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Abstract

In the future, an increased food demand together with restrictions of pesticide use will require new options of disease management within agriculture. Usage of biological control agents (BCAs) is a feasible alternative. For example, BCAs may reduce pathogen attacks in plants by induced resistance. In this strategy the BCA initially elicits defence responses in the plant. Signalling hormones such as salicylic acid (SA) and jasmonic acid (JA) are synthesised and down-stream defence genes expressed. This interaction leads to faster and stronger defence response of the plant against later pathogen attack.

Clonostachys rosea strain IK726 is a BCA under development. It has been reported to endophytically colonize the roots of cucumber and to elicit the expression of defence-related genes in wheat and canola.

Induced resistance by *C. rosea* against grey mould, *Botrytis cinerea*, was studied for greenhouse tomato and the model plant *Arabidopsis thaliana*. *C. rosea* was found to colonize the roots of tomato and *A. thaliana,* both on the surface and endophytically. This interaction with the plant also triggered defence responses, in tomato SA-related defence gene *CHI9* was induced, while in *A. thaliana* expression of JA-related defence gene *PDF 1.2* and camalexin biosynthesis gene *PAD3* were induced. Yet, these responses were only weakly expressed, and when tomato and *A. thaliana* were later challenged with *B. cinerea* there was no visible suppression of the infection. In summary, *C. rosea* was able to colonize both tomato and *A. thaliana* endophytically and to induce defence-related gene expression changes, but did not promote plant growth or induce systemic resistance against infection of *B. cinerea* in the leaves.

Keywords: biological control agent, *Clonostachys rosea*, endophytic colonization, induced resistance

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1 Background

Diseases caused by plant pathogens usually result in crop losses within agriculture. These losses range between 11-21% for staple crops such as rice, wheat, maize and potatoes (Oerke 2006). However, the absence of crop protection would result in an even larger loss. Human population is estimated to reach nine billion people by 2050 (UN 2014), and in order to secure food safety production needs to increase by 100-110% (Tilman et al. 2011). The closing of yield gaps, rather than expansion of agricultural land, is suggested as one solution to this future demand (Godfray et al. 2010; Beed 2014), and the reduction of plant diseases is a way to achieve this (Flood 2010). In addition, Integrated Pest Management (IPM) and additional directives recently implemented in the EU, restrict the use of chemical pesticides in crop protection (Birch et al. 2011). Therefore, alternative methods to manage disease control are important to improve food production.

Biological control is a complement and alternative for disease management in agriculture, it is described as the inhibition of a pathogen by reduction of inoculum or disease by other organisms (Cook & Baker 1983 cited in Alabouvette et al. 2006). These organisms are antagonists such as bacteria or fungi that weaken or kill the plant pathogen. Antagonists used in biological control are called biological control agents (BCAs).

The strategies used by antagonists are the results of direct or indirect interactions with the pathogen. For example, mycoparasitism, secretion of toxic secondary metabolites and competition for nutrient and space are direct interactions. On the other hand, induced resistance in host plants and plant-growth-promotion are indirect interactions (Harman et al. 2004; Gerbore et al. 2014). To inhibit a plant pathogen the antagonist may use one or several of these strategies.

Mycoparasitism is a strategy described for various *Trichoderma spp.* (Druzhinina et al. 2011). It is suggested that to detect other fungi these antagonists secrete protease enzymes, which release oligopeptides from the cell wall of nearby fungi. The oligopeptides are perceived by *Trichoderma*, which grows towards the

targeted fungi and coils around and penetrates the hypha (dos Reis Almeida et al. 2007; Druzhinina et al. 2011). Similarly, *Pythium oligandrum* parasites on *Fusarium*, and coils around the target hypha and forms a papilla-like structure to aid penetration (Benhamou et al. 1999). Antagonists can also parasite on sclerotia, which is a resting structure that can persist in the soil for several years. It is a source of inoculum, hence reduction of sclerotia would result in reduced germination of the pathogen (Ikeda et al. 2012; Hu et al. 2013; Geraldine et al. 2013).

