruning, foliar stunting and significant yield losses (Hood and Shew, 1996). *T. basicola* was originally classified as a necrotroph because it causes classical root necrosis of tobacco roots (Mims et al., 2000). However, because biotrophic and necrotrophic stages sequentially exist at different steps of the interaction with the root, it has been reclassified as a hemibiotroph (Hood and Shew, 1997) and is best described as a hemibiotrophic-necrotrophic pathogen (Mims et al., 2000). *T. basicola* is haploid and reproduces via two forms of asexual spores: hyaline, cylindrical phialospores (endoconidia) and thick dark-walled chlamydospores, the latter being responsible for its long survival in soil (Nah Raj and Kendrick, 1975). For the present work, *T. basicola* isolate DSM No.: 63050 from the German Resource Centre for Biological Material was used.

II.2- Results

II.2.1- Pathogen selection

Pathogenicity is defined as the ability of an organism to cause a disease on a putative host (Horsfall and Dimond, 1960) while aggressiveness refers to the disease severity of an isolate on different hosts or on hosts of different ages. Therefore, all four pathogens were tested for their pathogenicity to *P. hybrida* seedlings *in vitro* and their disease severity was evaluated in *P. hybrida* plants *in vivo*.

II.2.1.1- Pathogenicity tests *in vitro*

Petunia seedlings grown on M-medium for 4 weeks were challenged against each fungal pathogen separately to observe pathogen development and its ability to cause symptoms. For each treatment 2 plates with 2 seedlings were prepared.

Only *F. oxysporum* and *T. basicola* gave obvious symptoms on petunia seedlings two weeks after inoculation. *F. oxysporum* mycelium grew fast towards the seedlings, infected the roots and then developed into shoot parts to attack leaves (Fig. II-1b). In the same way, germinated *T. basicola* conidia infected roots and then covered all seedlings (Fig. II-1c). Control seedlings remained healthy with green leaves (Fig. II-1a).

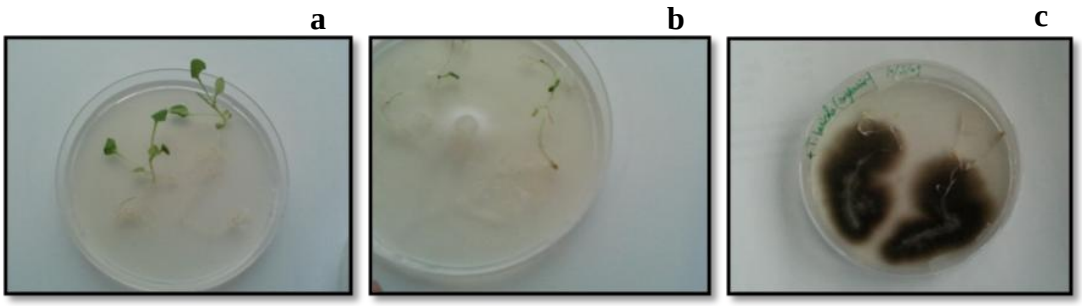


Figure II-1. Four week-old *P. hybrida* seedlings grown *in vitro,* 2 weeks after pathogen inoculation. Control seedlings (a) are compared to seedlings inoculated with *Fusarium oxysporum* (b) and *Thielaviopsis basicola* (c).

II.2.1.2- Pathogenicity tests *in vivo*

Petunia seedlings, grown under the same conditions as for seed germination, were inoculated with each fungal pathogen four weeks after transplanting into pots. The experiments were done independently for each pathogen with four biological repetitions for each treatment.

All four pathogens had no significant influence on petunia plant biomass (t-test, *P=0.05, n=4*) (Fig. II-2a) and inoculated plants showed no obvious symptoms in the shoots. Disease symptoms were also not observed in the roots, except for *T. basicola*, where brown lesions were detected. These lesions were associated with mycelium and chlamydospores, visible by light microscopy without staining (Fig. II-2b). Despite its presence in roots, *T. basicola* was never detected in any other part of the plant. Only *F. oxysporum* grew from surface disinfected collar pieces placed on

M-medium (Fig. II-2c). Neither *P. aphanidermatum* nor *R. solani* gave any symptoms of infection on petunia seedlings.

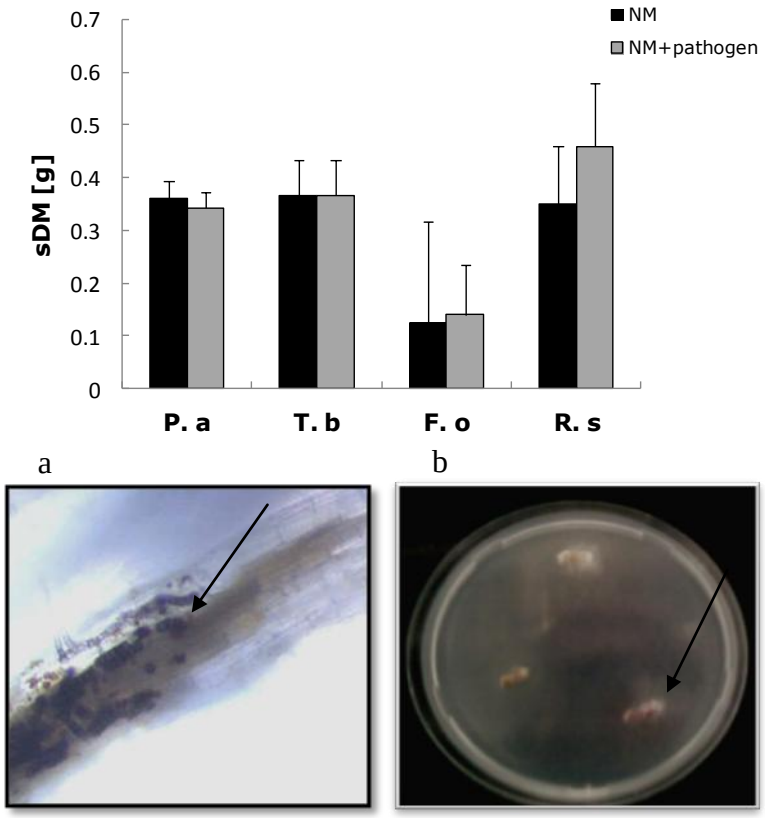


Figure II-2. Shoot dry mass (sDM) and disease symptoms of six week old *Petunia hybrida* plants. sDM was compared between two treatments (control: black, pathogen-inoculated: gray) with four different pathogens (P.a: *Pythium aphanidermatum*, T. b: *Thielaviopsis basicola*, F. o: *Fusarium oxysporum* and R. s: *Rhizoctonia solani*) (Bars = standard errors). (a) Root browning (arrow) caused by *T. basicola* is associated with the presence of chlamydospores. (b) Typically pigmented purple *F. oxysporum* mycelium (arrow) growing from collar parts one week after incubation on M-medium.

II.2.2- Time course infection with *T. basicola*

T. basicola was retained for the pathosystem in petunia. To better define this, disease symptoms and fungal development were monitored after inoculation of young plants.

II.2.2.1- Root necrosis and leaf symptoms

The time course of infection by *T. basicola* in non-mycorrhizal plants inoculated three weeks after transplanting showed no root necrosis and/or leaf discoloration at early stages 24 h and 36 h (Table II-1), whilst such symptoms were obvious after 1 and 2 weeks (Fig. II-3).

Table II-1. Symptoms accompanying *Thielaviopsis basicola* development 24 h, 36 h, 1 week and 2 weeks after inoculation of 3 week-old *Petunia hybrida* plants.

After inoculation	Root necrosis	Leaf discoloration
24 h	—	—
36 h	—	—
1 week	✓	—
2 weeks	✓	✓

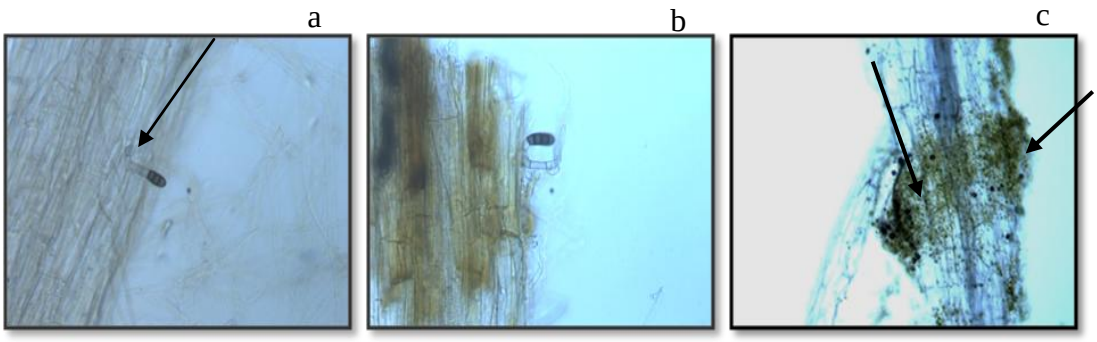


Figure II-3. Different stages of *Thielaviopsis basicola* root infection. First contact of chlamydospores with *Petunia hybrida* roots forming similar structures to appressoria (arrow) (a) without or (b) with typical root browning 1 week after inoculation, and (c) more advanced infection development 4 weeks after inoculation (arrows) (c).

II.2.2.2- Molecular detection of *T. basicola*

Although symptoms were absent at 24 h and 36 h after root inoculation with *T. basicola*, RT-PCR using the primer pair specific for the pathogen revealed the presence of the fungus in the roots at these time points (Fig. II-4). With further development of the pathogen, detection was accentuated 1 and 2 weeks after inoculation when necrotic symptoms were observed on roots. *T. basicola* DNA from pure fungal cultures was used as positive control and water as negative control in all amplifications.

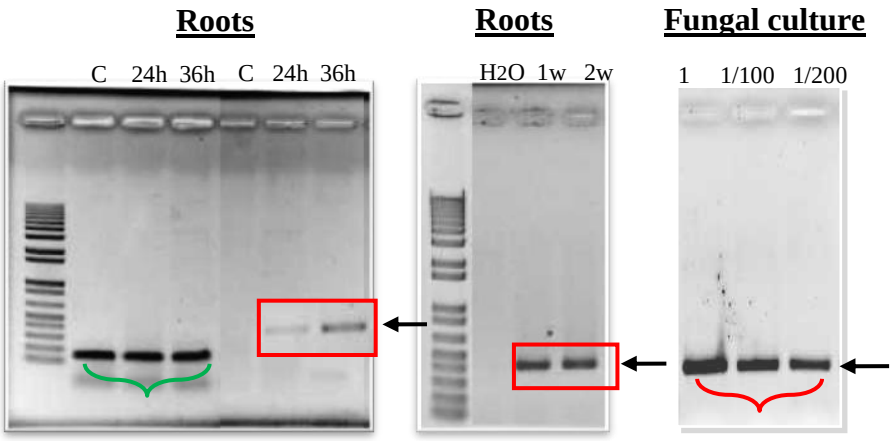


Figure II-4. RT-PCR showing the presence of 500 bp (black arrow) fragments in roots of inoculated *P. hybrida* plants (red square), corresponding to *Thielaviopsis basicola* LSU rRNA gene fragments that are absent from non-inoculated control plants (C). *T. basicola* detection was compared at 24 h, 36 h, 1 w and 2 weeks after inoculation. *Petunia hybrida* ubiquitin transcripts were used to control the quality of the RNA extractions (green bracket). *T. basicola* LSU rDNA at different dilutions (non-diluted, 1/100, 1/200) extracted from pure fungal cultures was used as positive control (red bracket) and H_2O as negative control.

II.3- Discussion

Among the four root fungal pathogens, only *F. oxysporum* and *T. basicola* infected petunia seedlings *in vitro*. Clear disease symptoms on roots could only be observed *in vivo* after inoculation with *T. basicola* which caused typical browning of petunia roots. *T. basicola* is a common pathogen of petunia in production systems where it causes root pruning, foliar stunting and severe yield losses (Johnson, 1916). No petunia cultivars resistant to this fungal pathogen are known and fungicide application is the only current control strategy used in greenhouses (David and Ortiz, 1980; Kessler, 2004) which underlines the interest of using AM fungi in order to enhance petunia bioprotection. *T. basicola* infection is known to be divided into four main steps (Hood and Shew, 1997):

i. spore germination
ii. exogenous signal recognition to initiate host penetration
iii. differentiation of fungal structures similar to haustoria that invaginate the plasma membrane of the living host cell: defined as biotrophic phase

iv. intracellular hyphae growth from haustoria structures and invade host cell causing cell infraction and necrosis: defined as necrotrophic phase

In order to better define the petunia pathosystem, it was necessary to follow *T. basicola* development under our experimental conditions to identify early (absence of root necrosis) and late (presence of root necrosis) stages of infection for studies of AM bioprotection. Although the fungus was already detected by RT-PCR in association with *P. hybrida* roots at 24 h after inoculation, no obvious symptoms were observed before 1 week of infection. Therefore, 24 h and 36 h can represent early stages of root-pathogen interactions, whilst 1 week and 2 weeks after inoculation reflect later, more advanced stages of infection.

Chapter III:

Mycorrhiza-induced bioprotection of *Petunia hybrida* Mitchell against *Thielaviopsis basicola*

III.1- Introduction

As already mentioned in the general introduction, mycorrhizal plants respond quite differently to various biotrophic or necrotrophic, leaf or root pathogens. Compared to the corresponding non-mycorrhizal plants, they can be more resistant or more susceptible to attack, and more tolerant or more sensitive to consequences of pathogen development (Pozo and Azcón-Aguilar, 2007). Therefore, we first investigated whether there exists a protective effect of mycorrhization in *P. hybrida* roots against *T. basicola* and whether this is dependent on the AM fungus involved. In a second step, an experimental system was optimised in order to analyse the molecular basis of mycorrhizal bioprotection.

III.2- Results

III.2.1- Comparison of the effect of three AM fungi in the petunia/*T. basicola* pathosystem

The effectiveness of *G. mosseae, Gig. rosea* and *G. intraradices* against *T. basicola*, as well as on shoot biomass and P content of petunia, was assessed four weeks after challenging five week-old mycorrhizal seedling plants with the pathogen.

Differences in root colonization parameters were observed between *G. mosseae*, *Gig. rosea* and *G. intraradices* (Table III-1), and the influence of *T. basicola* inoculation on these differed between the three AM fungi. As compared to *G. intraradices*, *G. mosseae* and *Gig. rosea* showed lower root colonisation (M%, m%) and arbuscule development (A%, a%) inside petunia roots. For *G. mosseae,* colonization parameters were slightly, but not significantly, enhanced by *T. basicola*, whilst they remained unaffected for *Gig. rosea;* in contrast, the presence of *T. basicola* significantly reduced the development of *G. intraradices* within petunia roots to a level similar to *G. mosseae* (Table III-1).

Neither *Gig. rosea* nor *G. intraradices* reduced disease severity (DS) caused by *T. basicola* infection (Table III-1). Only the presence of *G. mosseae* in roots resulted in a significant lower DS value (reduced from 0.49 to 0.065). This reduction in *T. basicola* DS by *G. mosseae* at four weeks after pathogen inoculation was confirmed at two weeks after *T. basicola* inoculation in a second experiment under the

same conditions, where DS significantly decreased from 0.88 in non-mycorrhizal plants (NM+Tb) to 0.22 in mycorrhizal plants (Gm+Tb) (Table III-2).

Table III-1. Mycorrhizal colonization and disease severity in *Petunia hybrida* roots 5 weeks after inoculation with 3 different AM fungi: *Glomus mosseae* (Gm), *Gigaspora rosea* (Gr) and *Glomus intraradices* (Gi) and an additional 4 weeks after inoculation with *Thielaviopsis basicola* (+Tb). Mycorrhizal colonization parameters are compared between the 6 different treatments: Gm, Gr, Gi, Gm+Tb, Gr+Tb and Gi+Tb. Different letters in columns indicate significant differences between values using ANOVA followed by the Tukey-test (P=0.05, n=3). Disease severity (DS) of *T. basicola* was compared between treatments in absence of the AM fungus (NM+Tb), or in plants colonized by *G. mosseae* (Gm+Tb), *Gig. rosea* (Gr+Tb) and *G. intraradices* Gi+Tb. Significant difference in DS of *G. mosseae*-colonized roots to the control (t-test, P=0.05, n=3) is indicated by asterisks. ± means standard error.

	F%	M%	A%	m%	a%	DS
Gm	100 ±0 a	13 ±2 bc	10 ±2 bc	13 ±2 bc	77 ±6 acd	
Gm+Tb	96 ±3 a	18.7 ±1 b	15.7 ±1 b	19 ±1 ab	83 ±1 cd	**0.065 ±0.005***
Gr	61 ±9 b	4.0 ±3 c	3.0 ±2 c	5[c] ±3	46 ±18 ab	
Gr+Tb	37 ±8 c	3.0 ±2 c	2.0 ±1 c	2 ±0 c	31 ±5 b	**0.240 ±0.2**
Gi	100 ±0 a	32.0 ±3 a	29.5 ±3 a	32 ±1 a	92 ±3 cd	
Gi+Tb	97 ±2 a	19.0 ±3 b	14.0 ±3 bc	13 ±4 bc	80 ±4 acd	**0.320 ±0.1**
NM+Tb						**0.490 ±0.2**

Table III-2. Mycorrhizal colonization and disease severity in *Petunia hybrida* roots 5 weeks after inoculation with *Glomus mosseae* (Gm) and an additional 2 weeks after inoculation with *Thielaviopsis basicola* (+Tb). Mycorrhizal colonization parameters are compared between the 2 different treatments: Gm, and Gm+Tb. No significant effect was determined using ANOVA (P=0.05, n=4). Disease severity (DS) of *T. basicola* was compared between treatments in absence of the AM fungus (NM+Tb), or in plants colonized by *G. mosseae* (Gm+Tb). Significant difference in DS of *G. mosseae*-colonized roots to the control (t-test, P=0.05, n=4) is indicated by asterisks. ± means standard error.

	F%	M%	A%	m%	a%	DS
Gm	95	10	3	10	45	
Gm+Tb	77	6	3	8	41	**0.22 ±0.04***
NM+Tb						**0.88 ±0.1**

Nine weeks after inoculation, shoot dry mass and water content were not significantly affected by mycorrhization with *G. mosseae* in the absence of *T. basicola*, but P contents were significantly higher (Fig. III-1). Neither *Gig. rosea* nor *G. intraradices* significantly influenced these plant growth parameters.

Infection by *T. basicola* affected growth, shoot water content or P content of petunia plants but to different extents depending on the treatment (Fig. III-1). Shoot dry mass was reduced by the pathogen to a lesser extent in *G. mosseae*-mycorrhizal (5%) than non-mycorrhizal plants (15%). Shoot water content and P contents were not significantly affected by pathogen development in *G. mosseae*-colonized plants. *T. basicola* inoculation of *Gig. rosea*-colonized roots also reduced shoot dry mass of petunia plants but not water or P content as compared to non-mycorrhizal plants. Shoot dry mass, water and P content were not significantly affected by the presence of *G. intraradices* alone. However, when root systems were infected by *T. basicola*, P content was significantly higher in *G. intraradices* mycorrhizas compared to control roots infected by the pathogen.

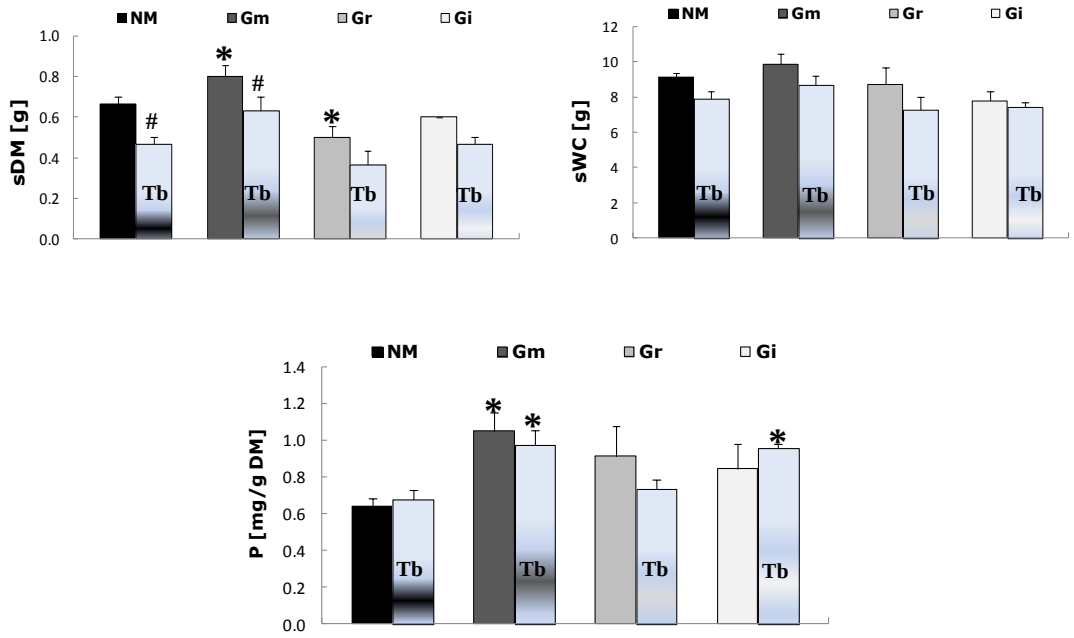


Figure III-1. Effects of interactions between three AM fungi and *Thielaviopsis basicola* on *Petunia hybrida* shoot dry mass (sDM), water content (sWC) and phosphorus content (P) 5 weeks after inoculation of seedlings with AM fungus and a further 4 weeks after infection with *T. basicola*: control (NM), inoculated with the pathogen (NM+Tb), inoculated with *Glomus mosseae* (Gm), *Gigaspora rosea* (Gr) or *Glomus. intraradices* (Gi), and inoculated with the AM fungi and the pathogen (Gm+Tb,

Gr+Tb and Gi+Tb). Two-way ANOVA (P=0.05, n=3) revealed no interaction between the factors 'AM fungus' (Gm, Gr or Gi) and *T. basicola* (Tb). Significant differences between mycorrhizal plants and the corresponding controls among the pathogen-inoculated or the pathogen-free plants are indicated by asterisks. Among the mycorrhizal plants or the corresponding controls, significant differences between pathogen-inoculated plants and pathogen-free plants are indicated by hashed icons (Tukey–test, P=0.05, n=3). Bars = standard errors.