Mycoparasitism is also closely linked to production of enzymes and secondary metabolites. These are often involved in degradation of the pathogen's cell wall. Enzymes such as glucanases, chitanases and proteases were identified in *Trichoderma spp.* These enzymes break down the cell wall components chitin, polysaccharides and β-glucans, and proteases deactivate the pathogen's hydrolytic enzymes (Howell 2003; Geraldine et al. 2013; Steindorff et al. 2014). Both chitinases and glucanases are produced by the mycoparasite *Clonostachys rosea*, which were confirmed to degrade the cell walls of plant pathogens *Pythium* and *Fusarium* (Chatterton & Punja 2009). Furthermore, secondary metabolites that are toxic to fungi may be secreted during antagonist-pathogen interactions as well. Toxic compounds such as polyketides, terpenoids (viridiol) and non-ribosomal peptides (NRPs) such as gliotoxin and peptaibol are described for *Trichoderma* (Druzhinina et al. 2011; Mukherjee et al. 2012). NRPs were also identified in *C. rosea*, where peptaibol metabolites were produced during interaction with the pathogen *Sclerotinia sclerotium* (Rodríguez et al. 2011).

Competition for nutrients and space in the rhizosphere are also important strategies of the antagonist. For example, antagonist *Trichoderma harzianium* changed the composition of exudates produced by cucumbers, which reduced germination of plant pathogen *Fusarium oxysporum* forma specialis (f. sp.) *cucumerinum* (Zhang et al. 2013). *Trichoderma spp.* was also reported to reduce pathogen germination by competing for seed exudates that triggered its germination (Howell 2002).

Moreover, the establishment of these antagonists also have indirect results. Several fungi promote plant growth, for example *Trichoderma virens* (Contreras-Cornejo et al. 2009), *C. rosea* (Ravnskov et al. 2006), arbuscular mycorrhizal fungi *Funneliformis mosseae* and *Acaulospora laevis* (Tanwar et al. 2013) among others. This growth promotion is likely to occur through the production of phytohormones and beneficial secondary metabolites. Harzianolide, a secondary metabolite was identified for *T. harzianium* (Vinale et al. 2008; Cai et al. 2013) and triptamine, an auxin hormone, was identified for *P. oligandrum* (Le Floch et al. 2003a). Auxin compounds were also detected for *T. virens* (Contreras-Cornejo et al. 2009). However, similar auxin compounds found in *Pythium* promoted growth

in *P. oligandrum* but inhibited growth in two different *Pythium* species (Le Floch et al. 2003a).

Likewise, induced resistance is also an indirect result of interaction with the antagonist. It is a defence response of the plant towards the antagonist, which results in a faster defence response towards subsequent pathogen attack. For example, interaction with *Trichoderma spp.* reduces infection from various pathogenic fungi and viruses in several plant species (Harman et al. 2004). This is also the case for genera *Pythium*, *Fusaria*, *Rhizoctonia* and *Clonostachys*, which contain non-pathogenic strains that induce resistance (Le Floch et al. 2003b; Fravel et al. 2003; Jabaji-Hare & Neate 2005; Roberti et al. 2008).

1.1 Plant defence

Plant defence against pathogens or other organisms is illustrated by the zigzag model of Jones and Dangl (2006). This model describes two main responses, PAMP-triggered immunity (PTI) and effector-trigged immunity (ETI). PTI is an initial immunity, whereby receptors at the cell membrane induce plant response when pathogen/microbial/damage-associated-molecular-patterns (PAMP, MAMP, DAMP) are recognized. Within fungi these are, for example, xylanases, β-glucans, chitin and ergostol (Nürnberger et al. 2004). In contrast, ETI is triggered from receptors within the cell that detect a pathogen's effector molecules. These effectors are secreted directly into the cytoplasm and can suppress PTI signalling (de Jonge et al. 2010). However, PTI is also impeded if PAMP/MAMP/DAMPs are undetected (Bardoel et al. 2011). ETI is a stronger and faster response than PTI (Jones & Dangl 2006), although activation of both PTI and ETI receptors lead to a defence signalling cascade and a local or systemic response.

Some of the signals produced during defence signalling are, for example, salicylic acid (SA), jasmonic acid (JA) and ethylene (ET). These phytohormones regulate the expression of defence genes (Bari & Jones 2009). Antagonists may trigger synthesis of these phytohormones in the plant and induce defence responses (Hase et al. 2008; Salas-Marina et al. 2011; Kojima et al. 2013).

SA is the main signalling element in systemic acquired resistance (SAR), while JA and ET are important for induced systemic resistance (ISR). SAR provides a broad spectrum protection that is trigged by synthesis of SA or its analogs 2,6-dichloroisicotinic (INA) and benzothiadiazole (BTH)(White 1979; Métraux et al. 1991; Görlach et al. 1996). After SA is synthesized it interacts with transcription cofactor NPR1. High levels of SA result in degradation of NPR1 and induction of local cell death, while lower levels result in increased concentrations of NPR1 and expression of defence genes. These lower levels can be found in uninfected tissues

of the attacked plant (Fu & Dong 2013). Methyl-salicylate (MeSA), a volatile and inactive metabolite, is suggested as a long-distance signal for SA (Park et al. 2007). Other mobile signals involved in acquisition of SA in uninfected tissues are lipid-transfer protein (DIR1; Maldonado et al. 2002) and azelaic acid (AZA) (Jung et al. 2009).

Similarly, ISR also leads to faster defence response during pathogen attacks. However, this response is associated to JA and ET signalling instead. JA is synthesized through the oxylipin pathway, and further metabolized and transformed into JA-Ile (jasmonyl-isoleucine) (Fonseca et al. 2009). This compound is highly active and binds to the SCF^{COI1} complex, which allows degradation of JAZ, a JA signalling inhibitor (Pauwels & Goossens 2011). Following JAZ degradation, two branches of JA- responsive genes are activated: ERF (ethylene response factor 1), an ethylene dependent signalling pathway, and MYC. However, the ERF branch is associated with pathogen attacks and activates defence genes such as *PDF1.2* (*plant defensin 1.2*), while the MYC branch is associated with insect attacks, and activates defence gene *VSP2* (*vegetative storage protein 2*)(Lorenzo & Chico 2004).

Several studies show that SA and JA signalling pathways interact with each other. These signalling pathways are described as antagonistic as well as synergistic (van Wees et al. 2000; De Vos et al. 2005; Mur et al. 2006). However, much is still unknown of how this interaction occurs. Expression of these pathways is also influenced by other plant hormones such as auxin, gibberellin (GA) and abscisic acid (ABA) (Bari & Jones 2009; Pieterse et al. 2012).

1.2 Biocontrol agent *Clonostachys rosea* IK726

Clonostachys rosea (Link: Fr.) Schroers, Samuels, Seifert & Gams is the asexual stage of teleomorph *Bionectria ochroleuca* (Schw.) Shroers & Samuels. It belongs to the family *Bionectriaceae* and was previously described as *Gliocladium roseum* (Schroers et al. 1999).

C. rosea is a common soil fungus that occurs in a broad range of habitats (Sutton et al. 1997). It is a saprophyte and antagonist to several plant pathogens. For example, it decreases symptoms of *Plasmodiophora brassicae* in canola roots (Lahlali & Peng 2013), *Pythium tracheiphylum* in chinese cabbage (Moller et al. 2003) and of *Botrytis cinerea* in several species (Sutton et al. 1997; Cota et al. 2008; Nobre et al. 2005). The antagonistic strategies used by *C. rosea* are mycoparasitism, production of chitanase and glucanase enzymes as well as production of secondary metabolites (peptides) (Xue 2003; Pisi et al. 2006; Roberti et al.

2008; Chatterton & Punja 2009; Rodríguez et al. 2011). It is also reported to grow endophytically in cucumber (Chatterton et al. 2008); and to induce expression of defence genes in wheat and canola (Roberti et al. 2008; Lahlali & Peng 2013).

Currently, two biocontrol products based on *C. rosea* are available in Europe. Prestop (Verdera Oy) which is based on *C. rosea* f. *catenulata* and GlioMix (Verdera Oy) which is a mix of *Clonostachys* fungi. An additional biocontrol product is also under development for strain IK726 of *C. rosea* (Jensen et al. 2007).

1.3 Defence reactions in tomato and *Arabidopsis* against grey mould infection

Grey mould, *B. cinerea*, is a widely spread disease in various agricultural species. It is a necrotrophic fungus that causes large losses in greenhouse crops. This fungus infects fruits, leaves, stems and flower tissues, and, less often, plant tissue below ground (Williamson et al. 2007).