III.2.2- Effect of *G. mosseae* on cuttings in the petunia/*T. basicola* pathosystem

There was no significant difference in the colonization parameters between rooted cuttings inoculated with *G. mosseae* or in mycorrhizal rooted cuttings challenged 4 weeks with *T. basicola* inoculation (Table III-2). *G. mosseae* inoculation had a positive effect on growth of the petunia cuttings and this was not affected by the presence of *T. basicola.* As for plants propagated from seedlings, DS caused by *T. basicola* was significantly decreased by the presence of *G. mosseae* in root systems (Table III-3).

Table III-3. Mycorrhizal colonization (M% and A%), shoot dry mass (sDM%) and disease severity (DS) of 4 week-old *Petunia hybrida* rooted cuttings with an additional 2 weeks after *Thielaviopsis basicola* inoculation compared between four different treatments : control non-mycorrhizal (NM), control inoculated with *T. basicola* (NM+Tb), plants inoculated with *Glomus mosseae* (Gm) and plants in presence with both fungi (Gm+Tb). Different letters in columns indicate significant differences between values using ANOVA followed by Tukey-test (P=0.05, n=4). Significant difference of *G. mosseae*-colonized roots inoculated with *T. basicola* to their corresponding control according to t-test (P=0.05, n=4) is indicated by asterisks. ± means standard error.

	F%	M%	A%	m%	a%	sDM (g)	DS
NM						0.35 ±0.05 a	
NM+Tb						0.4 ±0.2 a	**0.86 ±0.04***
Gm	100 ±0 a	11 ±3 a	1 ±0.3 a	11± 3 a	12 ±2 a	1 ±0.5 b	
Gm+Tb	99 ±2 a	8 ±2 a	1 ±0.3 a	8 ±3 a	16 ±3 a	0.9 ±0.07 b	0.22 ± 0.2

III.2.3- Optimization of *G. mosseae*-induced bioprotection against *T. basicola*

Mycorrhization by *G. mosseae* in petunia roots was first monitored at different time points in order to determine maximum AM development and optimize the bioprotective effect of the AM fungus against *T. basicola*. Four week-old petunia seedlings were inoculated with *G. mosseae* using two different methods. Inoculum was either placed directly under the seedlings (MT) or mixed throughout the substrate (MM).

At 2 and 5 weeks after inoculation, no significant difference was observed between MT and MM treatments in the intensity of mycorrhizal colonization (M%) or arbuscule abundance (A%) in the root system. However, a significant difference between the two methods of inoculation was observed at the third and fourth week: M% and A% values remained low in the MT treatment whilst they were generally higher in the MM treatment and reached a maximum at week 3 and 4 (Fig. III-2).

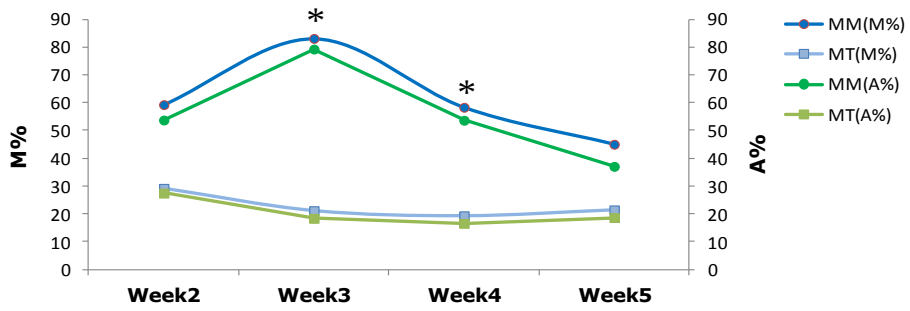


Figure III-2. Colonization parameters (M% and A%) of *Petunia hybrida* roots are compared between two methods of *Glomus mosseae* inoculation (MM and MT) at four time points (weeks 2-5). Asterisks indicate significant differences between MM and MT according to ANOVA ($P = 0.05$; $n = 4$).

The more rapid and greater mycorrhiza development by *G. mosseae* using the inoculation method MM resulted in a significant increase in shoot (sFM) and in root (rFM) fresh mass 5 weeks after inoculation, whilst a significant effect was not observed when the MT method of inoculation was used (Fig. III-3).

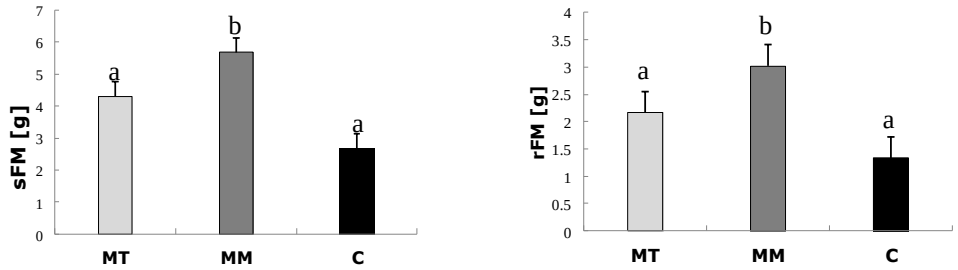


Figure III-3. Effect of inoculation by placing *Glomus mosseae* under a seedling (MT) or mixing it with the substrate (MM) on shoot (sFM) and root (rFM) fresh mass of *Petunia hybrida* compared to non-inoculated control plants (C). Different letters above columns indicate significant difference between treatments according to one-way ANOVA at (P = 0.05; n = 4). Bars = standard errors.

Based on these results and the observations on *T. basicola* development (Chapter II), an experimental system for analyzing mycorrhiza-induced resistance in petunia was established. Petunia seedlings were inoculated or not with *G. mosseae*. Three weeks later, mycorrhiza development was estimated and half of the remaining mycorrhizal and control plants were inoculated with *T. basicola*. Plants were harvested at an early pathogen infection stage (24 and 36 hours after inoculation, hai) to monitor bioprotection by *G. mosseae* against *T. basicola* before disease symptoms appeared.

Mycorrhization parameters and total plant biomass showed no significant differences between different treatments at 24 and 36 hai (Table III-4), and a lower transcript abundance (10 and 28 fold, respectively) of the *T. basicola* LSU rRNA gene was detected at 24 and 36 hai in roots of mycorrhizal (M+Tb) as compared to non-mycorrhizal plants (NM+Tb) in the absence of root necrosis (Fig. III-4). This indicates that the bioprotective effect in mycorrhizal roots of petunia as compared to non-mycorrhizal plants, previously observed four weeks after *T. basicola* inoculation based on disease severity reduction, is already expressed at early stages of pathogen-root interactions (24 hai, 36 hai).

Table III-4. Mycorrhizal root colonization parameters (M% and A%) and total plant biomass (g) of three week-old *Petunia hybrida* plants at 24 h and 36 h after inoculation (hai) with *Thielaviopsis basicola*. Values are compared between four treatments: control (NM), inoculated with *Glomus mosseae* alone (M), inoculated with *T. basicola* (NM+Tb), and in presence of both fungi (M+Tb). No significant effects of *G. mosseae* inoculation were observed according to ANOVA ($P = 0.05$; $n = 4$).

		NM	NM+Tb	M	M+Tb
M%	**24 hai**			17	13
	36 hai			16	15
A%	**24 hai**			13	10
	36 hai			12	11
Biomass	**24 hai**	5	6	5	6
	36 hai	7	6	5	6

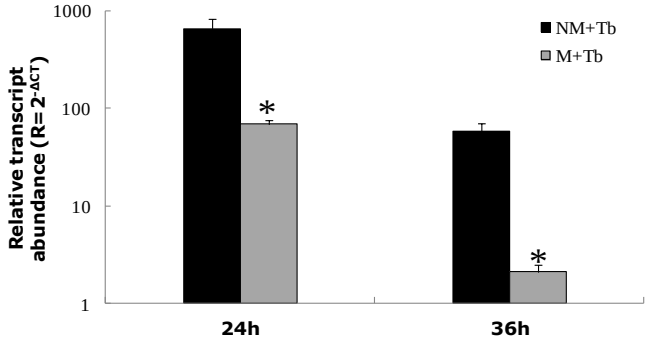


Figure III-4. Transcript abundance of the *Thielaviopsis basicola* LSU rRNA gene in *Petunia hybrida* roots colonized (M+Tb) or not (NM+Tb) by *G. mosseae* at 24 h and 36 h after pathogen inoculation. Asterisks indicate significant differences between treatments (t-test, $P = 0.05$, $n = 3$). *P. hybrida* ubiquitin gene expression was used for normalization. Bars = standard errors.

Prior to molecular investigations, a second experiment was performed to confirm the results concerning the mycorrhiza-induced bioprotective effect at early stages of *T. basicola* infection (24 hai and 36 hai) and to extend analyses to a later stage with root necrosis (1 wai) (Table III-5). Parameters of root colonization with *G. mosseae* (M%, A%) and biomass three weeks after inoculation again did not show any significant difference between plants with or without *T. basicola* at 24 hai, 36 hai and 1 wai (Table III-3). However, in this experiment, total petunia biomass was significantly greater at 24 hai in mycorrhizal as compared to non-mycorrhizal plants, independent of *T. basicola* inoculation, although no difference was detected between treatments at 36 hai and 1 wai (Table III-5**).**

Table III-5. Mycorrhizal root colonization parameters (M% and A%), total plant biomass (g) and root necrosis (RN) of three week-old *Petunia hybrida* plants at 24 h, 36 h and 1 week after inoculation (hai and wai respectively) with *Thielaviopsis basicola.* Values are compared between four treatments: control (NM), inoculated with *Glomus mosseae* alone (M), inoculated with *T. basicola* (NM+Tb), and in presence of both fungi (M+Tb). Asterisks indicate significant effects of *G. mosseae* inoculation according to ANOVA ($P = 0.05$; $n = 4$).

		NM	NM+Tb	M	M+Tb
M%	**24 hai**			19	19
	36 hai			16	22
	1 wai			20	15
A%	**24 hai**			15	16
	36 hai			13	19
	1 wai			17	10
biomass	**24 hai**	2	1	4*	5*
	36 hai	4	5	4	5
	1 wai	9	7	7	9
RN	**24 hai**				
	36 hai				
	1 wai		✓		✓

Disease symptoms were only observed on root systems 1 week after *T. basicola* inoculation. However, the higher transcript abundance of *T. basicola* LSU rRNA in *T. basicola*-inoculated non-mycorrhizal roots (NM+Tb) than in pathogen-challenged mycorrhizal roots (M+Tb) confirmed development of *T. basicola* at all time points (Fig. III-5). In this experiment, the detection level of *T. basicola* increased by 10 fold in non-mycorrhizal roots at 24 and 36 hai, and by 4 fold at 1 wai.

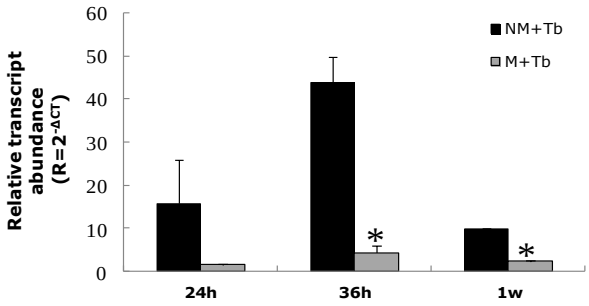


Figure III-4. Transcript abundance of the *Thielaviopsis basicola* LSU rRNA gene in *Petunia hybrida* roots colonized (M+Tb) or not (NM+Tb) by *Glomus mosseae* at 24 h, 36 h and 1 week after *T. basicola* inoculation. Asterisks indicate significant differences between mycorrhizal and non-mycorrhizal plants (t-test, $P = 0.05$, $n = 3$). *P. hybrida* ubiquitin gene expression was used for normalization. Bars= standard errors.

III.3- Discussion

The potential of three AM fungi to affect plant growth and induce bioprotection was investigated for the first time in petunia under soilless conditions. Petunia showed a greater mycorrhizal response to *G. mosseae* than to *G. intraradices*, an observation which concords with the previously reported better capacity of *G. mosseae* compared to *G. intraradices* in supplying nutrients to petunia plants (Shamshiri et al., 2011). In contrast to the *Glomus* isolates, *Gig. rosea* had a negative influence on plant biomass. Such negative effects of this AM fungal species have been observed before (Grunwald et al., 2009) and may be based on the higher carbohydrate sink strength of this fungus (Lerat et al., 2003).

Infection by *T. basicola* reduced shoot dry mass in non-mycorrhizal petunia whilst biomass, water and mycorrhiza-enhanced P contents were unaffected by pathogen development in *G. mosseae*-colonised plants. *G. intraradices* and *Gig. rosea* did not show such bioprotective effects, and the presence of *T. basicola* significantly reduced the development of *G. intraradices* within petunia roots. Such negative effects on AM fungal development have been observed in the interaction between pea and *Aphanomyces euteiches* (Bodker et al., 2002).

Mycorrhization in *G. mosseae*-colonised petunia plants was barely affected by inoculation with *T. basicola*, but disease symptoms were significantly reduced by the presence of this AM fungus at two and four weeks after pathogen infection. It has consistently been shown that bioprotection needs a high level of mycorrhization (Graham and Menge, 1982; Caron et al., 1986; Cordier et al., 1998; Khaosaad et al., 2007), but in petunia the bioprotective effect against *T. basicola* was induced despite low mycorrhization levels compared to other plants. The consistent bioprotective effect of *G. mosseae* against *T. basicola* in petunia represents an additional interest for horticulture. In greenhouses, the survival of *T. basicola* propagules poses a potentially serious problem to subsequent crops (Copes and Hendrix, 1996). *G. mosseae* inoculation into a soilless substrate may reduce infection levels by *T. basicola* so compensating for greenhouse chemical disinfestation insufficiency and contributing to control measures of contamination.

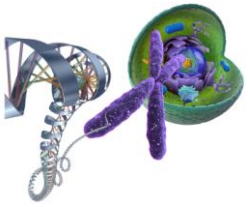
Following optimization of AM development using the MM method of inoculation, bioprotection by *G. mosseae* was better defined in the petunia/*T. basicola* pathosystem at earlier stages after *T. basicola* inoculation and before any root necrosis symptoms (24 hai and 36 hai). No effect of the presence of *T. basicola* on *G. mosseae* colonization levels in petunia roots was observed at 24 h, 36 h and 1 week after inoculation. In addition, an important reduction in *T. basicola* development (bioprotection), based on the expression of the *T. basicola* LSU rRNA gene, was already observed in *G. mosseae*-colonized roots at the two earliest time points, before root necrosis.

Although mycorrhiza-induced resistance has been reported for different AM fungi in different plants (Whipps et al., 2004), direct comparisons have always shown that *G. mosseae* is the most effective for members of the Solanaceae (e.g. Pozo et al., 1999; Garmendia et al., 2004). The basis for such differences is largely unknown, but the mycorrhization level at the moment of pathogen inoculation does not appear to play a role because this was highest for *G. intraradices* in the present study. This lack of correlation between levels of mycorrhiza development and bioprotection has also been reported in the pea-*A. euteiches* pathosystem (Bodker et al., 2002). Differences in mycorrhiza-induced bioprotection could be based on the expression of particular plant genes or proteins. For example, a β-1,3-glucanase was only expressed after *P. parasitica* inoculation of tomato roots colonised by *G. mosseae* but not by *G. intraradices* (Pozo et al., 1999).

The optimized bioprotection by *G. mosseae* root colonization against *T. basicola* sets the basis for molecular investigations in order to reveal plant genes that could be involved in mycorrhiza-induced resistance against the pathogen. As already indicated in chapter II, *T. basicola* is classified as a hemibiotrophic pathogen and infection must require complex signalling networks before and after physical contact with the host plant (Coumans et al., 2011) which lead to root necrosis in a susceptible host plant or restrict infection sites in resistant varieties like those identified in tobacco and cotton (Clayton, 1969; Niu et al., 2008). Therefore, investigation of petunia genes modulated by *G. mosseae* or *T. basicola* independently and/or by both fungi may help in understanding the molecular mechanisms induced in the AM-induced bioprotection.

Chapter IV:

Molecular investigations of mycorrhiza-induced resistance against Thielaviopsis basicola in Petunia hybrida



IV.1- Introduction

There have been very few molecular studies of plant tissue modifications related to the bioprotective effect of AM against plant pathogens, called mycorrhiza-induced resistance (MIR). One of the first analyses was carried out in tomato where callose appositions at sites of *Phytophthora parasitica* infection were only observable when the root system was colonised by an AM fungus (Cordier et al., 1998). Data from a *G. intraradices/*nematode/grapevine interaction also pointed to the so-called 'priming' as a mechanism involved in the protection or maintenance of tissue integrity in pathogen-challenged mycorrhizal roots (Hao et al., 2012). Priming is defined as an enhanced capacity for rapid and effective activation of cellular defence responses to effectively combat pathogen attack (van der Ent et al., 2009; Conrath et al., 2006). It is a common feature of induced systemic resistance (ISR) by beneficial PGPF and PGPR (Pieterse et al., 1998). Further evidence for MIR being similar to ISR is the fact that jasmonate, the phytohormone central for ISR, shows enhanced accumulation in mycorrhizal roots (Isayenkov et al., 2005). A study focusing on the JA signalling pathway provided evidence that MIR against take-all disease of wheat is independent of systemic accumulation of SA (Khoasaad et al., 2007), the basis for systemic acquired resistance (SAR). An early proteomic study on tomato roots showed reduced accumulation of PR proteins in *G. mosseae/P. parasitica* interactions as compared to non-mycorrhizal roots challenged with the pathogen, which already suggested that MIR may involve plant mechanisms independent of SA signalling (Dassi et al., 1996). However, transient AM priming of SA-dependent genes (*PR2* and *GST1*) was observed at early and late stages of *G. intraradices/R. solani/*potato interactions (Gallou, 2011). Alternatively, it has been proposed that the weak activation of defence-related genes or proteins by AM fungi in different plants conditions mycorrhizal roots for enhanced resistance when they are challenged with a pathogen (Gianinazzi and Gianinazzi-Pearson, 1990; Benhamou et al., 1994; Yao et al., 2003; Lee et al., 2005). This hypothesis is supported by the fact that arbuscule-containing cells are the site of defence-related gene up-regulation in mycorrhizal roots (Gianinazzi-Pearson et al., 1996; Cordier et al., 1998; Dumas-Gaudot et al., 2000) and that functional arbuscules are a prerequisite for MIR (Slezack et al., 2000).

For the present investigations of mechanisms underlying MIR against *T. basicola* in petunia, defence-related genes regulated by JA or SA were analysed in order to elucidate if the molecular basis of MIR is more related to ISR or SAR. These experiments were also targeted to the question whether priming plays a role in this system or if the constitutive expression of defence-related genes in mycorrhizal roots may play a determining role. In addition, the expression of plant genes previously shown to be modulated specifically in AM was analysed to monitor symbiosis functionality during interactions with the fungal pathogen.

IV.2- AM-related plant genes

As an indication of AM functionality, three petunia genes encoding plasma membrane transporters were selected based on results from a previous *P. hybrida* array analysis (Breuillin et al., 2011): phosphate transporter genes *PT3* and *PT4*, and the potassium transporter gene *KT*. *PT3* is mycorrhiza up-regulated and *PT4* expression is mycorrhiza-specific. The potassium transporter gene was included because its expression was found to be specific for AM in petunia (Breuillin et al., 2011) and because of the reported role of K in plant defence responses and resistance to diseases (Perrenoud, 1990).

Three other genes with reported AM-induced expression in other plants were selected based on their relation to defence responses and their regulation by different signalling pathways: i) AM-activated *PR10a*, coding for class 10 PR protein with RNase activity and a potential marker for SA accumulation (Ruiz-Lozano, 1999; Siciliano et al., 2007, Gutjahr and Paskowski, 2009), ii) AM-specific *Chit3*, encoding a member of the class III PR3 proteins, a JA-induced group of chitinases (Salzer et al., 2000), and iii) the AM-specific *GST*, a member of the glutathione-S-transferase gene family involved in plant detoxification of diverse exogenous and endogenous substrates in plants (Coleman et al., 1997; Müller et al., 2000).

IV.3- SA- and JA- regulated plant defence genes

In SAR, PR proteins are coded by a well known family of marker genes for the SA signalling pathway which plays a central role in both local and systemic induction of resistance (Durner et al., 1997; van Loon et al., 2006). Three PR petunia

genes were chosen to investigate the implication of SA in *G. mosseae*-induced bioprotection against *T. basicola*: *PR2* (endo-1,3-beta-glucanase), *PR5* (thaumatin-like) and *PR6* (proteinase inhibitor). Although, most of these PR proteins are induced by chemicals such as SA, a special class of *PR* inducers are hormones that include JA and ET (Edreva, 2005).

In particular, the proteinase inhibitor (PI) family of PRs is found JA-dependent in Solanaceae plants, petunia and tomato (Zahn et al., 2005; Hondo et al., 2007). However, a more recent study by Melvin and Muthukumaran (2008) showed that exogenous application of SA or JA induce similarly the enzyme activity of PI; whilst a mixture of both highly reduce the protein activity as compared to control tomato leaves (Melvin and Muthukumaran, 2008). Therefore and based on these facts, a cross-talk between signalling pathways mediated by these secondary messages could also operate the expression of *PR* genes (Edreva, 2005).

A number of plant genes known to be JA-related and activated during ISR by PGPR (Raymond and Farmer, 1998) were also selected: two class I PR3 chitinases (*Chit1a* and *Chit1b*), phenylalanine ammonia lyase (*PAL1*), and chalcone synthase (*CHS*) involved in the pathway of phenylpropanoid biosynthesis. To study the influence of AM on jasmonate production during bioprotection, genes encoding two enzymes involved in the jasmonate biosynthetic pathway were studied: 13-lipoxygenase (*LOX*) and allene oxide cyclase (*AOC*). *LOX* belongs to a multigene family and is the first enzyme in the octadecanoid pathway for biosynthesis of jasmonic acid starting from α-linolenic acid. *AOC* encodes a later enzyme in the same pathway which catalyses formation of the JA precursor and first oxilipin product, 12-oxo-phytodienoic acid, OPDA.

IV.4- Plant defence genes with other functions

Three other defence-related petunia genes were selected in order to investigate their induction by *T. basicola* and their possible role in AM-induced bioprotection: an enhanced disease resistance 1 gene (*EDR*), a callose synthase gene (*CAL*), and a NADPH: cytochrome P450 reductase (*P450*). In *Arabidopsis thaliana*, the *EDR* gene encodes a kinase protein involved in disease resistance, stress response signalling and cell death regulation (Tang et al., 2005). This kinase seems to negatively affect JA as

well SA signalling (Wawrzynska et al., 2010**).** Callose is typically induced by PAMPs during relatively early stages of pathogen invasion and associated with the formation of cell-wall opposition barriers (papilla structures) (Brown et al., 1998; Gomez-Gomez et al., 1999; Luna et al., 2011), like those linked to AM-related ISR against *P. parasitica* in tomato (Cordier et al., 1998). Callose synthase activity in *A. thaliana* interestingly activates SA, but inhibits SA defence pathways (Nishimura et al., 2003). The P450 enzymes family mediates the synthesis of a subset of secondary metabolites (allelochemicals) using a pathway other than phenylpropanoids, which leads to the synthesis of terpenoids, natural products including many plant defence compounds (monoterpenes and sesquiterpenes), as well as accessory pigments (carotenoids) and hormones (GAs and ABA) (McGarvey and Croteau, 1995). Most of eukaryotic P450s are not self-sufficient enzymes, and their catalytic activities rely strictly on the electron donor NADPH:cytochrome P450 reductase (Lu et al., 1969).

IV.5- Results

PCR primers for the petunia genes related to AM functioning and/or defence were designed from published petunia gene sequences (Table 1, Materials and Methods). The potential implication of the selected genes in mechanism(s) underlying AM bioprotection was investigated by quantifying their transcripts (RT-qPCR) in petunia roots from two independent experiments 24 hai, 36 hai or 1 wai. Transcript abundance was normalized using the *UBQ* housekeeping gene and, for clarity, expression of the different genes in petunia roots inoculated with *G. mosseae* and/or *T. basicola* is presented relative to non-inoculated control plants, according to the procedure of Pfaffl (2001).

IV.5.1- Expression of AM-related plant genes

Relative expression of all the three petunia genes involved in AM functionality at the level of membrane nutrient transfer (*PT3*, *PT4* and *KT*) was highest in mycorrhizal roots (M) compared to non-mycorrhizal control plants (NM) at all time points (24 hai, 36 hai and 1 wai). The presence of *T. basicola* did not significantly affect their gene expression and no interaction between both fungi (*G. mosseae/T. basicola)* was detected in the M+Tb treatment. An exception was *PT3* expression at 1

wai, which was significantly decreased by *T. basicola*; this tendency was maintained in *G. mosseae*-colonized roots (M+Tb) but to a lesser extent and without affecting *G. mosseae*-enhanced expression as compared to non-inoculated-control plants (Fig. IV-1).

The three genes belonging to the defence category which may also reflect mycorrhiza functionality due to their specific expression (*Chit3*, GST) or up-regulation in AM interactions (*PR10a*) were also highly induced by *G. mosseae* root colonization as compared to non-mycorrhizal controls, independent of the presence of *T. basicola* (treatment M or M+Tb) at all time points (Fig. IV-1). Although the expression of *Chit3* and *GST* was slightly decreased by the presence of *T. basicola* (M+Tb) at 24 hai, this did not cause a significant difference between the treatments M and M+Tb as compared to non-inoculated control plants. The significant interaction between both fungi at 24 hai was related to a negative effect of *T. basicola* early inoculation on the expression of *Chit3* and *GST*, as showed in the NM+Tb treatment. No significant interaction between both fungi in the treatment M+Tb was detected at the later time points (36 hai, 1 wai). The expression of *PR10* was not affected by the pathogen in mycorrhizal and non-mycorrhizal roots at any time point (Fig. IV-1).

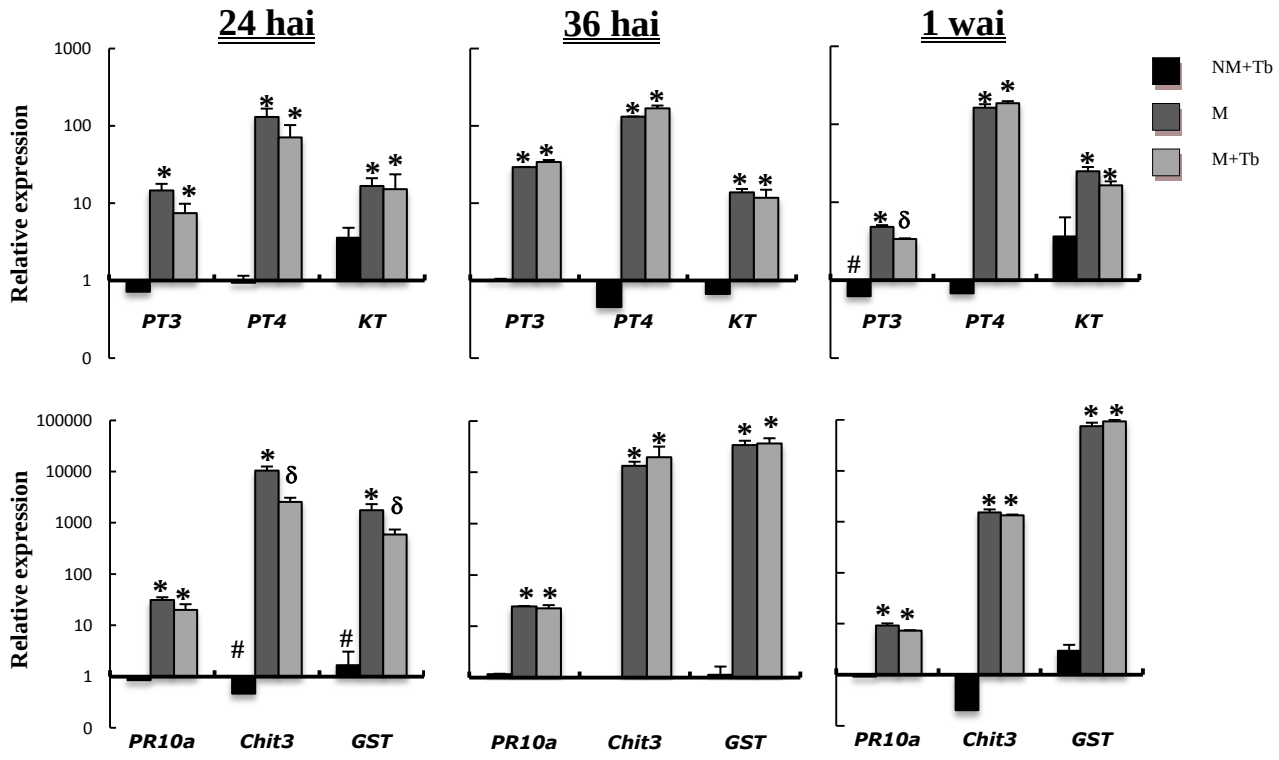


Figure IV-1. Transcript accumulation in *Petunia hybrida* root systems of AM-related genes encoding nutrient transporters and defence-related proteins. *P. hybrida* plants were inoculated with *Glomus mosseae* and challenged with *Thielaviopsis basicola* 3 weeks later. Values for gene expression normalized with the *UBQ* reference gene are shown at 24 h, 36 h and 1 week after pathogen inoculation. Values obtained from the 3 treatments: NM+Tb (inoculated with *T. basicola*), M (mycorrhizal), M+Tb (in presence of both fungi) are shown as ratios to values obtained in control non-inoculated (NM) plants (value 1). Delta (δ) above columns indicates a significant interaction between *G. mosseae* and *T. basicola* according to two-way ANOVA (P=0.05, n=3). Asterisks or hashed icons (#) above columns indicate significant effect of *G. mosseae* or *T. basicola*, respectively. *PT3:* phosphate transporter 3; *PT4*: phosphate transporter 4, *KT:* potassium transporter, *PR10:* pathogenesis related protein 10a; *chit3:* chitinase class III, *GST:* glutathione-S-transferase and *UBQ:* ubiquitin. Bars= standard errors.

IV.5.2- Expression of SAR or ISR-related defence genes

Expression of the three investigated *PR* genes (*PR2*, *PR5* and *PR6)* tended to decrease in roots colonized by *G. mosseae* alone at all time points as compared to non-inoculated control plants (24 hai, 36 hai, 1 wai). This effect was significant for *PR2* and *PR6* 24 hai, and *PR5* and *PR6* 36 hai (Fig. IV-2). Pathogen inoculation of *G. mosseae*-colonized petunia roots led to a significant interaction between both fungi 36

hai in the treatment M+Tb for all three genes (Fig. IV-2). This interaction resulted in a decrease in the reduced relative values of *PR2*, *PR5* and *PR6* expression as compared to non-inoculated control plants (closer to level 1 of expression). This tendency continued to 1 wai, where significant enhanced expression of the *PR6* gene was observed in the treatment M+Tb.

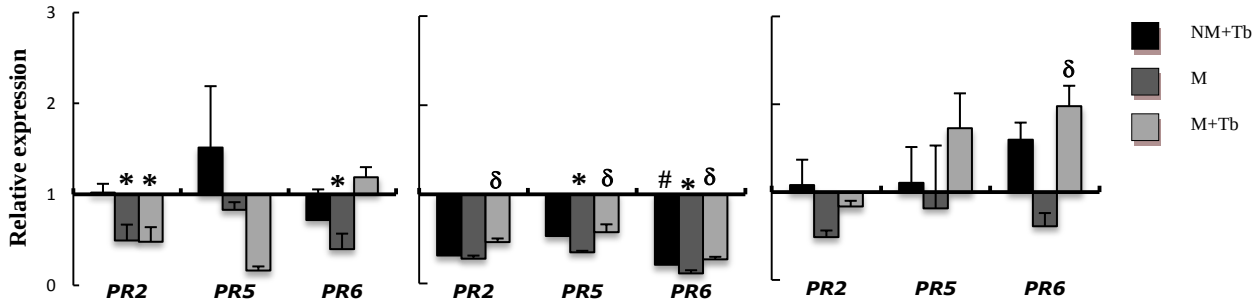


Figure IV-2. Transcript accumulation in *Petunia hybrida* root systems of defense genes related to salicylic acid (SA) signaling pathway. *P. hybrida* plants were inoculated with *Glomus mosseae* and challenged with *Thielaviopsis basicola* 3 weeks later. Values for gene expression normalized with the *UBQ* reference gene are shown at 24 h, 36 h and 1 week after pathogen inoculation. Results from 3 treatments: NM+Tb (inoculated with *T. basicola*), M (mycorrhizal), M+Tb (in presence of both fungi) are expressed relative to control non-inoculated (NM) plants (value 1). Delta (δ) above columns indicates a significant interaction between *G. mosseae* and *T. basicola* according to two-way ANOVA (*P*=0.05, *n*=3). Asterisks or hashed icons (#) above columns indicate significant effect of *G. mosseae* or *T. basicola*, respectively. *PR2:* pathogenesis related protein 2, *PR5:* pathogenesis related protein 5, *PR6:* pathogenesis related protein 6, *UBQ:* ubiquitin. Bars= standard errors.

Six defence genes involved in JA biosynthesis or related to its signalling pathway were studied: *AOC, LOX, Chit1a, Chit1b, PAL1* and *CHS.* The two genes encoding enzymes in JA biosynthesis responded differently to *G. mosseae* or *T. basicola* (Fig. IV-3). While *AOC* tend to be unaffected (24 hai, 36 hai) or even down-regulated (1 wai) by *G. mosseae,* the selected isoform of *LOX* appeared to be up-regulated by AM at 24 hai and 36 hai. This effect for *LOX* disappeared at 1 wai where no significant difference was detected between mycorrhizal and non-mycorrhizal plants. *T. basicola* down-regulated *AOC* expression over the whole experimental period, but down-regulation of *LOX* by the pathogen at 24 hai switched to up-regulation at later time points. A significant interaction between *G. mosseae* and *T. basicola* was detected for *LOX* at the early time points where mycorrhizal up-

regulation of the gene was diminished in presence of the pathogen and for *AOC* at the late date where an additive effect was detected (Fig. IV-3).

For the JA-induced PR3 chitinase genes, two family members were studied due to their important role in plant defence against fungal pathogens (*Chit1a, Chit1b*). While *Chit1a* was AM-induced, but not affected by the pathogen, across the time points, *Chit1b* showed down-regulation by both fungi and this was significant for mycorrhiza at 36 hours. Interestingly, a significant interaction between both fungi resulted in a compensation of this reduction and in an expression level as obtained in control roots (Fig. IV-3). The genes encoding the two enzymes related to secondary metabolite production, *PAL1* (general furanocoumarins) and *CHS* (flavonoids in particular), showed no significant regulation any treatment. Only *G. mosseae* induced *Chs* expression at one wai, but to a low extent (around two-fold).

IV.5.3- Expression of defence genes with different functions

In addition to genes clearly involved in the SA or JA pathway, *EDR*, *CAL* and *P450* show more complex functions during plant defence responses (see above). No significant variations were detected between treatments (NM+Tb, M, M+Tb), or compared to non-mycorrhizal control plants for *EDR* at all sampling times (Fig. IV-3). However, *CAL* expression was significantly down-regulated at 1 wai by *G. mosseae* (M) or by pathogen infection (NM+Tb). *P450* expression, was increased in mycorrhizal roots at 36 hai, but decreased at 1 wai in the presence of both fungi. The interaction between *G. mosseae* and *T. basicola* led to an even more intense down-regulation of *P450* than when they were singly present in the root system.

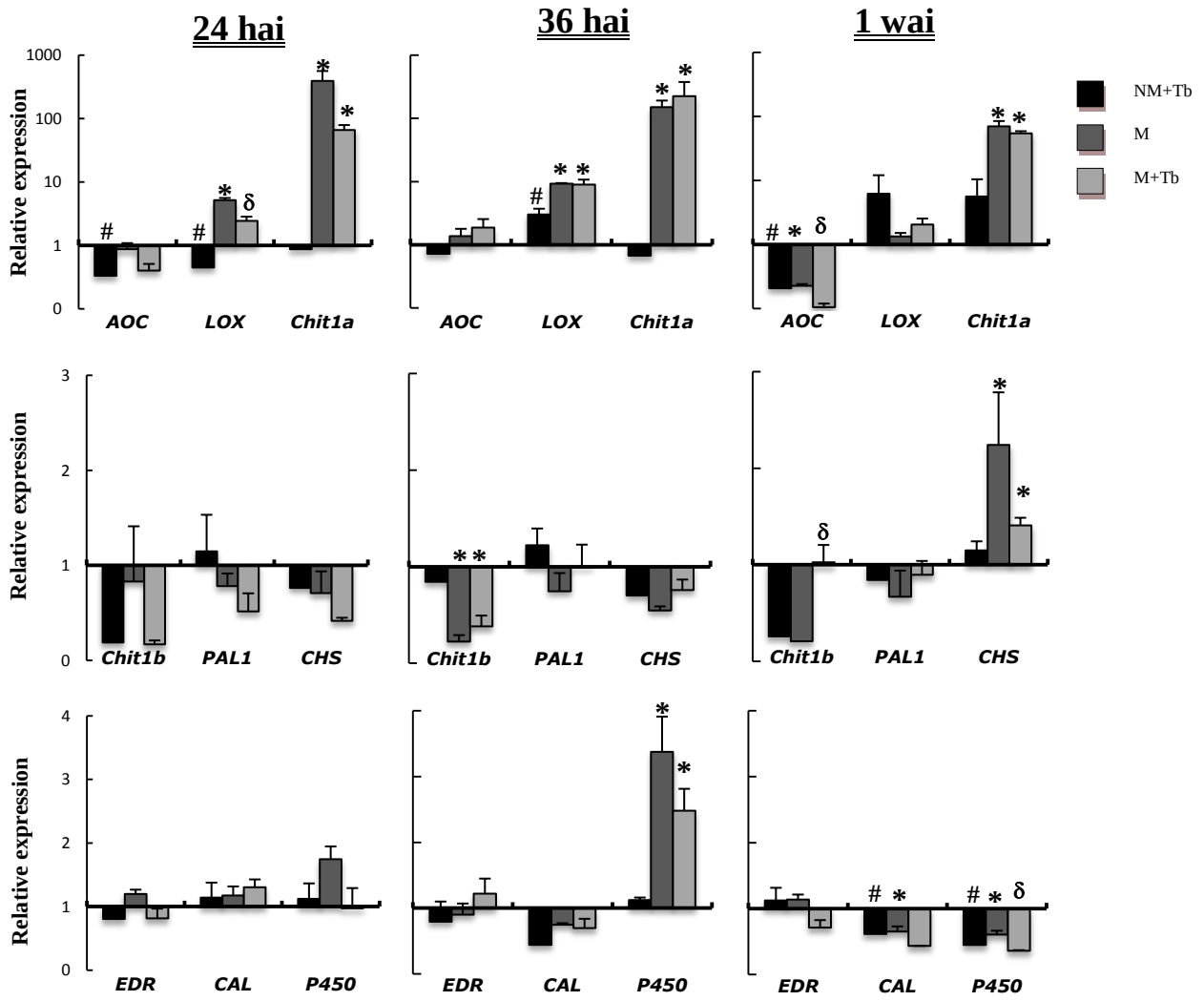


Figure IV-3. Transcript accumulation in *Petunia hybrida* root systems of defense genes related to jasmonic acid (JA) signaling pathway. *P. hybrida* plants were inoculated with *Glomus mosseae* and challenged with *Thielaviopsis basicola* 3 weeks later. Values for gene expression normalized with the *UBQ* reference gene are shown at 24 h, 36 h and 1 week after pathogen inoculation. Results from 3 treatments: NM+Tb (inoculated with *T. basicola*), M (mycorrhizal), M+Tb (in presence of both fungi) are expressed relative to control non-inoculated (NM) plants (value 1). Delta (δ) above columns indicates a significant interaction between *G. mosseae* and *T. basicola* according to two-way ANOVA (P=0.05, n=3). Asterisks or hashed icons (#) above columns indicate significant effect of *G. mosseae* or *T. basicola*, respectively. *AOC:* allene oxide cyclase, *LOX:* lipoxygenase; *Chit1a*: chitinase class Ia, *Chit1b:* chitinase class Ib; *PAL1*: phenylalanine ammonia lyase 1; *CHS:* chalcone synthase; *EDR1:* enhanced disease resistance; *CAL:* callose; *P450:* NADPH: cytochrome P450 reductase; *UBQ:* ubiquitin. Bars=standard errors.

IV.4- Discussion

Genes linked to mycorrhizal function and to defence responses of plants were targeted in order to evaluate symbiont-pathogen interactions and the molecular basis of MIR. The nutritional benefit of mycorrhiza is well known as the main reason for improved growth of host plants (Gerdeman, 1968; Mosse, 1973; Rhodes, 1980). Molecular analyses targeting this central function have identified a number of mycorrhiza-regulated macronutrient transporters, mainly inorganic phosphate (Pi), in both partners of the symbiosis (Balestrini and Lanfranco 2006). Phosphorus is an indispensable nutrient for plant growth and AM fungi are able to transport Pi from soils to plant roots resulting in a 3 to 5 times increased P flux compared to non-mycorrhizal roots (Smith and Read, 1997). Another important macro-nutrient is potassium and a mycorrhiza-regulated K transporter has been identified in petunia (Breuillin et al., 2010). Hence, the gene expression for two Pi transporters and one K transporter was analysed in order i) to confirm the functionality of the petunia/*G. mosseae* mycorrhizal system, and ii) to reveal any significant influence of *T. basicola* on this mycorrhizal functionality. In addition to the expression of mineral nutrient transporters, genes encoding proteins implicated in plant stress responses are also induced during AM fungal colonisation of plant roots and it has been postulated that such a phenomenon may be related to MIR (Gianinazzi-Pearson et al., 1996; Dumas-Gaudot et al., 2000; Pozo and Azcon-Aguilar, 2007). Consequently, expression of the AM-related defence genes encoding a chitinase (Salzer et al., 2000), a glutathione S-transferase (Wulf et al., 2003; Brechenmacher et al., 2004) and the pathogenesis-related protein PR10 (Ruiz-Lozano et al., 1999) was also monitored during petunia/*G. mosseae*/T. *basicola* interactions. Root expression profiling of these different categories of genes showed that AM development in petunia roots was functional at two levels: mineral nutrient transfer (*PT3*, *PT4* and *KT*) and modulation of defence-related responses (*Chit3, GST* and *PR10*). In addition, the development of *T. basicola* in mycorrhizal roots of petunia did not affect AM functionality at any stage of pathogen infection (24 hai, 36 hai and 1 wai). These results are in agreement with those reported in Chapter IV where no significant differences in the colonisation patterns between mycorrhizal plants inoculated or not with the pathogen were observed.