Necrotrophic pathogens such as *B. cinerea* often induce JA/ET defence signalling pathway in the plant, while biotrophs or hemibiotroph fungi trigger SA defence signalling pathways (Glazebrook 2005; Stout et al. 2006). In *Arabidopsis thaliana* JA-deficient mutants showed increased susceptibility against *B. cinerea* infection (Thomma et al. 1998). Similarly, JA/ET dependent genes were up-regulated during interactions with this pathogen (Thomma et al. 2001; Zimmerli et al. 2001; Ferrari et al. 2003). Yet cross-talk with the SA signalling pathway was observed by Zimmerli et al. (2001). In addition, Ferrari et al. (2003) proposed that SA was involved in local response, while JA/ET was involved in systemic responses against *B. cinerea*. When *A. thaliana* was pre-treated with the BCA *T. harzianum*, systemic defences were induced by the JA pathway (Korolev et al. 2008).

In tomato, basal defence against *B. cinerea* is dependent on SA synthesis (Achuo et al. 2004), and infection by *B. cinerea* up-regulates both SA- and JA/ET-dependent genes (Harel et al. 2014). Tomato plants pre-treated with *T. atroviride* or *T. harzianium* induced expression of JA-dependent genes during *B. cinerea* infection (Tucci et al. 2011; Martínez-Medina et al. 2013). But *T. harzianum* also up-regulates SA-dependent genes (Harel et al. 2014). Similarly to *A. thaliana*, it is hypothesized that JA is a main signalling pathway during *B. cinerea* infection in tomato, and that the SA signalling pathway is also elicited but to a lesser extent. Additionally, a recent study reported that resistance against *B. cinerea* in tomato

may also be independent of SA and JA/ET signalling pathways (Beyers et al. 2014).

Further, *B. cinerea* also manipulates the defence response of tomato by eliciting the SA pathway, which in turn suppresses JA signalling (El Oirdi et al. 2011).

An additional defence response reported in *A. thaliana* is biosynthesis of camalexin (Contreras-Cornejo et al. 2011; Salas-Marina et al. 2011). Production of this phytoalexin is triggered by recognition of MAMPs or abiotic stress and contributes to a basal resistance against pathogens such as *B. cinerea* (Ahuja et al. 2012).

The aim of this study is to investigate if *C. rosea* can induce resistance in tomato and *A. thaliana* against the plant pathogen *B. cinerea*. The following questions are addressed: (1) can *C. rosea* grow endophytically in tomato and *A. thaliana*?, (2) does *C. rosea* elicit defense responses in tomato and *A. thaliana*?, (3) does *C. rosea* induce systemic resistance in tomato and *A. thaliana*?, (4) which signalling pathway(s) are responsible for induced resistence in tomato and *A. thaliana*?

2 Methods

2.1 Plant material, fungal strains and cultivation conditions

We used tomato seeds of variety Harzfeuer (F1 generation) from Weibulls, and *A. thaliana* seeds ecotype Columbia. The tomato seeds were sterilized with 1 ml of 2% sodium hypochlorite (NaOCl) for 5 minutes and then rinsed three times with 1 ml sterile water. The seeds were directly spread on petri-dishes of 1% water agar (WA) medium (for recipe see appendix 1) and sealed with parafilm. To induce germination, the seeds were left in the dark overnight at 25°C.

A. thaliana seeds were sterilized with 500 µl of 95% ethanol for 5 minutes and with 500 µl bleach solution for an additional 5 minutes. The seeds were rinsed three times with 1 ml sterile water. To induce germination the seeds were left in the dark at 4^{o}C for 48 hours before placing them on Murashige and Skoog (MS) medium (Duchefa Biochemie; Murashige & Skoog 1962). Both tomato and *A. thaliana* seeds were left to germinate in a climate chamber at 22^{o}C.

C. rosea strain IK726, *Trichoderma atroviride* strain IMI206040 and *B. cinerea* strain B05.10 were grown on potato dextrose agar (PDA; Sigma Aldrich) media at 25^{o}C in darkness. To induce conidiation of *C. rosea*, the plates where left in light for 30 minutes. Conidia suspensions were made by pipetting sterile water several times on the *C. rosea* plates. The concentration was determined and adjusted with a hemacytometer (Hausser Scientific).

2.2 Endophytic colonization

Colonization of tomato and *A. thaliana* by *C. rosea* was tested both *in vitro* (on MS plates) and in soil. Tomato and *A. thaliana* seedlings were transferred to MS plates, and inoculated with 10 µl conidia suspension (1x10^{7} conidia/mL) onto the

roots. *C. rosea* was allowed to colonize the seedlings during 4 days. A root sample was collected from each replicate of control and *C. rosea* treatment, and divided into four subsamples. Two of these were rinsed with distilled water and the remaining two were surface sterilized with 2% NaOCl for 1 minute and subsequently rinsed 2 times with distilled water (Dubey et al. 2014). All samples were placed on rose Bengal (Chroma Gesselschaft) selective media at 25°C for 4 days. This dye decreases the occurrence of fast growing bacteria and fungi at concentrations between 0.01-0.03 g/L (Vargas Gil et al. 2009). A total of 10 biological replicates were sampled for tomato and 6 biological replicates for *A. thaliana*. In addition, seedlings of tomato and *A. thaliana* were inoculated with *T. atroviride* as positive control.

For experiments in soil, seedlings of tomato and *A. thaliana* were inoculated with 25 µl conidia suspension ($1x10^7$ conidia/mL) on each root when transferred into pots. The soil used was *S-jord* from Hasselfors Garden (see appendix 1 for soil composition data). It was autoclaved twice and to recover physical and chemical properties it was left to air for 1-2 weeks. Samples from leaves, stem and roots were collected four weeks after inoculation. These samples were rinsed from soil with distilled water and surface sterilized with 2% NaOCl as described earlier. All samples were placed on rose Bengal media, and checked for growth of *C. rosea* after 6 days. Four biological replicates of control and *C. rosea* treatment were inoculated in soil conditions for tomato and *A. thaliana*.

In addition, molecular markers were used to detect endophytic colonization in *A. thaliana* inoculated in soil. DNA was isolated from leaves, roots and stem samples after surface sterilization. All samples were homogenized in 3% CTAB and left to lyse during 1.5 hour at 60°C. The lysate was extracted twice in chloroform, and DNA was precipitated with isopropanol for 30 min at -20°C. The pellet was washed with 70% ethanol and dissolved in 25-50 µl milliQ water. In the PCR amplification the final concentrations used were 0.06 U/µl of DreamTaq DNA polymerase, 1x of DreamTaq buffer, 1 mM of dNTPs and 0.2 pmol/µl of primers. Primers CRnA and CRnB (Wang 2012) were used, which amplified a 124 bp long region of non-coding DNA in the *C. rosea* IK726 genome. The amplification conditions were set to denaturation at 95°C for 3 min followed by 35 cycles of 30 sec denaturation at 95°C, 20 sec annealing at 60°C and 30 sec elongation at 72°C, with a final elongation at 72°C for 5 min. Presence of amplification product was checked on a 1.2% electrophoresis gel with a positive control of *C. rosea* as reference. A total of 6 biological replicates from control and 10 biological replicates from *C. rosea* treatment were tested for *A. thaliana*.

2.3 Effects of *C. rosea* treatment for tolerance against *B. cinerea*

To test if *C. rosea* root colonization resulted in systemic resistance against *B. cinerea* in tomato and *A. thaliana*, seedlings were inoculated on MS plates as described above. Tomato was inoculated with 10 µl conidia suspension ($1x10^6$ coniadia/mL) and *A. thaliana* with 2.5 µl conidia suspension ($1x10^6$ coniadia/mL). The seedlings were transferred into autoclaved soil 3 days after inoculation and *C. rosea* was allowed to colonize for an additional 15 days.

Before infecting with *B. cinerea* on the leaves, the humidity level was increased overnight. The plants were placed in closed plastic boxes (*A. thaliana*) or sealed with plastic film (tomato). Four replicates of tomato and *A. thaliana* were made. Their leaves were slightly damaged before placing a mycelia plug of *B. cinerea*. All plugs were of uniform size and placed on the edge of the leaves. In each replicate 8 leaves from control and *C. rosea* treatment were infected for tomato and 12 leaves for *A. thaliana*. Before sealing the replicates the plants were sprayed with water. The lesion area was measured after 6 days of pathogen inoculation, using DeltaPix camera and software (DeltaPix, Denmark). A random root sample from each biological replicate was surface sterilized and placed on selective medium with rose Bengal. After four days the occurrence of *C. rosea* was checked.