Although MIR in root systems was reported early in mycorrhizal research, for example for tobacco and cotton (Baltruschat and Schönbeck, 1975; Schönbeck and Dehne, 1977), molecular investigations are rare. Previous proteomic studies on *G. mosseae/P. parasitica* interactions in tomato, and of mycorrhizal *M. truncatula* roots inoculated with the root pathogen *Aphanomyces euteiches* indicate that MIR may involve plant mechanisms other than the classical defence responses (Dassi et al., 1996; Colditz et al., 2005). In another approach targeting genes in bean plants encoding enzymes involved in flavonoid biosynthesis (Guillon et al., 2002), all genes were induced by *Rhizoctonia solani* but none by an AM fungus. For the present study on petunia/*G. mosseae/T. basicola* interactions, a more systematic approach was adopted to identify candidate genes playing a role in MIR by targeting SA-induced genes involved in SAR and JA-induced genes involved in ISR, the two pathways of systemic plant protection against pathogens. In this context, a recent study on *G. intraradices/R. solani/*potato interactions has reported transient AM priming of SA-dependent genes (*PR2* and *GST1*) at early and late stages (Gallou, 2011).

The expression of three defence genes reported to be related to SA signalling pathway (*PR2, PR5, PR6*) was down-regulated or unaffected by *G. mosseae* inoculation, and no enhanced expression in *T. basicola*-inoculated plants was observed at any time point except for the proteinase inhibitor-encoding gene *PR6*. Expression of this gene was significantly up-regulated in mycorrhizal roots 1 wai with the pathogen, although only two-fold. Since protection of mycorrhizal roots against the spread of *T. basicola* was already observed at the early time points (24 and 36 hai), it is doubtful that this gene is involved in MIR. Contrary to *G. intraradices/R. solani/*potato interactions (see above), it would appear that the SA signalling pathway is not involved in *G. mosseae*-induced resistance in the petunia/*T. basicola* pathosystem and that mechanisms underlying MIR are not to related to those driving SAR.

Concerning ISR, a number of genes were selected which on the one hand encodes enzymes involved in JA biosynthesis, and on the other hand are regulated by JA. In the pathway of JA synthesis, *LOX* encodes a lipoxygenase which catalyses the conversion of alpha-linoleic acid derived from membrane lipids to hydroxyperoxylinoleic acid, while *AOC* encoding allene oxid cyclase is involved in a

later step converting epoxylinoleic acid to oxophytodienoic acid. Interestingly, while *LOX* is clearly mycorrhiza-upregulated at the early (24 and 36 hours) time points, AOC is not significantly affected and is even down-regulated at 1 wai. This is in agreement with a finding in mycorrhizal tomato plants where gene expression and phytohormone analysis also suggested that JA is not accumulating, but one of the precursor oxylipins (López-Ráez et al., 2010). Such an oxylipin could be involved in the induction of genes being involved in MIR as *T. basicola* represses *LOX* expression at 24 hours and this repression is overbalanced by the AM fungus. Further analyses have to specifically identify this oxylipin and to show if it has phytohormone-like function similar to JA.

Concerning the plant defence genes described as modulated by JA, a PR3 chitinase class I gene (*Chit1a*) responded in a similar way to the other AM-upregulated defence-related genes. Also like *PR10, GST* and *Chit3,* it is not affected by *T. basicola*. In contrast, expression of *Chit1b*, as well as the other investigated defence genes *PAL1, CHS, EDR, CAL* and *P450,* showed a similar tendency to *PR* genes. Their expression was mainly not significantly affected, in some cases it was mycorrhiza-upregulated, as for *CHS* at 36 hours or *P450* after one week, or down-regulated by the presence of both fungi in roots, as for *CAL* or *P450* at the latest sampling date. None of these patterns seemed to be related to MIR and could therefore not contribute to the understanding of this phenomenon.

None of the studied genes showed ‘priming’, but a number of defence-related genes were mycorrhiza-induced and not further affected by the pathogen. Therefore, *G. mosseae*-induced resistance to *T. basicola* in petunia could simply be based on the constitutive expression of defence-related genes in mycorrhizal roots. In addition the expression pattern of *LOX* indicates that a yet unknown compound in the JA pathway might be involved in the induction of such genes in the mycorrhizal symbiosis.

Chapter V:

Investigation of systemic bioprotection by Glomus mosseae against Thielaviopsis basicola

V.1- Introduction

Previous studies have provided evidence that mechanisms similar to ISR by plant growth promoting rhizobacteria (PGPR) are also involved in the bioprotection against pathogens by AM fungi, called MIR (Conrath et al., 2006; Pozo and Azcon-Aguilar, 2007). Colonisation of plant roots by AM fungi is not only able to induce plant resistance in whole root systems as described in the previous chapter, but also systemically either in shoots or between different parts of a same root system. The systemic effect on shoots was observed by an enhanced tolerance of mycorrhizal plants when challenged with several necrotrophic leaf pathogens (review, Whipps, 2004), whilst systemic MIR in root systems has been consistently shown against different fungal pathogens and nematodes using experimental split-root systems (Rosendahl, 1985; Cordier *et al.*, 1998; Slezack *et al.*, 1999; Zhu and Yao, 2004; Khaosaad *et al.*, 2007; Elsen *et al.*, 2008).

V.2- Results

A split-root system was set up to investigate whether bioprotection is also systemically induced by *G. mosseae* against *T. basicola* in petunia. Root system halves of petunia shoot cuttings rooted in Ferty 8 (0.5 mM KH_2PO_4) for 3 weeks were planted into two juxtaposed pots compartments containing 400 g vermiculite/sand (1:1/v:v). One half of the root system was inoculated (M) or not (NM) with the AM fungus *G. mosseae*. The pathogen *T. basicola* was added 5 weeks later to the other halves of the root systems. Hence two treatments were obtained: (I) one root system half inoculated with the pathogen and half with *G. mosseae* (M/NM+Tb)or (II) one root system half inoculated with the pathogen and one half free of either fungus (NM/NM+Tb) (Fig. V-1).

Plants were harvested 48 h after *T. basicola* inoculation and mycorrhization parameters were estimated. In addition RNA was extracted for real-time RT-PCR to monitor pathogen development based on LSU rRNA gene transcript abundance and the expression of petunia genes related to AM functioning (*PT3, PT4, KT, Chit3, GST, PR10a*) and/or to defense (*AOC, PAL1, Chit1a, Chit1b, EDR, CAL*).

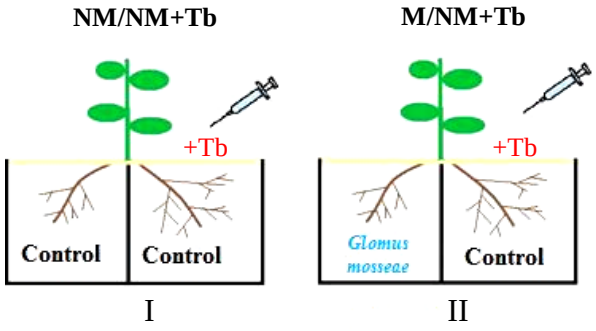


Figure V-1. Petunia split-root system to investigate systemic bioprotection against *Thielaviopsis basicola* induced by *Glomus mosseae:* NM, control non-mycorrhizal; NM+Tb, control *T. basicola*-inoculated; M, *Glomus mosseae*-inoculated.

V.2.1- Petunia growth, mycorrhizal colonization and *T. basicola* development

No significant differences were observed in petunia shoot growth between treatments (Table V-1). Root fresh mass was lower in both halves of the M/NM+Tb than in the NM/NM+Tb split root system. Mycorrhizal colonization intensity and arbuscule abundance in *G. mosseae*-inoculated root compartments were comparable to the previous values on mycorrhization of cuttings (Chapter I), while no AM fungal structures could be detected in the other three compartments.

Table V-1. Petunia shoot fresh mass (sFM), root fresh mass (rFM) and mycorrhization colonization parameters (M% and A%) in split-root halves: control (NM), control inoculated with *Thielaviopsis basicola* (NM+Tb) or inoculated with *Glomus mosseae* (M) in two different treatments (I and II). No significant differences were observed between treatments according to one-way ANOVA ($P = 0.05$, $n = 2$). ± means standard error.

	I		**II**	
	NM	NM+Tb	M	M+Tb
sFM(g)	13.1 ±1	13.1 ±1	12.5 ±0	12.5 ±0
rFM(g)	7.8 ±0	6.5 ±0.3	4.7 ±1.3	4.8 ±0.3
rFM/sFM	0.6	0.5	0.4	0.4
M%	-	-	18.2 ± 8.6	-
A%	-	-	13.8 ± 5.6	-

T. basicola rRNA transcript abundance was 3.5 fold lower in the inoculated root halves of the Gm/Tb treatment as compared to the NM/NMTb root systems (Fig. V-2). *T. basicola* could not be detected in non-inoculated compartments of either system (data not shown).

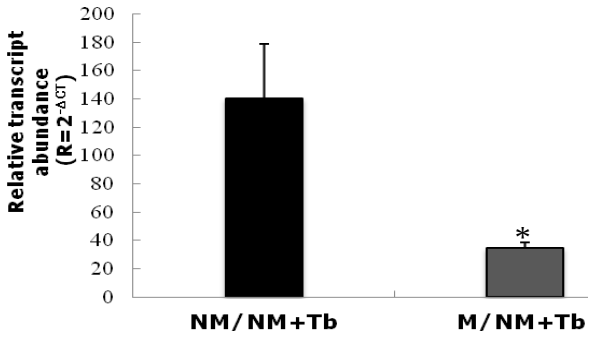


Figure V-2. Transcript abundance of the *Thielaviopsis basicola* LSU rRNA gene related to petunia ubiquitin gene expression in petunia plants in pathogen inoculated halves of split-root systems: control/control inoculated with *Thielaviopsis basicola* (NM/NM+Tb), *Glomus mosseae* colonized/control inoculated with *T. basicola* (M/NM+Tb). Asterisks indicate significant differences between treatments (t-test, $P = 0.05$, $n = 2$). Bar= standard error.

V.2.3- Petunia gene expression

AM-specific activation of the petunia genes *PT3, PT4, KT, Chit3, GST* and *PR10a* was induced by *G. mosseae* root colonisation (Fig. V-3). This occurred locally in the mycorrhizal half of the split-root systems and the presence of *T. basicola* 48 hai had no significant effect on the expression of these genes.

For the SA-dependent defence genes, transcript accumulation of *PR2* was greater in both *G. mosseae* and *T. basicola*-inoculated root system halves of the M/NM+Tb treatment as compared to either root system half in the NM/NM+Tb treatment (Fig. V-4). Neither fungus had a clear effect on *PR5* expression in root system halves across treatments. A significant increase in transcripts was detected for the *PR6* gene in *T. basicola*-inoculated halves of the root system in the treatment M/NM+Tb, compared to all the other root system halves.

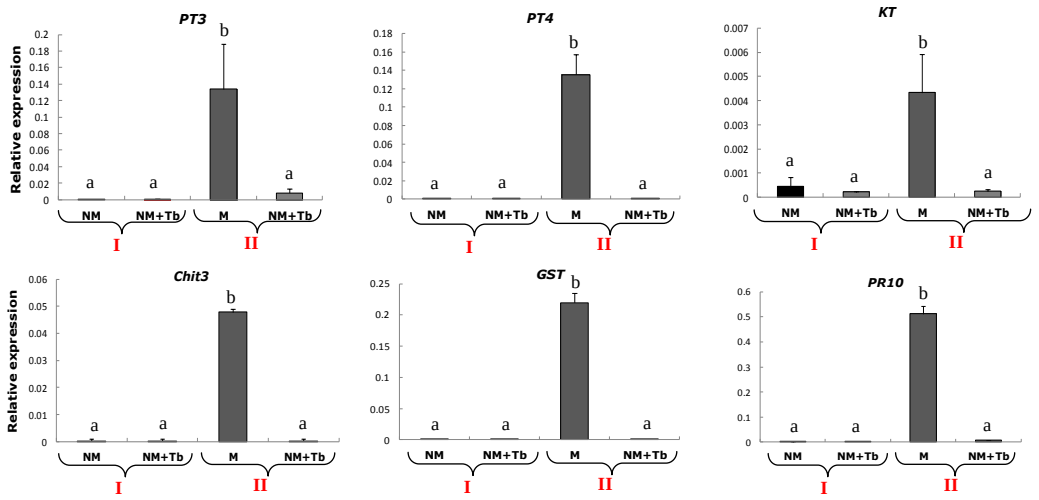


Figure V-3. Relative expression of *Petunia hybrida* mycorrhiza-regulated genes. Expression of the phosphate transporters (*PT3*, *PT4*), potassium transporter (*KT*), chitinase class III (*chit3)*, glutathione-S-transferase (*GST*) and PR protein 10a (*PR10*) genes, normalized by the ubiquitin gene (*UBQ*), was quantified in all four compartments of the two split-root systems (I and II). NM: control, NM+Tb: inoculated with *Thielaviopsis basicola*, M: inoculated with *Glomus mosseae.* Different letters above columns indicate significant differences between treatments according to one-way ANOVA (P = 0.05; n = 2).

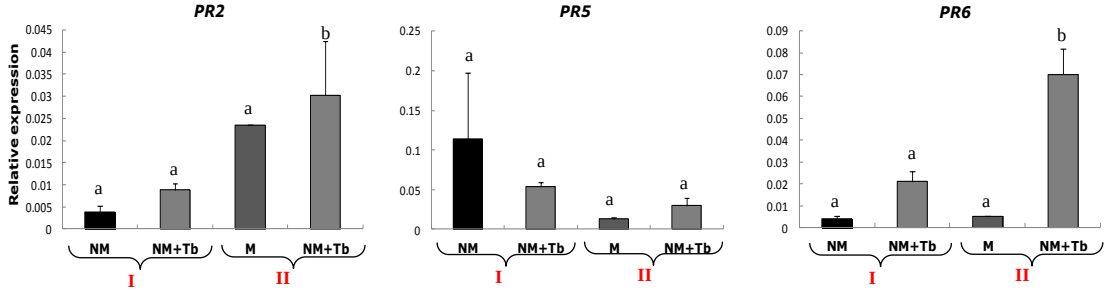


Figure V-4. Relative expression of *Petunia hybrida* genes related to salicylic acid (SA) signalling pathways. Expression of three pathogenesis related protein encoding genes (*PR2*, *PR5* and *PR6*), normalized by the ubiquitin gene (*UBQ*), was quantified in all four compartments of the two split-root systems (I and II). NM: control, NM+Tb: inoculated with *Thielaviopsis basicola*, M: inoculated with *Glomus mosseae.* Different letters above columns indicate significant difference between treatments according to one-way ANOVA (P = 0.05; n = 2).

Expression of the defence genes *PAL1* and *Chit1b* implicated in JA-related signalling pathways was not significantly affected by the presence of *T. basicola* or *G. mosseae* in one half of the petunia root system as compared to the control root system halves (Fig. V-5). Concerning *LOX* and *AOC* implicated in JA-biosynthesis, *Lipox* expression was too low to be detected except in the mycorrhizal compartment of the split-root system M/NM+Tb. However, a significant effect was detected on the

gene implicated in JA oxylipin biosynthesis (*AOC*) in the *T. basicola*-inoculated root system half of the M/NM+Tb treatment as compared to NM/NM+Tb root systems. The *Chit1a* gene was induced by *G. mosseae* specifically in AM root system halves whilst no significant effect was shown in any treatments for the two JA-regulated genes, *CAL* and *EDR1*.

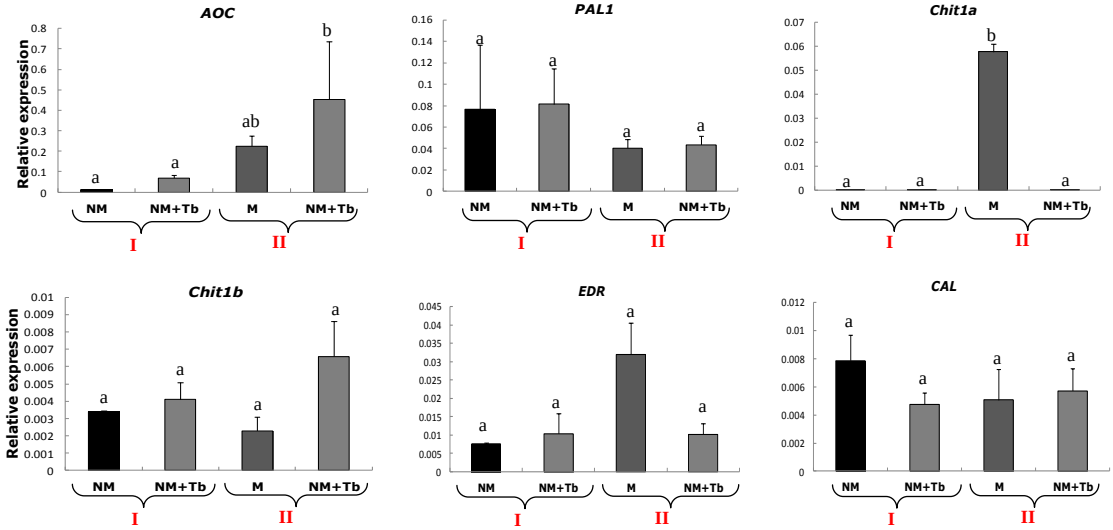


Figure V-5. Relative expression of *Petunia hybrida* genes related to jasmonic acid (JA) signalling pathways. Gene expression, normalized by the ubiquitin gene (*UBQ*), was quantified in all four compartments of the two split-root systems (I and II). NM: control, NM+Tb: inoculated with *Thielaviopsis basicola*, M: inoculated with *Glomus mosseae.* Different letters above columns indicate significant difference between treatments according to one-way ANOVA (P = 0.05, n = 2). *AOC:* allene oxide cyclase, *PAL1:* phenylalanine ammonia lyase 1, *Chit1a:* chitinase class Ia, *Chit1b*: chitinase class I b, *EDR*: enhanced disease resistance, *CAL*: callose synthase.

V.3- Discussion

A split-root system that is connected via the shoots was successfully established using petunia cuttings in a soilless substrate to investigate systemic MIR by *G. mosseae* against *T. basicola.* Whilst petunia shoot fresh mass was comparable across treatments, the presence of mycorrhiza appeared to negatively affect development of the whole root system. Such negative effects have been reported before as, for example, in the interaction between *M. truncatula* and *G. rosea* (Grunwald et al., 2009), and it was suggested that the sink strength of the AM fungus could not be balanced by the carbohydrate supply of the plant. However, in the present split-root system, biomass was affected not only in the *G. mosseae*-inoculated

half but also in the non-inoculated half. A possible explanation for this might come from a very recent study where, by applying proteomics and transcriptomics on single cell level, the expression of genes involved in transport, carbohydrate and lipid metabolism in arbuscule-containing cells and non-arbuscule-containing cells of mycorrhizal roots was analysed (Gaude et al., 2011). This showed that carbohydrates are mobilised in the non-colonised cells and transported to cells containing the fungal arbuscules. Mobilisation of these carbohydrates in the non-colonised root parts and their transport over long distances towards the mycorrhizal root half could contribute to the systemic reduction of root biomass in the split root system.

G. mosseae BEG12 was able to systemically induce bioprotection against *T. basicola* in petunia root systems in the vermiculite/sand soilless substrate. The decrease in *T. basicola* development in one half of the petunia split-root system by mycorrhization in the second half indicates the existence of a mobile signal. Such a systemic inhibitory effect by mycorrhiza on sequential inoculation of the juxtaposed compartment is not exclusive to pathogens. In fact, an enhanced systemic suppression was also reported by *G. mosseae* BEG 12 against AM fungi when subsequently inoculating the non-mycorrhizal root half and similar to bioprotection against pathogens, this suppression is more efficient with a fully established mycorrhizal system (Vierheilig, 2004).

All the AM-related petunia genes were highly expressed in the mycorrhizal halves of the split-root system. Transcripts were absent from non-mycorrhizal root compartments and no systemic effect could be observed. This shows that the genes are only locally induced and do not seem to play a role in the systemic activation of MIR in petunia plants. This was not only true for the transporter genes, but also for those encoding the defence-related proteins *Chit3, GST, PR10* and *Chit1a*. In agreement with this finding, transcripts of *Chit3* and *GST* have been localized specifically in arbusculated cells of potato (Strittmatter et al., 1996; Franken et al., 2000), *M. truncatula* (Wulf et al., 2003; Elfstrand et al., 2005) and of pea mycorrhiza (Kutnetsova et al., 2010). This means that even if mycorrhiza-induced expression of these defence-related genes plays a role in the resistance of arbuscule-containing root tissues against the pathogen, as discussed in the previous chapter, they cannot be responsible for the bioprotection in the non-mycorrhizal half of the split root system.

In contrast, enhanced *GST* expression was detected in non-mycorrhizal potato root halves challenged with *R. solani* in a mycorrhizal split-root system, suggesting a role of the gene in AM systemic MIR in this experimental system (Gallou, 2011). In grapevine inoculated with *G. intraradices* and a nematode as pathogen, however, the gene was also only locally induced (Hao et al., 2012).

Concerning SA induced genes, *PR5* shows a trend of being repressed similar to the pattern described for whole root systems in Chapter IV. *PR2* and *PR6*, however, are not repressed by *G. mosseae* or *T. basicola*, as in the whole root system experiments described before. One reason could be that splitting a root causes an additional abiotic stress which affects the expression patterns. Another possibility is that a strict separate colonisation of the same root system has a different effect from that if both fungi are present in close proximity. Interestingly, *PR2* and *PR6* indicate a priming effect by G. mosseae. Both genes are highest induced in the pathogen-infected root half, if the AM fungus is present in the other half. Hence, while SA-signalling and priming cannot be observed when both fungi are in the same parts of a root system, these two mechanisms could play a role in long distance MIR. *PR2* and *PR6* have been described before as players in MIR. *PR2* codes for one of the hydrolytic enzymes (beta-1,3-glucanase) that has been reported to be enhanced during systemic AM bioprotection in tomato and potato root systems (Pozo et al., 2002; Gallou, 2011), and the proteinase inhibitor-encoding *PR6* gene was found to be down-regulated in mycorrhizal grapevine roots but up-regulated in nematode challenged non-mycorrhizal halves of mycorrhizal root systems (Hao et al., 2012). In addition, *PR6* was reported to contribute to ISR by *Pseudomonas aeruginosa* in tomato leaves against *Spodoptera exigua* (Melvin and Muthukumaran, 2008).

Although the present results on petunia/*G. mosseae*/*T. basicola* interactions, may reflect a potential similarity between SAR and MIR in a split root system, this does not exclude the involvement of functions known for ISR. In fact, *AOC* was systemically induced in *T. basicola*-challenged non-mycorrhizal parts of mycorrhizal petunia root systems (M/NM+Tb). This suggests a putative role of JA as a mobile signal between both compartments in the system M/NM+Tb that is able to induce for example JA-dependent *PR6* gene expression (Zahn et al., 2005). This obvious effect on *AOC* expression in the split-root system could not however be observed in whole

root systems (results from chapter IV). Consequently, primed synthesis of JA may not occur when *G. mosseae* and *T. basicola* colonise the same part of the root. A similar phenomenon has been observed for the root-colonising PGPR *Bacillus cereus*. Full induced resistance against a bacterial pathogen could only be achieved if both SAR and ISR were active (Niu et al., 2011). Hence, the synergistic activity of SA and JA signalling pathways, together with priming, could also be a mechanism for MIR when an AM fungus and a fungal pathogen are spatially separated as in split root systems. Further investigations are still necessary to better elucidate the role of each phytohormone in MIR.

4 -

Concluding Remarks and Perspectives

Many countries are developing strategies to decrease pesticide application in crop production. The new European Union (EU) legislation* on pesticides obliges all EU member states to establish so-called National Action Plans (NAPs) on the sustainable use of pesticides. In France, for example, a plan of action is in progress (Ecophyto2018) to identify and mainstream means enabling reductions in pesticide use to 50% by 2018 (ENDURE, 2010). Similar actions are also developed in Germany where different protection associations, like Pesticide action Network (PAN), Nature and Biodiversity Conservation Union (NABU) and Greenpeace, collaborate to improve protection of the environment as well as nature conservation.

In this context, the management and exploitation of the beneficial effects of the AM symbiosis on plant performance may provide an alternative strategy to ensure plant production and quality in emerging systems of sustainable agriculture aimed at reducing chemical inputs (as fertilizers or pesticides). There are numerous reports that AM fungi improve not only plant mineral nutrition but also induce protection against plant pathogens under controlled conditions and in the field or greenhouse (Gianinazzi *et al.*, 2010). Whilst the biological processes underlying improved mineral nutrition by AM fungi are well characterized, research into the molecular mechanisms of AM-induced bioprotection is ongoing and no clear hypothesis has yet been defined to explain the phenomenon.

Thielaviopsis basicola (Berk. And Broom) Ferraris is a serious problem for petunia production in nurseries since no petunia variety has been identified to be resistant against this root pathogen and its control requires the use of fungicides. Alternative sustainable methods of pathogen control therefore need to be developed and AM-induced bioprotection against plant disease is a promising possibility. For this reason, my thesis work has focused on the mycorrhizal system *Petunia hybrida* Mitchell - *Glomus mosseae* BEG12 in a commonly used soilless horticultural substrate (vermiculite/sand), in order to determine the existence of an AM-induced bioprotective effect against *T. basicola*. The main results were:

*1 September 2008, Reg. (EC) No 396/2005
http://ec.europa.eu/food/plant/protection/evaluation/new_reg_ppp_en.htm

i) In the interaction between petunia roots and *G. mosseae,* mycorrhiza development had a significant positive effect on plant biomass and the phosphorus content of shoots, which could be mimicked by fertilization with 0.5 mM KH_2PO_4.

ii) *G. mosseae* induced bioprotection in a petunia/*T. basicola* pathosystem. *T. basicola* development and disease severity caused by the pathogen decreased significantly in roots of *G. mosseae*-colonized plants, as compared to non-mycorrhizal plants or plants inoculated with the AM fungal species *Gigaspora rosea* and *Glomus intraradices*.

iii) **The pathosystem *G. mosseae*/petunia/*T. basicola* was optimized.** Time course experiments showed that the highest level of mycorrhization occurred at 3 weeks after inoculation. Since it is known from other pathosystems that an established mycorrhiza is necessary for bioprotection, *T. basicola* was inoculated at this time point. Pathogen detection by RT-PCR in non-mycorrhizal roots showed rapid development of *T. basicola* 24 and 36 hours after inoculation (hai), before root necrosis symptoms.

iv) **MIR against *T. basicola* petunia showed early and systemic induction**. *T. basicola* development significantly decreased in mycorrhizal root systems well before disease symptoms appeared. This phenomenon was also active in non-mycorrhizal roots of mycorrhizal plants showing that MIR acts also through systemic mechanisms. This suggests the implication of a long distance plant-mediated signal.

Based on this information, **molecular investigations of MIR against** *T. basicola* **were undertaken** in order to contribute to the understanding of the underlying molecular processes in this petunia pathosystem. The working hypothesis was that mechanisms involved either phytohormon-based signalling pathways like those described for salicylic acid (SA)-dependent systemic acquired resistance (SAR) and JA-related induced systemic resistance (ISR) in plants, or an intrinsic AM programme which may enhance root resistance and maintain symbiotic activity. The petunia genes which were selected for their implication in SA- or JA- related defence, or their activation during AM symbiotic interactions are summarized in Table 4-1 together with the effect of *T. basicola* (NM+Tb), *G. mosseae* (M) or *G. mosseae*-induced MIR (M+Tb) on their expression.

Table 4-1. Summary of the effect of *Thielaviopsis basicola* infection (NM+Tb), *Glomus mosseae* mycorrhization (M) or *G. mosseae*-induced MIR (M+Tb) on the expression of *Petunia hybrida* genes belonging to different categories. Expression is significantly up-regulated (+) or repressed (-), as compared to non-inoculated control plants. δ indicates a significant interactive effect on expression in the treatment with both fungi as compared to each alone.

	PT3	*PT4*	*KT*	*PR10a*	*Chit3*	*GST*	*AOC*	*LOX*	*Chit1a*	*Chit1b*	*PAL1*	*CHS*	*EDR1*	*CAL*	*P450*	*PR2*	*PR5*	*PR6*
24 hai																		
NM+Tb					-	-	-	-										
M	+	+	+	+	+	+		+	+							-		-
M+Tb	+	+	+	+	+	+		+	+							-		
					δ	δ		δ										
36 hai																		
NM+Tb								+										
M	+	+	+	+	+	+		+	+	-					+		-	-
M+Tb	+	+	+	+	+	+		+	+	-					+	-	-	-
								δ								δ	δ	δ
1 wai																		
NM+Tb	-						-							-	-			
M	+	+	+	+	+	+	-		+			+		-	-			
M+Tb	+	+	+	+	+	+	-		+			+			-			+
	δ						δ			δ					δ			δ

In pot cultures where all treatments were analysed in whole root systems (local MIR), gene expression results showed a clear induction of genes described before to be AM-regulated in *G. mosseae*-inoculated petunia plants at all time points. This was true for those encoding mineral element transporters (*PT3, PT4, KT*) and defence-related proteins (*PR10a, Chit3, GST, Chit1a*). The presence of *T. basicola* did not affect this induction even if there was a significant (negative) interaction, such as for *Chit3* and *GST* at 24 hai and *PT3* at 1 wai. High expression levels were maintained with no significant differences between root systems of M and M+Tb treatments, as compared to control non-mycorrhizal plants.

Concerning the SA signalling pathway, none of the selected genes seemed to be induced. In contrast all genes described before as SA-induced (*PR2, PR5, PR6*) were rather down-regulated by the presence of both *G. mosseae* and *T. basicola* at time points where bioprotection was evident. Consequently, the hypothesis that SA-signalling is involved and that local MIR is related to SAR does not appear valid in this pathosystem in petunia.

Among plant genes being described as JA-regulated, petunia *Chit1a* showed an expression pattern similar to the other AM-induced defence-related genes. The other genes, if at all regulated, were not induced at the time point where bioprotection by *G. mosseae* against *T. basicola* already occurred, or were repressed. Interestingly, while *LOX* encoding an enzyme in the oxylipin pathway showed induction by both

fungi, *AOC* involved in the final steps of JA biosynthesis was repressed. If indeed a signal other than JA is involved in local MIR has to be further investigated. However, MIR to *T. basicola* in petunia does not seem to be related to ISR and does not involve ISR-related priming.

When systemic MIR was analysed in a split-root system, the same AM-regulated genes where also locally induced in mycorrhizal roots and seemed not to be further affected by the pathogen (Table 4-2). In contrast to whole root systems without separation of the two fungi *G. mosseae* and *T. basicola*, however, *PR2, PR6* and *AOC* were mycorrhiza-induced in the pathogen infected half of the root system and to an even greater extent than in presence of the AM fungus alone. Hence, there appears to be a long distance induction and priming of the two SA-regulated genes and of JA biosynthesis.

Table 4-2. Summary of the effect of *Thielaviopsis basicola* infection compared between non-mycorrhizal system (NM/NM+Tb) and *Glomus mosseae* mycorrhizal system (M/NM+Tb) on the expression of *Petunia hybrida* genes belonging to different categories. Expression is significantly up-regulated (+) or repressed (-), as compared to non-inoculated control plants. δ indicates a significant systemic effect of *G. mosseae* inoculation on expression in the non-mycorrhizal compartment inoculated with *Thielaviopsis basicola*.

	PT3	***PT4***	***KT***	***PR10a***	***Chit3***	***GST***	***AOC***	***LOX***	***Chit1a***	***Chit1b***	***PAL1***	***CHS***	***EDR1***	***CAL***	***P450***	***PR2***	***PR5***	***PR6***
48 hai																		
NM																		
NM+Tb																		
M	+	+	+	+	+	+			+									
NM+Tb							+									+		+
							δ									δ		δ

These different observations raise the question of the role of the AM-related marker genes in MIR. The expression patterns of the nutrient transporter genes (*PT3*, *PT4* and *KT*) indicate that mycorrhizal functionality was not affected by *T. basicola* infection so maintaining overall nutrient supply to, and fitness of, the host plant. However, P and K may also be more directly involved in MIR since P has been reported to induce defence responses in pea via an inorganic phosphate signaling pathway (Kawahara et al., 2006), and the role of K in plant resistance to many diseases has been suggested (Perrenoud, 1990).

Concerning the category of AM-specific defence related genes, the *PR10a* gene belongs to the PR protein family known to be involved in induced plant

resistance against pathogens (Liu and Ekramodoullah, 2006). The class III chitinase encoded by *Chit3* could directly attack the chitin-containing cell wall of *T. basicola* or release chitin derivatives, like chitosan known to induce ISR (Iriti and Faoro, 2008), from the symbiotic fungal wall which could act as signal molecules for MIR (Dumas-Gaudot et al., 2000). Finally, activation of the *GST* gene could contribute to maintaining the symbiotic functionality by protecting pathogen-challenged roots against oxidative stress and cell death.

In conclusion, ***G. mosseae*** **BEG12** is suitable for **reducing phosphate fertiliser levels in petunia production** at least during early growth periods, and its use can be envisaged to **increase resistance against root pathogens**. Hence, this AM fungal strain constitutes a **useful biological agent to make petunia production in soilless substrates more sustainable** and to meet the consumers' demands for ecologically-produced ornamental crops. *G. mosseae* BEG12 is, however, not efficient in inducing salt tolerance of petunia and screening of other isolates is necessary to cover this facet of bioprotection by AM. In addition, the differential interaction between petunia, *G. mosseae* and *G. intraradices* can provide a novel system to analyse the molecular basis of different activities of AM fungi in interactions with plant pathogens.

Results from the present thesis work demonstrate that bioprotection against *T. basicola* in AM petunia plants involves local and systemic MIR. **Local MIR** by *G. mosseae* against *T. basicola* in petunia root systems **is related neither to SAR nor to ISR**. The possibility that **constitutive expression of AM-specific genes** in mycorrhiza is the basis for the mycorrhiza-induced local bioprotection merits further attention and analyses. The implication of defence mechanisms inherent to the mycorrhizal symbiosis in bioprotection against *T. basicola* raises the question of how plant defence is modulated to permit AM fungal development whilst protecting the host plant against fungal pathogen invasion. In contrast to local MIR, **systemic MIR in this pathosystem could include elements of both SAR** (*PR2* and *PR6* induction) **and ISR** (*AOC* induction and priming).

The hypothesis that mycorrhiza-integral mechanisms rather than the induction of plant basal defence mechanisms may underly AM bioprotection prompts **new perspectives for further investigations into the cellular basis of MIR**. In particular, the use of recently developed petunia microarrays (Breuillin et al., 2010) to perform non-targeted gene expression analyses will provide broader information about the network of plant genes regulated during MIR and identify those specific to AM-induced bioprotection against *T. basicola* in petunia by:

i) distinguishing between genes responsive to P and those specifically regulated during bioprotection

ii) studying the expression of genes in pathogen-challenged petunia roots colonized by *G. intraradices* or *Gig. rosea* to pinpoint those related to bioprotection by *G. mosseae* rather than mycorrhiza development

iii) determining the implication of a functional AM using petunia mutants altered in the mycorrhiza phenotype (Sekhara Reddy et al., 2007)

In addition:

iv) fine tuning of basal defense gene expression at earlier time points of AM bioprotection against *T. basicola* (before 24h) will clarify whether priming occurs at very early stages of the interactions

v) cell/tissue localization of plant gene products specific to MIR will contribute to the spatio-temporal comprehension of mechanisms involved

vi) use of RNAi to down-regulate particular AM bioprotection-related genes to determine their direct influence on MIR

5 -REFERENCES

Adams, S. G. C. 1988. *Thanatephorus cucumeris* (*Rhizoctonia solani*), a species complex of wide host range. ***Advance in Plant Pathology***, 6: 535-552.

Adie, B. A., Perez-Perez, Perez-Perez, M. M., Godoy, M., Sanchez-Serrano, J. J., Schmelz, E. A., & Solano, R. 2007. ABA Is an essential signal for plant resistance to pathogens affecting JA biosynthesis and the activation of defenses in *Arabidopsis*. ***The Plant Cell Online***, 19: 1665-1681.

Agrios, G. N. 1997. ***Plant Pathology*** (fourth edition). Academic Press, San Diego, pp 635.

Ahkami, A. H., Lischewski, S., Haensch, K. T., Porfirova, S., Hofmann, J., Rolletschek, H., Melzer, M., Franken, P., Hause, B., Druege, U., & Hajirezaei, M. R. 2009. Molecular physiology of adventitious root formation in *Petunia hybrida* cuttings: involvement of wound response and primary metabolism. ***New Phytologist***, 181: 613-625.

Akiyama, K., Matsuzaki, K., & Hayashi, H. 2005. Plant sesquiterpenes induce hyphal branching in arbuscular mycorrhizal fungi. ***Nature***, 435: 824-827.

Al-Karaki, G. N. 2000. Growth of mycorrhizal tomato and mineral acquisition under salt stress. ***Mycorrhiza***, 10: 51-54.

Al-Karaki, G. N., Hammad, R., & Rusan, M. 2001. Response of two tomato cultivars differing in salt tolerance to inoculation with mycorrhizal fungi under salt stress. ***Mycorrhiza***, 11: 43-47.

Al-Karaki, G. N. 2006. Nursery inoculation of tomato with arbuscular mycorrhizal fungi and subsequent performance under irrigation with saline water. ***Scientia Horticulturae***, 109: 1-7.

Aloui, A., Recorbet, G., Gollotte, A., Robert, F., Valot, B., Gianinazzi-Pearson, V., Aschi-Smiti, S., & Dumas-Gaudot, E. 2009. On the mechanisms of cadmium stress alleviation in *Medicago truncatula* by arbuscular mycorrhizal symbiosis: a root proteomic study. ***Proteomics***, 9: 420-433.

Anderson, N.A. 1982. The genetics and pathology of *Rhizoctonia solani*. ***Annual Review of Phytopathology,*** 20: 329-347.

Augé, R. M., Stodola, A. J. W., Tims, J. E., & Saxton, A. M. 2001. Moisture retention properties of a mycorrhizal soil. ***Plant and Soil***, 230: 87-97.

Ausubel, F. M. 2005. Are innate immune signaling pathways in plants and animals conserved? ***Nature Immunology***, 6: 973-979.

Azcón-Aguilar, C. & Barea, J. M. 1997. Arbuscular mycorrhizas and biological control of soil-borne plant pathogens: an overview of the mechanisms involved. ***Mycorrhiza***, 6: 457-464.

Bago, B., Shachar-Hill, Y. & Pfeffer, P. E. 2000. Dissecting carbon pathways in arbuscular mycorrhizas with NMR spectroscopy. In: Podila GK, Douds DD (eds) ***Current advances in mycorrhizae research.*** APS, St. Paul, USA, pp 111–126

Balestrini, R. & Lanfranco, L. 2006. Fungal and plant gene expression in arbuscular mycorrhizal symbiosis. ***Mycorrhiza***, 16: 509-524.

Barea, J. M., Pozo, M. J., Azcon, R., & Azcon-Aguilar, C. 2005. Microbial co-operation in the rhizosphere. ***Journal of Experimental Botany***, 56: 1761-1778.

Bari, R. & Jones, J. 2009. Role of plant hormones in plant defence responses. ***Plant Molecular Biology***, 69: 473-488.

Barker, S. J., Stummer, B., Gao, L., Dispain, I., O. G., Connor, P. J., & Smith, S. E. 1998. A mutant in *Lycopersicon esculentum* Mill. with highly reduced VA mycorrhizal colonization: isolation and preliminary characterisation. ***The Plant Journal***, 15: 791-797.

Bärtschi, H., Gianinazzi-Pearson, V., & Vegh, I. 1981. Vesicular arbuscular mycorrhiza formation and root rot disease *Phytophthora cinnamomi* development in *Chamaecyparis lawsonia*. ***Phytopathologische Zeitschrift***, 102:213–218.

Baltruschat, H. & Schönbeck, F. 1972. The influence of endotrophic mycorrhiza on the infestation of tobacco by *Thielaviopsis basicola.* ***Phytopathology***, 84: 172–188.

Baltruschat, H. & Schönbeck, F. 1975. Untersuchungen über den Einfluß der endotrophen Mycorrhiza auf den Befall von Tabak mit Thielaviopsis basicola. ***Phytopathologische Zeitschrift***, 84:172-188.

Bonfante, P. & Genre, A. 2010. Mechanisms underlying beneficial plant-fungus interactions in mycorrhizal symbiosis. ***Nature Commununications*** (online), doi/10.1038/ncomms1046.

Benhamou, N., Lafontaine, P. J., & Nicole, M. 1994. Induction of systemic resistance to *Fusarium* crown and root rot in tomato plants by seed treatment with Chitosan. ***Phytopathology***, 84: 1432-1444.

Benhamou, N., Kloepper, J. W., & Tuzun, S. 1998. Induction of resistance against *Fusarium* wilt of tomato by combination of chitosan with an endophytic bacterial strain: ultrastructure and cytochemistry of the host response. ***Planta***, 204: 153-168.

Besserer, A., Puech-Pages, V., Kiefer, P., Gomez-Roldan, V., Jauneau, A., Roy, S., Portais, J. C., Roux, C., Becard, G., & Sejalon-Delmas, N. 2006. Strigolactones stimulate arbuscular mycorrhizal fungi by activating mitochondria. ***PLoS Biology***, 4: e226.

Berta, G., Sampo, S., Gamalero, E., Massa, N., & Lemanceau, P. 2005. Suppression of *Rhizoctonia* root-rot of tomato by *Glomus mossae* BEG12 and *Pseudomonas fluorescens* A6RI is associated with their effect on the pathogen growth and on the root morphogenesis. ***European Journal of Plant Pathology***, 111: 279-288.

Blee, K. A. & Anderson, A. J. 1998. Regulation of arbuscule formation by carbon in the plant. ***The Plant Journal***, 16: 523-530.

Blee, K. A. & Anderson, A. J. 2000. Defense responses in plants to arbuscular mycorrhizal fungi. In: Podila G. K., Douds D. D. (eds) ***Current advances in mycorrhizae research.*** Minnesota, USA: The American Phytopathological Society: 27–44.

Bodker, L., Kjoller, R., Kristensen, K., & Rosendahl, S. 2002. Interactions between indigenous arbuscular mycorrhizal fungi and *Aphanomyces euteiches* in field-grown pea. ***Mycorrhiza***, 12: 7-12.

Boldt, K., Pörs, Y., Haupt, B., Bitterlich, M., Kühn, C., Grimm, B. & Franken, P. 2011. Photochemical processes, carbon assimilation and RNA accumulation of sucrose transporter genes in tomato arbuscular mycorrhiza. ***Journal of Plant Physiology***, 168: 1256-1263.

Bonfante, P. & Requena, N. 2011. Dating in the dark: how roots respond to fungal signals to establish arbuscular mycorrhizal symbiosis. ***Current Opinion in Plant Biology***, 14: 451-457.

Bonfante, P. & Perrotto, S. 1995. Tansley Review No. 82 Strategies of arbuscular mycorrhizal fungi when infecting host plants. ***New Phytologist***, 130: 3-21.

Bossolini, E., Klahre, U., Brandenburg, A., Reinhardt, D., & Kuhlemeier, C. 2011. High resolution linkage maps of the model organism *Petunia* reveal substantial synteny decay with the related, genome of tomato. ***Genome***, 54: 327-340.

Bowler, C. & Fluhr, R. 2000. The role of calcium and activated oxygens as signals for controlling cross-tolerance. ***Trends in Plant Science***, 5: 241-246.

Brechenmacher, L., Weidman, S., van Tuinen, D., Chatagnier, O., Gianinazzi, S., Franken, P., & Gianinazzi-Pearson, V. 2004. Expression profiling of up-regulated plant and fungal genes in early and late stages of *Medicago truncatula-Glomus mosseae* interactions. ***Mycorrhiza,*** 14: 253-262.

Breuillin, F., Schramm, J., Hajirezaei, M., Ahkami, A., Favre, P., Drüge, U., Hause, B., Bucher, M., Kretzschmar, T., Bossolini, E., Kuhlemeier, C., Martinoia, E., Franken, P., Scholz, U., & Reinhardt, D. 2010. Phosphate systemically inhibits development of arbuscular mycorrhiza in *Petunia hybrida* and represses genes involved in mycorrhizal functioning. ***The Plant Journal***, 64: 1002-1017.

Brown, I., Trethowan, J., Kerry, M., Mansfield, J., & Bolwell, G. P. 1998. Localization of components of the oxidative cross-linking of glycoproteins and of callose synthesis in papillae formed during the interaction between non-pathogenic strains of *Xanthomonas campestris* and French bean mesophyll cells. ***The Plant Journal***, 15: 333-343.

Bucher, M. 2007. Functional biology of plant phosphate uptake at root and mycorrhiza interfaces. ***New Phytologist***, 173: 11-26.

Cameron, R. K., Paiva, N. L., Lamb, C. J., & Dixon, R. A. 1999. Accumulation of salicylic acid and PR-1 gene transcripts in relation to the systemic acquired resistance (SAR) response induced by *Pseudomonas syringae* pv. tomato in *Arabidopsis*. ***Physiological and Molecular Plant Pathology***, 55: 121-130.

Carling, D. E., Baird, R. E., Gitatis, R. D., Brainard K. A., & Kuninaga, S. 2002. Characterization of AG-13, a newly reported anastomosis group of *Rhizoctonia solani*. ***Phytopathology,*** 92:893-899.

Caron, M., Fortin, J. A., & Richard, C. 1986. Effect of inoculation sequence on the interaction between *Glomus intraradices* and *Fusarium oxysporum* f. sp. radicis-lycopersici in tomatoes. ***Canadian Journal of Plant Pathology,*** 64: 552-556.

Chalot, M., Blaudez, D., & Brun, A. 2006. Ammonia: a candidate for nitrogen transfer at the mycorrhizal interface. ***Trends in Plant Science***, 11: 263-266.

Chavez, W., Di Benedetto, A., Civeira, G., & Lavado, R. l. 2008. Alternative soilless media for growing *Petunia hybrida* and *Impatiens wallerana*: Physical behavior, effect of fertilization and nitrate losses. ***Bioresource Technology***, 99: 8082-8087.

Clayton, E. E. 1969. The study of resistance of the black root rot disease of tobacco. ***Tobacco Science,*** 13: 30-37.

Colditz, F., Niehaus, K., & Krajinski, F. 2007. Silencing of PR-10-like proteins in *Medicago truncatula* results in an antagonistic induction of other PR proteins and in an increased tolerance upon infection with the oomycete *Aphanomyces euteiches*. ***Planta***, 226: 57-71.

Coleman, J., Blake-Kalff, M., & Davies, E. 1997. Detoxification of xenobiotics by plants: chemical modification and vacuolar compartmentation. ***Trends in Plant Science***, 2: 144-151.

Conner, A. J., Albert, N. W., & Deroles, S. C. 2009. Transformation and regeneration of Petunia. In T. Gerats & J. Strommer (eds) ***Petunia: evolutionary, developmental and physiological genetics.*** Springer New York, 395-409.

Conrath, U., Beckers, G. J. M., Flors, V., García-Agustín, P., Jakab, G., Mauch, F., Newman, M. A., Pieterse, C. M., Poinssot, B., Pozo, M. J., Pugin, A., Schaffrath, U., Ton, J., Wendehenne, D., Zimmerli, L., & Mauch-Mani, B. 2006. Priming: Getting ready for battle. ***Molecular Plant-Microbe Interactions***, 19: 1062-1071.

Copes, W. E. & Hendrix, F. F. 1996. Chemical disinfestation of greenhouse growing surface materials contaminated with *Thielaviopsis basicola*. ***Plant Disease***, 80:885-886.

Cordier, C., Pozo, M. J., Barea, J. M., Gianinazzi, S., & Gianinazzi-Pearson, V. 1998. Cell defense responses associated with localized and systemic resistance to *Phytophthora parasitica* induced in tomato by an arbuscular mycorrhizal fungus. ***Molecular Plant-Microbe Interactions***, 11: 1017-1028.

Coumans, J. V., Harvey, J., Backhouse, D., Poljak, A., Raftery, M. J., Nehl, D., Katz, M. E., & Pereg, L. 2011. Proteomic assessment of host-associated microevolution in the fungus *Thielaviopsis basicola*. ***Environmental Microbiology***, 13: 576-588.

Dangl, J. L. & Jones, J. D. G. 2001. Plant pathogens and integrated defence responses to infection. ***Nature***, 411: 826-833.

Dassi, B., Dumas-Gaudot, E., Asselin, A., Richard, C., & Gianinazzi, S. 1996. Chitinase and beta-1,3-glucanase isoforms expressed in pea roots inoculated with arbuscular mycorrhizal or pathogenic fungi. ***European Journal of Plant Pathology***, 102: 105-108.

David-Schwartz, R., Badani, H., Smadar, W., Levy, A. A., Galili, G., & Kapulnik, Y. 2001. Identification of a novel genetically controlled step in mycorrhizal colonization: plant resistance to infection by fungal spores but not extra-radical hyphae. ***The Plant Journal***, 27: 561-569.

David-Schwartz, R., Gadkar, V., Wininger, S., Bendov, R., Galili, G., Levy, A. A., & Kapulnik, Y. 2003. Isolation of a premycorrhizal infection (pmi2) mutant of tomato, resistant to arbuscular mycorrhizal fungal colonization. ***Molecular Plant-Microbe Interactions***, 16: 382-388.

David, C. H., & Ortiz, M., JR. 1980. Suppression of *Thielaviopsis basicola* by two fungicides applied to sandy loam soils in New Mexico. ***Plant Disease***, 64:1011-1012.

Davis, R. M. & Menge, J. A. 1980. Influence of *Glomus fasciculatus* and soil phosphorus on Phytophthora root rot of citrus. ***Phytopathology***, 70: 447-452.

de la Noval, B., Perez, E., Martinez, B., Leon, O., Martinez-Gallardo, N., & Delano-Frier, J. 2007. Exogenous systemin has a contrasting effect on disease resistance in mycorrhizal tomato (*Solanum lycopersicum*) plants infected with necrotrophic or hemibiotrophic pathogens. ***Mycorrhiza***, 17: 449-460.

Dehne, H. W. & Schönbeck, F. 1975. The influence of endotrophic mycorrhizae on the *Fusarium* wilt of tomato. ***Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz,*** 82:630-632.

Dehne, H. W. & Schönbeck, F. 1979. Untersuchungen zum Einflux der endotrophen Mycorrhiza auf Pflanzenkrankheiten. ***Journal of Phytopathology***, 95: 105-110.

Dehne, H. W. 1982. Interaction between vesicular-arbuscular mycorrhizal fungi and plant pathogens. ***Phytopathology***, 72: 1115-1119.

Dong X. 2004. NPR1, all things considered. ***Current Opinion in Plant Biology***, 7: 547-552.

Dreistadt, S.H. 2001. Integrated pest management for floriculture and nurseries. ***University of California, Davis, CA.***

Dudal, R., Nachtergayele, F. O., & Purnell, M. F. 2002. The human factor of soil formation. ***Transactions 17 World Congress of Soil Science, Bangkok,*** 2: 93.

Dumas-Gaudot, E., Gollotte, A., Cordier, C., Gianinazzi, S. & Gianinazzi-Pearson, V. 2000. Modulation of host defense systems. In: Kapulnik Y., Douds D. D. Jr. (eds) ***Arbuscular Mycorrhizas: Physiology and Function***. Kluwer Academy Publishers: 173-200.

Durner, J., Shah, J., & Klessig, D. F. 1997. Salicylic acid and disease resistance in plants. ***Trends in Plant Science***, 2: 266-274.

Durrant, W. E. & Dong, X. 2004. Systemic acquired resistance. ***Annual Review of Phytopathology***, 42: 185-209.

Edreva, A. 2005. Pathigenesis-related proteins: research progress in the last 15 years. ***General and Applied Plant Physiology,*** 31: 105-124.

Elfstrand, M., Feddermann, N., Ineichen, K., Nagaraj, V. J., Wiemken, A., Boller, T., & Salzer, P. 2005. Ectopic expression of the mycorrhiza-specific chitinase gene Mtchit 3-3 in *Medicago truncatula* root-organ cultures stimulates spore germination of glomalean fungi. ***New Phytologist***, 167: 557-570.

Elsen, A., Baimey, H., Swennen, R., & De Waele, D. 2003. Relative mycorrhizal dependency and mycorrhiza-nematode interaction in banana cultivars (*Musa* spp.) differing in nematode susceptibility. ***Plant and Soil***, 256: 303-313.

Elsen, A., Gervacio, D., Swennen, R., & De Waele, D. 2008. AMF-induced biocontrol against plant parasitic nematodes in *Musa* spp.: a systemic effect. ***Mycorrhiza***, 18: 251-256.

ENDURE. 2010. La protection intégrée dans l'agriculture européenne. ***INRA Press, 36 pp*** (www.endure-network.eu).

Engels, W. R. 1993. Contributing software to the internet: the Amplify program. ***Trends Biochemistry Science***, 18: 448-450.

Erstad, J. L. F. & Gislerod, H .R. 1994. Water uptake of cuttings and stem pieces as affected by different anaerobic conditions in the rooting medium. ***Scientia Horticulturae.,*** 58: 151–160.

Fakhro, A., Andrade-Linares, D., von Bargen, S., Bandte, M., Büttner, C., Grosch, R., Schwarz, D., & Franken, P. 2010. Impact of *Piriformospora indica* on tomato growth and on interaction with fungal and viral pathogens. ***Mycorrhiza***, 20: 191-200.

Feddermann, N., Muni, R. R., Zeier, T., Stuurman, J., Ercolin, F., Schorderet, M., & Reinhardt, D. 2010. The *PAM1* gene of petunia, required for intracellular accommodation and morphogenesis of arbuscular mycorrhizal fungi, encodes a homologue of VAPYRIN. ***The Plant Journal***, 64: 470-481.

Fire, A. 1999. RNA triggered gene silencing. ***Trends In Genetics,*** 15: 358-363.

Franken, P., Requena, N., Bütehorn, B., Krajinski, F., Kuhn, G., Laponin, L., Mann, P., Rhody, D. & Stommel, M. 2000. Molecular analysis of the arbuscular mycorrhiza symbiosis. ***Archives of Agronomy and Soil Science***: 271–286.

Franken, P. & George, E. 2006. Diversity of arbuscular mycorrhizal fungi. In: Benckiser G., Schnell S. (eds) ***Biodiversity in agricultural production systems. CRC*** Press, Boca Raton: 189–203.

Fravel, D.R., Olivan, C. & Alabouvette, C. 2003. *Fusarium oxysporum* and its biocontrol. ***New Phytologist,*** 157: 493-502.

Fritz, M., Jakobsen, I., Lyngkjaer, M. F., Thordal-Christensen, H., & Pons-Kuhnemann, J. 2006. Arbuscular mycorrhiza reduces susceptibility of tomato to *Alternaria solani*. ***Mycorrhiza***, 16: 413-419.

Gallou, A. 2011. Impact of an arbuscular mycorrhizal fungus and *Trichoderma harzianum* on the potato resistance against above- and below-ground pathogens. Thesis manuscript, UCL, Bruxelles, pp 264.

Gallou, A., Declerck, S., & Cranenbrouck, S. 2011a. Transcriptional regulation of defence genes and involvement of the WRKY transcription factor in arbuscular mycorrhizal potato root colonization. ***Functional & Integrative Genomics***, 12, DOI: 10.1007/s10142-011-0241-4, in press.

Gallou, A., Lucero Mosquera, H. P., Cranenbrouck, S., Suárez, J. P., & Declerck, S. 2011b. Mycorrhiza induced resistance in potato plantlets challenged by *Phytophthora infestans*. ***Physiological and Molecular Plant Pathology***, 76: 20-26.

García-Garrido, J. M. & Ocampo, J. A. 2002. Regulation of the plant defence response in arbuscular mycorrhizal symbiosis. ***Journal of Experimental Botany***, 53: 1377-1386.

Garmendia, I., Goicoechea, N., & Aguirreolea, J. 2004. Effectiveness of three *Glomus* species in protecting pepper (*Capsicum annuum L.*) against *Verticillium* wilt. ***Biological Control***, 31: 296-305.

Gaude, N., Bortfeld, S., Duensing, N., Lohse, M., & Krajinski, F. 2012. Arbuscule-containing and non-colonized cortical cells of mycorrhizal roots undergo extensive and specific reprogramming during arbuscular mycorrhizal development. ***The Plant Journal***, 69: 510-528.

Gaur, A. & Adholeya, A. 2000. Growth and flowering in *Petunia hybrida*, *Callistephus chinensis* and *Impatiens balsamina* inoculated with mixed AM inocula or chemical fertilizers in a soil of low P fertility. ***Scientia Horticulturae***, 84: 151-162.

Gaur, A. & Adholeya, A. 2005. Diverse response of five ornamental plant species to mixed indigenous and single isolate arbuscular-mycorrhizal inocula in marginal soil amended with organic matter. ***Journal Plant Nutrition,*** 28: 707-723.

Genre, A., Chabaud, M., Timmers, T., Bonfante, P., & Barker, D. G. 2005. Arbuscular mycorrhizal fungi elicit a novel intracellular apparatus in *Medicago truncatula* root epidermal cells before infection. ***The Plant Cell***, 17: 3489-3499.

Genre, A., Chabaud, M., Faccio, A., Barker, D. G., & Bonfante, P. 2008. Prepenetration apparatus assembly precedes and predicts the colonization patterns of arbuscular mycorrhizal fungi within the root cortex of both *Medicago truncatula* and Daucus carota. ***The Plant Cell***, 20: 1407-1420.

Gerats, T. & Vandenbussche, M. 2005. A model system comparative for research: Petunia. ***Trends in Plant Science***, 10: 251-256.

Gerats, A.G.M., Huits, H., Vrijlandt, E., Marana, C., Souer, E., & Beld, M. 1990. Molecular characterization of a nonautonomous transposable element (dTph1) of Petunia. ***The Plant Cell***, 2: 1121-1128.

Gerdemann, J. 1968. Vesicular-arbuscular mycorrhiza and plant growth. ***Annual Review of Phytopathology***, 6:397-418.

Gericke, S. & Kurmies, B. 1956. Manuring effects of magnesium phosphates and magnesium-containing phosphate fertilizers. ***Phosphorsaure***, 16: 121-139.

Gernns, H., Alten, H., & Poehling, H. M. 2001. Arbuscular mycorrhiza increased the activity of a biotrophic leaf pathogen - is a compensation possible? ***Mycorrhiza***, 11: 237-243.

Gianinazzi, S., Martin, C., & Vallee, J. C. 1970. Hypersensibilite aux virus, temperature et proteines solubles chez le Nicotiana Xanthi n.c. Apparition de nouvelles macromolecules lors de la repression de la synthese virale. ***Compte Rendu de l'Académie de Science D.,*** 270: 2382-2386.

Gianinazzi, S. & Gianinazzi-Pearson, V. 1990. Cellular interactions in vesicular arbuscular (VA) endomycorrhizae. The host's point of view. In: Nardon P., Gianinazzi-Pearson V., Grenier A. M., Margulis L., Smith D. C. (eds) ***Endocytobiology***. INRA Press: 83-90.

Gianinazzi-Pearson, V., Dumas-Gaudot, E., Gollotte, A., Tahiri-Alaoui, A., & Gianinazzi, S. 1996. Cellular and molecular defence-related root responses to invasion by arbuscular mycorrhizal fungi. ***New Phytologist***, 133: 45-57.

Gianinazzi-Pearson, V. & Dénarié, J. 1997. Red carpet genetic programmes for root endosymbioses. ***Trends in Plant Science***, 2: 371-372.

Gianinazzi, S., Gollotte, A., Binet, M. N., van Tuinen, D., Redecker, D., & Wipf, D. 2010. Agroecology: the key role of arbuscular mycorrhizas in ecosystem services. ***Mycorrhiza***, 20: 519-530.

Giovannetti, M., Tosi, L., Torre, G. D., & Zazzerini, A. 1991. Histological, Physiological and Biochemical Interactions between Vesicular-Arbuscular Mycorrhizae and Thielaviopsis basicola in Tobacco Plants. ***Journal of Phytopathology***, 131: 265-274.

Giovannetti, M., Sbrana, C., Avio, L., Citernesi, A. S., & Logi, C. 1993. Differential hyphal morphogenesis in arbuscular mycorrhizal fungi during pre-infection stages. ***The New Phytologist***, 125: 587-593.

Glazebrook, J. 2005. Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. ***Annual Review of Phytopathology***, 43: 205-227.

Gómez-Gómez, L., Felix, G., & Boller, T. 1999. A single locus determines sensitivity to bacterial flagellin in *Arabidopsis thaliana*. ***The Plant Journal***, 18: 277-284.

Gonzólez-Chavez, C., D'Haen, J., Vangronsveld, J., & Dodd, J. C. 2002. Copper sorption and accumulation by the extraradical mycelium of different *Glomus* spp. (arbuscular mycorrhizal fungi) isolated from the same polluted soil. ***Plant and Soil***, 240: 287-297.

González García, V., Portal Onco, M. A. & Rubio Susan, V. 2006. Biology and Systematics of the form genus Rhizoctonia. ***Spanish Journal of Agricultural Research***, 4: 55-79

Govindarajulu, M., Pfeffer, P. E., Jin, H., Abubaker, J., Douds, D. D., Allen, J. W., Bucking, H., Lammers, P. J., & Shachar-Hill, Y. 2005. Nitrogen transfer in the arbuscular mycorrhizal symbiosis. ***Nature***, 435: 819-823.

Graham, J. H. & Menge, J. A. 1982. Influence of vesicular-arbuscular mycorrhizae and soil phosphorous on take-all disease of wheat. ***Phytopathology***, 72: 95–98.

Grillas, S., Lucas, M., Bardopoulou, E. & Sarafopoulos, S. 2011. Perlite based soilless culture systems: Current commercial applications and prospects. ***Acta Horticulturae,*** 548: 105-113.

Grosch, R., Schneider, J. H. M., & Kofoet, A. 2004. Characterisation of *Rhizoctonia solani* anastomosis groups causing bottom rot in field grown lettuce in Germany. ***European Journal of Plant Pathology,*** 110: 53–62.

Gruda, N. 2009. Do soilless culture systems have an influence on product quality of vegetables? ***Journal of Applied Botany and Food Quality***, 82: 141-147.

Grunwald, U., Guo, W., Fischer, K., Isayenkov, S., Ludwig-Müller, J., Hause, B., Yan, X., Küster, H., & Franken, P. 2009. Overlapping expression patterns and differential transcript levels of phosphate transporter genes in arbuscular mycorrhizal, Pi-fertilised and phytohormone-treated *Medicago truncatula* roots. ***Planta***, 229: 1023-1034.

Güther, M., Balestrini, R., Hannah, M., He, J., Udvardi, M. K., & Bonfante, P. 2009. Genome-wide reprogramming of regulatory networks, transport, cell wall and membrane biogenesis during arbuscular mycorrhizal symbiosis in *Lotus japonicus*. ***New Phytologist,*** 182:200–212.

Guillon, C., St-Arnaud, M., Hamel, C., & Jabaji-Hare, S. H. 2002. Differential and systemic alteration of defence-related gene transcript levels in mycorrhizal bean plants infected with *Rhizoctonia solani*. ***Canadian Journal of Botany***, 80: 305-315.

Gutjahr, C. & Paszkowki, U. 2009. Weights in the balance: JA and SA signaling in root-biotroph interactions. ***Molecular Plant-Microbe Interactions,*** 22: 763-772.

Hahn, M. G., Darvill, A. G., & Albersheim, P. 1981. A fragment of plant cell wall polysaccharide that elicits phytoalexin accumulation in soybean. ***Plant Physiology***, 68: 1161-1169.

Hajiboland, R., Aliasgharzadeh, N., Laiegh, S. F., & Poschenrieder, C. 2010. Colonization with arbuscular mycorrhizal fungi improves salinity tolerance of tomato (*Solanum lycopersicum* L.) plants. ***Plant and Soil***, 331: 313-327.

Hao, Z., Fayolle, F., van Tuinen, T., Chatagnier, O., Li, X., Gianinazzi, S., & Gianinazzi-Pearson, V. 2012. Local and systemic mycorrhiza-induced protection against the ectoparasitic nematode *Xiphinema index* involves priming of defense gene responses in grapevine. ***Journal of Experimental Botany,*** in press.

Hase, S., Van Pelt, J. A., van Loon, L. C., & Pieterse, C. M. J. 2003. Colonization of *Arabidopsis* roots by *Pseudomonas fluorescens* primes the plant to produce higher levels of ethylene upon pathogen infection. ***Physiological and Molecular Plant Pathology***, 62: 219-226.

Hause, B. & Fester, T. 2005. Molecular and cell biology of arbuscular mycorrhizal symbiosis. ***Planta***, 221: 184-196.

Hause, B., Mrosk, C., Isayenkov, S., & Strack, D. 2007. Jasmonates in arbuscular mycorrhizal interactions. ***Phytochemistry***, 68: 101-110.

Hawkins, H. J., Johansen, A., & George, E. 2000. Uptake and transport of organic and inorganic nitrogen by arbuscular mycorrhizal fungi. ***Plant and Soil***, 226: 275-285.

Hayat, R., Ali, S., Amara, U., Khalid, R., & Ahmed, I. 2010. Soil beneficial bacteria and their role in plant growth promotion: a review. ***Annals of Microbiology***, 60: 579-598.

Heil, M. & Bostock, R. 2002. Induced systemic resistance (ISR) against pathogens in the context of induced plant defences. ***Annals of Botany***, 89: 503-512.

Helber, N., Wippel, K., Sauer, N., Schaarschmidt, S., Hause, B., & Requena, N. 2011. A versatile monosaccharide transporter that operates in the arbuscular mycorrhizal fungus *Glomus* sp is crucial for the symbiotic relationship with plants. ***The Plant Cell***, 23: 3812-3823.

Hendrix, F. F. & Campbell, W. A. 1970. Distribution of *Phytophthora* and *Pythium* species in soils in continental United-States. ***Canadian Journal of Botany***, 48: 377-384.

Herrera-Medina, M. J., Steinkellner, S., Vierheilig, H., Ocampo Bote, J. A., & García Garrido, J. M. 2007. Abscisic acid determines arbuscule development and functionality in the tomato arbuscular mycorrhiza. ***New Phytologist***, 175: 554-564.

Hondo D., Hase S., Kanayama Y., Yoshikawa N., Takenaka S. & Takahashi H. 2007. The LeALT6-associated ubiquitin/proteasome system may contribute to fungal elictor-activated defense response via the jasmonic acid-dependent signaling pathway in tomato. ***Molecular Plant-Microbe Interactions***, 20: 72–81.

Hood, M. E. & Shew, H. D. 1996. Pathogenesis of *Thielaviopsis basicola* on a susceptible and a resistant cultivar of burley tobacco. ***Phytopathology***, 86: 38-44.

Hood, M. E. & Shew, H. D. 1997. Initial cellular interactions between *Thielaviopsis basicola* and tobacco root hairs. ***Phytopathology***, 87: 228-235.

Horsfall, J. G. & Dimond, A. E. 1960. The pathogen: the concept of causality. In: Horsfall J. G., Dimond A. E. (eds) ***Plant pathology: an advanced treatise***. New York & London, Academic Press, 2: 1-18.

Huang, Z., He, C. X., He, Z. Q., Zou, Z. R., & Zhang, Z. B. 2010. The effects of arbuscular mycorrhizal fungi on reactive oxyradical scavenging system of tomato under salt tolerance. ***Agricultural Sciences in China***, 9: 1150-1159.

Iriti, M. & Faoro, F. 2008. Abscisic acid is involved in chitosan-induced resistance to tobacco necrosis virus (TNV). ***Plant Physiology and Biochemistry***, 46: 1106-1111.

Isayenkov, S., Mrosk, C., Stenzel, I., Strack, D., & Hause, B. 2005. Suppression of allene oxide cyclase in hairy roots of *Medicago truncatula* reduces jasmonate levels and the degree of mycorrhization with *Glomus intraradices*. ***Plant Physiology***, 139: 1401–1410.

Jeffries, P., Gianinazzi, S., Perotto, S., Turnau, K., & Barea, J. M. 2003. The contribution of arbuscular mycorrhizal fungi in sustainable maintenance of plant health and soil fertility. ***Biology and Fertility of Soils***, 37: 1-16.

Johnson, J. 1916. Host plants of *Thielavia basicola*. ***Journal of Agricultural Research,*** 7: 289-300.

Jones, J. D. & Dangl, J. L. 2006. The plant immune system. ***Nature***, 444: 323-329.

Jumpponen, A. R. I. & Trappe, J. M. 1998. Dark septate endophytes: a review of facultative biotrophic root-colonizing fungi. ***New Phytologist***, 140: 295-310.

Kabir, Z. & Koide, R. T. 2002. Effect of autumn and winter mycorrhizal cover crops on soil properties, nutrient uptake and yield of sweet corn in Pennsylvania, USA. ***Plant and Soil***, 238: 205-215.

Kang, J. G. & van Iersel, M. W. 2009. Managing fertilization of bedding plants: a comparison of constant fertilizer concentrations versus constant leachate electrical conductivity. ***Hortscience***, 44: 151-156.

Kessler, J. R. Jr. 2004. Growing and marketing bedding plants. ***Alabama Cooperative Extension System***, ANR-559.

Khaosaad, T., Garcia-Garrido, J. M., Steinkellner, S., & Vierheilig, H. 2007. Take-all disease is systemically reduced in roots of mycorrhizal barley plants. ***Soil Biology & Biochemistry***, 39: 727-734.

Kawahara, T., Namba, H., Toyoda, K., Kasai, T., Sugimoto, M., Inagaki, Y., Ichinose, Y., & Shiraishi, T. 2006. Induction of defense responses in pea tissues by inorganic phosphate. ***Journal of General Plant Pathology***, 72: 129-136.

Kistner, K. & Parniske, M. 2002. Evolution of signal transduction in intracellular symbiosis**.** ***TRENDS in plant science,*** 7: 511-518.

Kloepper, J. W., Ryu, C. M., & Zhang, S. 2004. Induced systemic resistance and promotion of plant growth by *Bacillus* ssp. ***Phytopathology***, 94: 1259-1266.

Klopotek, I., Haensch, K. T., Hause, B., Hajirezaei, M. R. & Drüge, U. 2010. Dark exposure of petunia cuttings strongly improves adventitious root formation and enhances carbohydrate availability during rooting in the light. ***Journal of Plant Physiology***, 7: 457-554.

Kloppholz, S., Kuhn, H., & Requena, N. 2011. A secreted fungal effector of *Glomus intraradices* promotes symbiotic biotrophy. ***Current Biology,*** 21: 1204-1209.

Koide, R. T., Landherr, L. L., Besmer, Y. L., Detweiler, J. M., & Holcomb, E. J. 1999. Strategies for mycorrhizal inoculation of six annual bedding plant species. ***Hortscience***, 34: 1217-1220.

Kosuta, S., Chabaud, M., Lougnon, G., Gough, C., Dénarié, J., Barker, D. G., & Bécard, G. 2003. A diffusible factor from arbuscularm fungi induces symbiosis-specific MtENOD11 expression in roots of *Medicago truncatula*. ***Plant Physiology***, 131: 952-962.

Krishna, K. R. & Bagyaraj, D. J. 1983. Interaction between *Glomus fasciculatum* and *Sclerotium rolfsii* in peanut. ***Canadian Journal of Botany***, 61: 2349-2351.

Kunkel, B. N. & Brooks, D. M. 2002. Cross talk between signaling pathways in pathogen defense. ***Current Opinion in Plant Biology***, 5: 325-331.

Kuznetsova, E., Seddas-Dozolme, P. M., Arnould, C., Tollot, M., van Tuinen, D., Borisov, A., Gianinazzi, S., & Gianinazzi-Pearson, V. 2010. Symbiosis-related pea genes modulate fungal and plant gene expression during the arbuscule stage of mycorrhiza with *Glomus intraradices*. ***Mycorrhiza***, 20: 427-443.

Leahy, R., M. 1998. Black root rot of pansies. ***Plant Pathology Circular*** No. 387**.**

Lee, C. S., Lee, Y. J., & Jeun, Y. C. 2005 Observations of infection structures on the leaves of cucumber plants pretreated with arbuscular mycorrhiza *Glomus intraradices* after challenge inoculation with *Colletotrichum orbiculare*. ***Plant Pathology Journal***, 21: 237–243

Leeman, M., Pelt, J. A., Ouden, F. M., Heinsbroek, M., Bakker, P. A. H. M., & Schippers, B. 1995. Induction of systemic resistance against *Fusarium* wilt of radish by lipopolysaccharides of *Pseudomonas fluorescens*. ***Phytopathology***, 85: 1021-1027.

Lerat, S., Lapointe, L., Gutjahr, S., Piche, Y. & Vierheilig, H. 2003. Carbon partitioning in a split-root system of arbuscular mycorrhizal plants is fungal and plant species dependent. ***New Phytologist***, 157: 589-595.

Li, H. Y., Yang, G. D., Shu, H. R., Yang, Y. T., Ye, B. X. & Nishida, I. 2006. Colonization by the arbuscular mycorrhizal fungus *Glomus versiforme* induces a defense response against the root-knot nematode *Meloidogyne incognita* in the grapevine (*Vitis amurensis* Rupr.), which includes transcriptional activation of the class III chitinase gene VCH3. ***Plant and Cell Physiology,*** 47: 154-163.

Lingua, G., D'Agostino, G., Massa, N., Antosiano, M., & Berta, G. 2002. Mycorrhiza-induced differential response to a yellows disease in tomato. ***Mycorrhiza***, 12: 191-198.

Lindhorst, H. J., van Loon, L. C., van Rossum, C. A. M., Mayer, A., Bol, J. F., van Roekel, J. S. C., Meulenhoff, E. J., & Cornelissen, B. J. C. 1990. Analysis of acidic and basic chitinases from tobacco and petunia and their constitutive expression in transgenic tobacco. ***Molecular Plant-Microbe Interactions,*** 3: 252-258.

Liu, R. J. 1995. Effect of vesicular-arbuscular mycorrhizal fungi on verticillium wilt of cotton. ***Mycorrhiza***, 5:293-297.

Liu, J. J. & Ekramoddoullah, A. K. M. 2006. The family 10 of plant pathogenesis-related proteins: Their structure, regulation, and function in response to biotic and abiotic stresses. ***Physiological and Molecular Plant Pathology***, 68: 3-13.

López-Ráez, J. A., Verhage, A., Fernández, I., García, J. M., Azcón-Aguilar, C. n., Flors, V., & Pozo, M. J. 2010. Hormonal and transcriptional profiles highlight common and differential host responses to arbuscular mycorrhizal fungi and the regulation of the oxylipin pathway. ***Journal of Experimental Botany***, 61: 2589-2601.

Lu, A. Y. H., Junk, K. W., & Coon, M. J. 1969. Resolution of the Cytochrome P-450-containing ω-hydroxylation system of liver microsomes into three components. ***Journal of Biological Chemistry***, 244: 3714-3721.

Ludwig-Müller, J. 2000. Hormonal balance in plants during colonization by mycorrhizal fungi**.** In Kapulnik Y., Douds D., (eds) ***Arbuscular mycorrhizas: physiology and function.*** Kluwer Academic Publishers, Amsterdam: 263–283.

Lumsden, R. D., Lewis, J. A., & Fravel, D. R. 1995. Formulation and delivery of biocontrol agents for use against soilborne plant pathogens. In: Hall, F. R. and Barry, J. W. (eds) ***Biorational Pest Control Agents: Formulation and Delivery.*** American Chemical Society, Washington DC: 166-187.

Luna, E., Pastor, V., Robert, J., Flors, V., Mauch-Mani, B., & Ton, J. 2010. Callose deposition: a multifaceted plant defense response. ***Molecular Plant-Microbe Interactions***, 24: 183-193.

Maillet, F., Poinsot, V., Andre, O., Puech-Pages, V., Haouy, A., Gueunier, M., Cromer, L., Giraudet, D., Formey, D., Niebel, A., Martinez, E. A., Driguez, H., Bécard, G., & Dénarié, J. 2011. Fungal lipochitooligosaccharide symbiotic signals in arbuscular mycorrhiza. ***Nature***, 469: 58-63.

Maria, M. G., Jolanda, R., Andre, J. C., Brenda, D. W., & Michael, J. W. 2006. Clonality in South African isolates and evidence for a European origin of the root pathogen *Thielaviopsis basicola*. ***Mycological Research***, 161: 306-311.

McGarvey, D. J. & Croteau R. 1995. Terpenoid metabolism**.** ***The Plant Cell***, 7: 1015-1026.

Mattews, V. D. Studies of the genus *Pythium*. 1931. ***University of North Carolina Press.*** 136 pp.

Matuo, T., & Ishigami, K. 1958. On the wilt of *Solanum melongena* L. and its causal fungus *Fusarium oxysporum* f. melongenae n. f. ***Annals of the Phytopathologicial Society of Japan***, 23: 189-192.

Maurhofer, M., Reimmann, C., Schmidli-Sacherer, P., Heeb, S., Haas, D., & Defago, G. 1998. Salicylic acid biosynthetic genes expressed in *Pseudomonas fluorescens* strain P3 improve the induction of systemic resistance in tobacco against tobacco necrosis virus. ***Phytopathology***, 88: 678-684.

Meding, S. M. & Zasoski, R. J. 2008. Hyphal-mediated transfer of nitrate, arsenic, cesium, rubidium, and strontium between arbuscular mycorrhizal forbs and grasses from a California oak woodland. ***Soil Biology and Biochemistry***, 40: 126-134.

Melvin, M. J. & Muthukumaran, N. 2008. Role of certain elicitors on the chemical induction of resistance in tomato against the leaf caterpillar *Spodoptera litura* Fab. ***Notulae Botanicae Horti Agrobotanici Cluj,*** 36: 71-75.

Mishiba, K. I., Ando, T., Mii, M., Watanabe, H., Kokubun, H., Hashimoto, G., & Marchesi, E. 2000. Nuclear DNA content as an index character discriminating taxa in the genus *Petunia* sensu Jussieu (Solanaceae). ***Annals of Botany,*** 85: 665−673.

Millet, Y. A., Danna, C. H., Clay, N. K., Songnuan, W., Simon, M. D., Werck-Reichhart, D., & Ausubel, F. M. 2010. Innate immune responses activated in *Arabidopsis* roots by microbe-associated molecular patterns. ***The Plant Cell***, 22: 973-990.

Miller, R. M. & Jasrow, J. D. 1990. Hierarchy of root and mycorrhizal fungal interactions with soil aggrcgation. ***Soil Biology & Biochemistry,*** 22: 579-584.

Mims, C. W., Copes, W. E., & Richardson, E. A. 2000. Ultrastructure of the penetration and infection of pansy roots by Thielaviopsis basicola. ***Phytopathology***, 90: 843-850.

Mitchell R. T. & Deacon J. W. 1986. Differential (host-specific) accumulation of zoospores of *Pythium* on roots of graminaceous and non-graminaceous plants. ***New Phytologist,*** 102: 113-122.

Moorman, G. W., Kang, S., Geiser, D. M., & Kim, S. H. 2002. Identification and characterization of *Pythium* species associated with greenhouse floral crops in Pennsylvania. ***Plant Disease***, 86: 1227-1231.

Mosse, B. 1973. Advances in the study of vesicular-arbuscular mycorrhiza. ***Annual Review of Phytopathology***, 11: 171-196.

Müller, L. A., Goodman, C. D., Silady, R. A., & Walbot, V. 2000. AN9, a petunia glutathione S-transferase required for anthocyanin sequestration, is a flavonoid-binding protein. ***Plant Physiology***, 123: 1561–1570.

Nagy, R., Karandashov, V., Chague, V., Kalinkevich, K., Tamasloukht, M., Xu, G., Jakobsen, I., Levy, A. A., Amrhein, N., & Bucher, M. 2005. The characterization of novel mycorrhiza-specific phosphate transporters from *Lycopersicon esculentum* and *Solanum tuberosum* uncovers functional redundancy in symbiotic phosphate transport in solanaceous species. ***The Plant Journal***, 42: 236-250.

Nag Raj, T.R. & W.B. Kendrick. 1975. A monograph of *Chalara* and allied genera. ***University of Waterloo, Ontario, Canada***, pp. 200.

Napoli, C., Lemieux, C., & Jorgensen, R.A. 1990. Introduction of a chimeric chalcone synthase gene into Petunia results in reversible co-suppression of homologous genes in trans. ***The Plant Cell***, 2: 279–289.

Nelson, P. E. 1981. Life cycle and epidemiology of Fusarium oxysporum. In Mace M. E., Bell A. A., and Beckman C. H. (eds) ***Fusarium Wilt Diseases of Plants***. Academic Press, New York: 51-80.

Neumann, E. & George, E. 2005. Does the presence of arbuscular mycorrhizal fungi influence growth and nutrient uptake of a wild-type tomato cultivar and a mycorrhiza-defective mutant, cultivated with roots sharing the same soil volume? ***New Phytologist***, 166: 601-609.

Newman, E. I. & Reddel, P. 1987. The distribution of mycorrhizas families of vascular plants. ***New Phytologist***, 106: 745-751.

Nishimura, M. T., Stein, M., Hou, B. H., Vogel, J. P., Edwards, H., & Somerville, S. C. 2003. Loss of a callose synthase results in salicylic acid-dependent disease resistance. ***Science***, 301: 969-972.

Niu, C., Lister, H. E., Nguyen, B., Wheeler, T. A., & Wright, R. J. 2008. Resistance to *Thielaviopsis basicola* in the cultivated A genome cotton. ***Theoretical and Applied Genetics***, 117: 1313-1323.

Niu, D. D., Liu, H. X., Jiang, C. H., Wang, Y. P., Wang, Q. Y., Jin, H. L., & Guo, J. H. 2011. The plant growth promoting rhizobacterium *Bacillus cereus* AR156 induces systemic resistance in *Arabidopsis thaliana* by simultaneously activating salicylate- and jasmonate/ethylene-dependent signaling pathways. ***Molecular Plant-Microbe Interactions***, 24: 533-542.

Nürnberger, T., Brunner, F., Kemmerling, B., & Piater, L. 2004. Innate immunity in plants and animals: striking similarities and obvious differences. ***Immunological Reviews***, 198: 249-266.

Okubara, P. & Paulitz, T. 2005. Root defense responses to fungal pathogens: a molecular perspective. ***Plant and Soil***, 274: 215-226.

Oliver, R. P. & Ipcho, S. V. S. 2004. Arabidopsis pathology breathes new life into the necrotrophs-vs.-biotrophs classification of fungal pathogens. ***Molecular Plant Pathology***, 5: 347-352.

Oyarzun, P., Gerlagh, M., & Hoogland, A. E. 1993. Pathogenic fungi involved in root-rot of peas in the netherlands and their physiological specialization. ***Netherlands Journal of Plant Pathology***, 99: 23-33.

Paré, P. W., Farag, M. A., Krishnamachari, V., Zhang, H., Ryu, C. M., & Kloepper, J. W. 2005. Elicitors and priming agents initiate plant defense responses. ***Photosynthesis Research***, 85: 149-159.

Park, Y., Chen, X., & Punja, Z. K. 2006. Molecular and biological characterization of a mitovirus in *Chalara elegans* (*Thielaviopsis basicola*). ***Phytopathology***, 96: 468-479.

Parniske, M. 2004. Molecular genetics of the arbuscular mycorrhizal symbiosis. ***Current Opinion in Plant Biology***, 7: 414-421.

Parniske, M. 2008. Arbuscular mycorrhiza: the mother of plant root endosymbioses. ***Nature Reviews/Microbiology***, 6: 763-775.

Paszkowski, U., Jakovleva, L., & Boller, T. 2006. Maize mutants affected at distinct stages of the arbuscular mycorrhizal symbiosis. ***The Plant Journal***, 47: 165-173.

Peer, R., Niemann, G. J., & Schippers, B. 1991. Induced resistance and phytoalexin accumulation in biological control of *Fusarium* wilt of carnation by *Pseudomonas* sp. strain WCS417r. ***Phytopathology***, 81: 728-734.

Perfect, S. E. & Green, J. R. 2001. Infection structures of biotrophic and hemibiotrophic fungal plant pathogens. ***Molecular Plant Pathology***, 2: 101-108.

Perrenoud, S. 1990. Potassium and plant health. ***International Potash Institute, Basel, Switzerland,*** 365 pages.

Pfaffl, M. W. 2001. A new mathematical model for relative quantification in real-time RT-PCR. ***Nucleic Acids Research*** 29, e45.

Phillips, J. M. & Hayman, D. S. 1970. Improved procedures for clearing roots and staining parasitic and vesicular-arbuscular mycorrhizal fungi for rapid assessment of infection. ***Transactions of the British Mycological Society***, 55: 158-161.

Pieterse, C. M., van Wees, S. C., van Pelt, J. A., Knoester, M., Laan, R., Gerrits, H., Weisbeek, P. J., & Van Loon, L. C. 1998. A novel signaling pathway controlling induced systemic resistance in *Arabidopsis*. ***The Plant Cell***, 10: 1571-1580.

Pieterse, C. M., Pelt, J. A., Ton, J., Parchmann, S., Mueller, M. J., Buchala, A. J., Metraux, J. P., & van Loon, L. C. 2000. Rhizobacteria-mediated induced systemic resistance (ISR) in *Arabidopsis* requires sensitivity to jasmonate and ethylene but is not accompanied by an increase in their production. ***Physiological and Molecular Plant Pathology***, 57: 123-134.

Pieterse, C. M. & van Loon, L. C. 2004. NPR1: the spider in the web of induced resistance signaling pathways. ***Current Opinion in Plant Biology***, 7: 456-464.

Pieterse, C. M., Leon-Reyes, A., Van der Ent, S., & van Wees, S. C. 2009. Networking by small-molecule hormones in plant immunity. ***Nature Chemical Biology***, 5: 308-316.

Postma, J., Schilder, M. T., Bloem, J., & van Leeuwen-Haagsma, W. K. 2008. Soil suppressiveness and functional diversity of the soil microflora in organic farming systems. ***Soil Biology and Biochemistry***, 40: 2394-2406.

Pozo, M. J., Azcón-Aguilar, C. N., Dumas-Gaudot, E., & Barea, J. M. 1999. [beta]-1,3-Glucanase activities in tomato roots inoculated with arbuscular mycorrhizal fungi and/or *Phytophthora parasitica* and their possible involvement in bioprotection. ***Plant Science***, 141: 149-157.

Pozo, M. J., Cordier, C., Dumas-Gaudot, E., Gianinazzi, S., Barea, J. M., & Azcón-Aguilar, C. 2002. Localized versus systemic effect of arbuscular mycorrhizal fungi on defence responses to *Phytophthora* infection in tomato plants. ***Journal of Experimental Botany***, 53: 525-534.

Pozo, M. J. & Azcón-Aguilar, C. 2007. Unraveling mycorrhiza-induced resistance. ***Current Opinion in Plant Biology***, 10: 393-398.

Rausch, C., Daram, P., Brunner, S., Jansa, J., Laloi, M., Leggewie, G., Amrhein, N., & Bucher, M. 2001. A phosphate transporter expressed in arbuscule-containing cells in potato. ***Nature***, 414: 462-470.

Rhodes, L. H. 1980. The use of mycorrhizae in crop systems. ***Outlook on Agriculture***, 10: 275-281.

Redecker, D., Morton, J. B., & Bruns, T. D. 2000. Ancestral lineages of arbuscular mycorrhizal fungi (Glomales). ***Molecular Phylogenetics and Evolution***, 14: 276-284.

Rémy, W., Taylor, T. N., Hass, H., & Kerp, H. 1994. Four hundred-million-year-old vesicular arbuscular mycorrhizae. ***Proceedings of the National Academy of Sciences***, 91: 11841-11843.

Reymond, P. & Farmer, E. E. 1998. Jasmonate and salicylate as global signals for defense gene expression. ***Current Opinion in Plant Biology***, 1: 404-411.

Ririe, K.M., Rasmussen, R.P., and Wittwer, C.T. 1997. Product differentiation by analysis of DNA melting curves during the polymerase chain reaction. ***Analytical Biochemistry,*** 245*:* 154-160.

Rivera-Becerril, F., Calantzis, C., Turnau, K., Caussanel, J. P., Belimov, A. A., Gianinazzi, S., Strasser, R. J., & Gianinazzi-Pearson, V. 2002. Cadmium accumulation and buffering of cadmium-induced stress by arbuscular mycorrhiza in three *Pisum sativum* L. genotypes. ***Journal of Experimental Botany***, 53: 1177-1185.

Rivera-Becerril, F., van Tuinen, D., Martin-Laurent, F., Metwally, A., Dietz, K. J., Gianinazzi, S., & Gianinazzi-Pearson, V. 2005. Molecular changes in *Pisum sativum* L. roots during arbuscular mycorrhiza buffering of cadmium stress. ***Mycorrhiza***, 16: 51-60.

Robert-Seilaniantz, A., Navarro, L., Bari, R., & Jones, J. D. 2007. Pathological hormone imbalances. ***Current Opinion in Plant Biology***, 10: 372-379.

Rosendahl, S. 1985. Interactions between the vesicular-arbuscular mycorrhizal fungus *Glomus fasciculatum* and *Aphanomyces euteiches* root rot of peas. ***Journal of Phytopathology***, 114: 31-40.

Rosendahl, C. N. & Rosendahl, S. 1990. The role of vesicular-arbuscular mycorrhiza in controlling damping-off and growth reduction in cucumber caused by *Pythium ultimum*. ***Symbiosis***, 9: 363-366.

Ruiz-Lozano, J. M., Azcon, R., & Palma, J. M. 1996. Superoxide dismutase activity in arbuscular mycorrhizal *Lactuca sativa* plants subjected to drought stress. ***New Phytologist***, 134: 327-333.

Ruiz-Lozano, J. M., Roussel, H., Gianinazzi, S., & Gianinazzi-Pearson, V**.** 1999. Defense genes are differentially induced by a mycorrhizal fungus and Rhizobium sp. in wild-type and symbiosis-defective pea genotypes. ***Molecular Plant-Microbe Interactions,*** 12: 976-984.

Ruiz-Lozano, J. M., Collados, C., Barea, J. M., & Azcon, R. 2001. Cloning of cDNAs encoding SODs from lettuce plants which show differential regulation by arbuscular mycorrhizal symbiosis and by drought stress. ***Journal of Experimental Botany***, 52: 2241-2242.

Ryu, C. M., Murphy, J. F., Mysore, K. S., & Kloepper, J. W. 2004. Plant growth-promoting rhizobacteria systemically protect *Arabidopsis thaliana* against cucumber mosaic virus by a salicylic acid and NPR1-independent and jasmonic acid-dependent signaling pathway. ***The Plant Journal***, 39: 381-392.

Salzer, P., Bonanomi, A., Beyer, K., Vogeli-Lange, R., Aeschbacher, R. A., Lange, J., Wiemken, A., Kim, D., Cook, D. R., & Boller, T. 2000. Differential expression of eight chitinase genes in *Medicago truncatula* roots during mycorrhiza formation, nodulation, and pathogen infection. ***Molecular Plant-Microbe Interactions***, 13: 763-777.

Schneider J. H. M., Salazar O., Rubio V., & Keijer J. 1997. Identification of *Rhizoctonia solani* associated with field-grown tulips using ITS rDNA polymorphism and pectic zymograms. ***European Journal of Plant Pathology***, 103: 607-622.

Schönbeck, F. & Dehne, H. W. 1977. Damage to mycorrhizal and nonmycorrhizal cotton seedlings by *Thielaviopsis basicola*. ***Plant Disease Reporter,*** 61: 266–267.

Schützendubel, A. & Polle, A. 2002. Plant responses to abiotic stresses: heavy metal-induced oxidative stress and protection by mycorrhization. ***Journal of Experimental Botany***, 53: 1351-1365.

Schüssler, A., Schwarzott, D., & Walker, C. 2001. A new fungal phylum, the Glomeromycota: phylogeny and evolution. ***Mycological Research***, 105: 1413-1421.

Schüßler, A. & Walker, C. 2010. The *Glomeromycota*. A species list with new families and new genera. ***The Royal Botanic Garden Kew, Botanische Staatssammlung Munich, and Oregon State University,*** https://www.createspace.com/3698603.

Sekhara, R. D. M. R., Schorderet, M., Feller, U., & Reinhardt, D. 2007. A petunia mutant affected in intracellular accommodation and morphogenesis of arbuscular mycorrhizal fungi. ***Plant Journal***, 51: 739-750.

Sekhara Reddy, D. M. R., Svistoonoff, S., Breuillin, F., Wegmüller, S., Bucher, M., & Reinhardt, D. 2009. Development and function of the arbuscular mycorrhizal symbiosis in petunia. In T. Gerats & J. Strommer (Eds) ***Petunia: evolutionary, developmental and physiological genetics.*** Springer, New York: 131-156.

Selosse, M. A., Richard, F., He, X., & Simard, S. W. 2006. Mycorrhizal networks: des liaisons dangereuses? ***Trends in Ecology & Evolution***, 21: 621-628.

Shamshiri, M. H., Mozafari, V., Sedaghati, E., & Bagheri, V. 2011. Response of petunia plants (*Petunia hybrida* cv. Mix) inoculated with *Glomus mosseae* and *Glomus intraradices* to phosphorus and drought stress. ***Journal of Agricultural Science and Technology,*** 13: 929-942.

Shaul, O., Galili, S., Volpin, H., Ginzberg, I., Elad, Y., Chet, I., & Kapulnik, Y. 1999. Mycorrhiza-induced changes in disease severity and PR protein expression in tobacco leaves. ***Molecular Plant-Microbe Interactions***, 12: 1000-1007.

Siciliano, V., Genre, A., Balestrini, R., Cappellazzo, G., de Wit, P. J. G. M., & Bonfante, P. 2007. Transcriptome analysis of arbuscular mycorrhizal roots during development of the prepenetration apparatus. ***Plant Physiology,*** 144: 1455-1466.

Slezack, S., Dumas-Gaudot, E., Rosendahl, S., Kjoller, R., Paynot, M., Negrel, J. & Gianinazzi S. 1999. Endoproteolytic activities in pea roots inoculated with the arbuscular mycorrhizal fungus *Glomus mosseae* and/or *Aphanomyces euteiches* in relation to bioprotection. ***New Phytologist,*** 142:517–529.

Slezack, S., Dumas-Gaudot, E., Paynot, M., & Gianinazzi, S. 2000. Is a fully established arbuscular mycorrhizal symbiosis required for bioprotection of *Pisum sativum* roots against *Aphanomyces euteiches*? ***Molecular Plant-Microbe Interactions***, 13: 238-241.

Smith, F. A. & Smith, S. E. 1997. Structural diversity in (vesicular)-arbuscular mycorrhizal symbiosis. ***New Phytologist,*** 137: 373-388.

Smith, S. E., & Read, D. J. ed. 1997. ***Mycorrhizal symbiosis.*** Academic Press, San Diego and London, pp 605.

Smith, S. E. & Read, D. J. ed. 2008. ***Mycorrhizal Symbiosis*** (Third Edition). Academic Press, London, pp 800.

Smith, S. E., Jakobsen, I., Gronlund, M., & Smith, F. A. 2011. Roles of arbuscular mycorrhizas in plant phosphorus nutrition: interactions between pathways of phosphorus uptake in arbuscular mycorrhizal roots have important implications for understanding and manipulating plant phosphorus acquisition. ***Plant Physiology***, 156: 1050-1057.

Song, Y. Y., Zeng, R. S., Xu, J. F., Li, J., Shen, X., & Yihdego, W. G. 2010. Interplant communication of tomato plants through underground common mycorrhizal networks. ***PloS One***, 5: e13324.

St-Arnaud, M., Vimard, B., Fortin, J. A., Hamel, C., & Caron, M. 1997. Inhibition of *Fusarium oxysporum* f-sp. dianthi in the non-VAM species *Dianthus caryophyllus* by co-culture with *Tagetes patula* companion plants colonized by *Glomus intraradices*. ***Canadian Journal of Botany***, 75: 998-1005.

Strittmatter, G., Gheysen, G., Gianinazzi-Pearson, V., Hahn C., Niebel, A., Rohde, W., & Tacke, E. 1996. Infections with various types of organisms stimulate transcription from a short promoter fragment of the potato gst1 gene. ***Molecular Plant-Microbe Interactions,*** 1: 68-73.

Tamasloukht, M., Sejalon-Delmas, N., Kluever, A., Jauneau, A., Roux, C., Becard, G., & Franken, P. 2003. Root factors induce mitochondrial-related gene expression and fungal respiration during the developmental switch from asymbiosis to presymbiosis in the arbuscular mycorrhizal fungus *Gigaspora rosea*. ***Plant Physiology***, 131: 1468-1478.

Tamasloukht, M., Waschke, A., & Franken, P. 2007. Root exudate-stimulated RNA accumulation in the arbuscular mycorrhizal fungus *Gigaspora rosea*. ***Soil Biology & Biochemistry***, 39: 1824-1827.

Tambascio, S. 2007. Yesterday, Today, Tomorrow. ***Greenhouse Grower, May 2007***: 1-8.

Tang, D., Ade, J., Frye, C. A., & Innes, R. W. 2005. Regulation of plant defense responses in *Arabidopsis* by EDR2, a PH and START domain-containing protein. ***The Plant Journal***, 44: 245-257.

Thordal-Christensen, H. 2003. Fresh insights into processes of non-host resistance. ***Current Opinion in Plant Biology,*** 4: 351-357.

Tollot, M., Wong Sak Hoi, J., van Tuinen, D., Arnould, C., Chatagnier, O., Dumas, B., Gianinazzi-Pearson, V., & Seddas, P. M. 2009. An STE12 gene identified in the mycorrhizal fungus *Glomus intraradices* restores infectivity of a hemibiotrophic plant pathogen. ***New Phytologist***, 181: 693-707.

Trouvelot, A., Fardeau, J. C., Plenchette, C., Gianinazzi, S., & Gianinazzi-Pearson, V. 1986. Nutritional balance and symbiotic expression in mycorrhizal wheat. ***Physiologie Vegetale***, 24: 300.

Truman, W., Bennett, M. H., Kubigsteltig, I., Turnbull, C., & Grant, M. 2007. Arabidopsis systemic immunity uses conserved defense signaling pathways and is mediated by jasmonates. ***Proceedings of the National Academy of Sciences***, ***USA,*** 104: 1075-1080.

van Der Ent, S., van Wees, S. C., & Pieterse, C. M. 2009. Jasmonate signaling in plant interactions with resistance-inducing beneficial microbes. ***Phytochemistry***, 70: 1581-1588.

van Loon, L. C. & Van Kammen, A. 1970. Polyacrylamide disc electrophoresis of the soluble leaf proteins from *Nicotiana tabacum* var. Samsun and Samsun NN: II. Changes in protein constitution after infection with tobacco mosaic virus. ***Virology***, 40: 199-211.

van Loon, L. C., Bakker, P. A., & Pieterse, C. M. 1998. Systemic resistance induced by rhizosphere bacteria. ***Annual Review of Phytopathology***, 36: 453-483.

van Loon, L. C., Rep, M., & Pieterse, C. M. 2006. Significance of inducible defense-related proteins in infected plants. ***Annual Review of Phytopathology***, 44: 135-162.

van Loon, L. 2007. Plant responses to plant growth-promoting rhizobacteria. ***European Journal of Plant Pathology***, 119: 243-254.

van Tuinen, D., Jacquot, E., Zhao, B., Gollotte, A., & Gianinazzi-Pearson, V. 1998. Characterization of root colonization profiles by a microcosm community of arbuscular mycorrhizal fungi using 25S rDNA-targeted nested PCR. ***Molecular Ecology***, 7: 879-887.

van Wees, S. C., Luijendijk, M., Smoorenburg, I., van Loon, L. C., & Pieterse, C. M. 1999. Rhizobacteria-mediated induced systemic resistance (ISR) in *Arabidopsis* is not associated with a direct effect on expression of known defense-related genes but stimulates the expression of the jasmonate-inducible gene Atvsp upon challenge. ***Plant Molecular Biology***, 41: 537-549.

Veit S., Wörle J. M., Nürnberger T., Koch W., & Ulrich Seitz H. 2001. A novel protein elicitor (PaNie) from *Pythium aphanidermatum* induces multiple defense responses in carrot, *Arabidopsis*, and tobacco. ***Plant Physiology***, 127: 832–841.

Verberne, M. C., Hoekstra, J., Bol, J. F., & Linthorst, H. J. M. 2003. Signaling of systemic acquired resistance in tobacco depends on ethylene perception. ***The Plant Journal***, 35: 27-32.

Verdonk, J. C., Haring, M. A., van Tunen, A. J., & Schuurink, R. C. 2005. *ODORANT1* regulates fragrance biosynthesis in Petunia flowers. ***The Plant Cell***, 17:1612-1624.

Verhagen, B. W., Glazebrook, J., Zhu, T., Chang, H. S., Van Loon, L. C., & Pieterse, C. M. 2004. The transcriptome of rhizobacteria-induced systemic resistance in arabidopsis. ***Molecular Plant-Microbe Interactions***, 17: 895-908.

Vessey, J. K. 2003. Plant growth promoting rhizobacteria as biofertilizers. ***Plant and Soil***, 255: 571-586.

Vierhielig, H. 2004. Further root colonization by arbuscular mycorrhizal fungi in already mycorrhizal plants is suppressed after a critical level of root colonization. ***Journal of Plant Physiology***, 161: 339-341.

Walters, D. R. & Boyle, C. 2001. Induced resistance and allocation costs: What is the impact of pathogen challenge? ***Physiological and Molecular Plant Pathology***, 66: 40-44.

Walters, D. R. 2011. Plant defense. Warding off attack by pathogens, herbivores, and parasitic plants. ***United Kingdom***: ***Blackwell Publishing Ltd,*** pp 236.

Wang, B. & Qiu, Y. L. 2006. Phylogenetic distribution and evolution of mycorrhizas in land plants. ***Mycorrhiza***, 16: 299-363.

Waschke, A., Sieh, D., Tamasloukht, M., Fischer, K., Mann, P., & Franken, P. 2006. Identification of heavy metal-induced genes encoding glutathione-S-transferases in the arbuscular mycorrhizal fungus *Glomus intraradices*. ***Mycorrhiza***, 17: 1-10.

Wawrzynska, A., Christiansen, K. M., Lan, Y., Rodibaugh, N. L. & Innes R. W. 2008. Powdery mildew resistance conferred by loss of the ENHANCED DISEASE RESISTANCE1 protein kinase is suppressed by a missense mutation in *KEEP ON GOING*, a regulator of abscisic acid signaling. ***Plant Physiology***, 148: 1510-1522.

Wegmüller, S., Svistoonoff, S., Reinhardt, D., Stuurman, J., Amrhein, N., & Bucher, M. 2008. A transgenic dTph1 insertional mutagenesis system for forward genetics in mycorrhizal phosphate transport of Petunia. ***The Plant Journal***, 54: 1115-1127.

Wei, G., Kloepper, J. W., & Tuzun, S. 1991. Induction of systemic resistance of cucumber to *Colletotrichum orbiculare* by select strains of plant growth-promoting rhizobacteria. ***Phytopathology***, 81: 1508-1512.

Weidmann, S., Sanchez, L., Descombin, J., Chatagnier, O., Gianinazzi, S., & Gianinazzi-Pearson, V. 2004. Fungal elicitation of signal transduction-related plant genes precedes mycorrhiza establishment and requires the dmi3 gene in *Medicago truncatula*. ***Molecular Plant-Microbe Interactions***, 17: 1385-1393.

Whipps, J. M. 2004. Prospects and limitations for mycorrhizas in biocontrol of root pathogens. ***Canadian Journal of Botany***, 82: 1198-1227.

Wright, E. R., Rivera, M. C., Asciutto, K., Gasoni, L., Barrera, V., & Kobayashi, K. 2004. First report of petunia root rot caused by *Rhizoctonia solani* in Argentina. ***Plant Disease***, 88: 86-86.

Wulf, A., Manthey, K., Doll, J., Perlick, A.M., Linke, B., Bekel, T., Meyer, F., Franken, P., Küster, H., & Krajinski, F. 2003. Transcriptional changes in response to arbuscular mycorrhiza development in the model plant *Medicago truncatula***.** ***Molecular Plant-Microbe Interactions,*** 16: 306–314.

Yao, M. K., Desilets, H., Charles, M. T., Boulanger, R., & Tweddell, R. J. 2003. Effect of mycorrhization on the accumulation of rishitin and solavetivone in potato plantlets challenged with *Rhizoctonia solani*. ***Mycorrhiza***, 13: 333-336.

Zahn, M., Wimalasekara, R., Gobel, C., Feussner, I., Holk, A., & Scherer, G. N. F. E. 2005. Expression of *Arabidopis* phospholipase A genes in *Petunia x hybrida*. Increased hypersensitive-like response after infection with *Botrytis cinerea* and *Pseudomonas syringae* pv. tomato DC3000 demonstrates a function for phospholipase A in pathogen defence. ***Physiological and Molecular Plant Pathology***, 67: 2-14.

Zhu, H. H. & Yao, Q. 2004. Localized and systemic increase of phenols in tomato roots induced by *Glomus versiforme* inhibit *Ralstonia solanacearum*. ***Journal of Phytopathology,*** *152: 537–542*.

Annexe 1

- **Ferty8 (enterprise, place; www.plantafert.com)**

Composition:		**Trace Elements:**	
Total Nitrogen:	**18**	Boron (B)	0.02
- nitrate nitrogen :	8.4	Copper (Cu)	0.03*
- ammonium nitrogen :	11.6	Iron (Fe)	0.075*
		Manganese (Mn)	0.05*
water-soluble Phosphate :	**--**	Molybdenum (Mo)	0.001
water-soluble Potassium Oxide:	**22**	Zinc (Zn)	0.01*
water-soluble Magnesium Oxide:	**3.3**		
		* chelated as EDTA	
		** chelated as EDTA and EDDHA	

A 10% stock solution of Ferty8 was prepared and diluted 100 fold for use. 1.757 (g) calcium sulfate ($CaSO_4$) was added and the final pH was adjusted to 5.5-6.

Annexe 2

- **Carrot agar medium**
    - Carrot juice extracted by grinding 30 g of fresh carrots and filtered using cloth sieve
    - 15 g agar (Roth, Karlsruhe, Germany)

Fill up with distilled water to 1000 ml. Adjust pH to 7. Autoclave the solution, cool it down (40-50°C) and add two antibiotics: Pimaricin (10 mg/L) (Sigma) and Penicillin (100 mg/L) (Sigma).

- **V-8 agar medium**
    - 200 ml of V-8 juice (Gemuesesaft, Penny, Germany)
    - 3 g of $CaCO_3$
    - 15 g agar (Roth, Karlsruhe, Germany)

Fill up with distilled water to 1000 ml. Adjust pH to 7.2. Autoclave the solution, cool it down (40-50°C) and add two antibiotics: Carnenicillin (100 mg) (Sigma) and Ampicillin (Sig