Similarly, seedlings of tomato and *A. thaliana* that were inoculated with *C. rosea* directly in soil were also tested for enhanced tolerance against *B. cinerea*. Inoculated seedlings were kept in the climate chamber during 27 days for tomato and 20 days for *A. thaliana*. The area of the lesion was measured 6 days after pathogen infection.

2.4 Analysis of plant growth promotion

Above and below ground biomass was measured in *A. thaliana* to detect if treatment with *C. rosea* resulted in changed growth. Seedlings were germinated on MS plates and inoculated with $5x10^4$ spores of *C. rosea* when transferred into autoclaved soil. All plants were left to grow in a climate chamber for 21 days. Both fresh and dry weight was measured. The samples were dried at room temperature for 72h before measuring dry weight.

In a second experiment *A. thaliana* seedlings were inoculated with 25 µl conidia suspension ($1x10^7$ conidia/mL) when transplanted into the soil. After 28 days the fresh weight was measured, and the dry weight was measured after 96h of drying. The samples were dried at 70°C and dry weight was stable already after 24h. Only data for above ground biomass was collected.

2.5 Analysis of gene expression

To assess if *C. rosea* elicited defence responses in tomato and *A. thaliana*, the expression levels of defence related genes were measured. In tomato these levels were measured 72h after inoculation and in *A. thaliana* 50h and 72h after inoculation. All seedlings were inoculated on MS plates with 10 µl of *C. rosea* conidia suspension ($1x10^5$ conidia/mL) for tomato and 2.5 µl of *C. rosea* conidia suspension ($1x10^5$ conidia/mL) for *A. thaliana*. The collected root samples were stored at -70°C until RNA isolation. A total of 3 biological replicates were sampled for tomato, and 2 biological replicates at 50h and 5 biological replicates at 72h were sampled for *A. thaliana.*

To isolate RNA the RNeasy Plant mini kit (QIAGEN) was used. The plant material was disrupted by grinding it with liquid nitrogen with a mortar and pestle before isolation. Both mortar and pestle were previously baked in aluminium during 4h at 240°C. The total RNA was diluted in 30 µl RNase free water (TOC 001), and the concentration was measured with Nanodrop (Thermo Scientific). All samples were treated with DNase, and the extraction quality was checked for the first four extractions with Bioanalyser (Agilent Technologies). Complementary DNA was synthesised with iScript cDNA Synthesis Kit (BioRad) and manufactures protocol was followed. All samples were diluted in 180 µl dH_2O and stored at -20° C for reverse transcription quantitative PCR (RT-qPCR).

The genes used as markers for SA and JA/ET signalling pathway in tomato and *A. thaliana* are summarised in Table 1. An additional marker for camalexin synthesis was added for *A. thaliana* and *Actin* was used as reference gene for both tomato and *A. thaliana*. To measure the PCR amplification efficiency of these markers a standard curve was made. DNA was used as template, which was purified from PCR products. A total of 75 µl PCR product for each marker was purified with chloroform. DNA was precipitated during 2h at -20°C with 95% ethanol and 3M NaOAc (pH 5.2), followed by centrifugation at 4°C, 14000 rpm for 15 min. The pellet was washed with 70% ethanol and diluted in 20 µl dH_2O. For *A. thaliana* primer efficiency was already available.

Transcript levels were quantified by RT-qPCR in an iQ5 qPCR System (Bio-Rad, Hercules, CA). The master mix consisted in 1x EvaGreen mix (Bio-Rad, Hercules, CA), 0.1875 pmol/µl primer for tomato or 0.2 pmol/µl primers for *A. thaliana* and dH_2O. Each reaction contained of 15 µl mastermix and 5 µl diluted cDNA. The amplification conditions were set to an initial denaturation at 98°C for 2 min, followed by 40 cycles of denaturation at 98°C for 5 sec and annealing and elongation at 60°C for 10 sec. A melt curve analysis was also run to check for amplification of primer dimers or nonspecific products. The melting curve was set to 61 cycles at 65°C - 95°C for 10 sec. After each cycle the temperature was in-

creased with 0.5°C. Three technical replicates were amplified for each sample. The CT values were collected and relative expression of each gene was calculated with the Pfaffl method (Pfaffl 2001).

In addition, the effect of *C. rosea* on gene expression was also crosschecked through a standard PCR that was visually checked for products on a 2% electrophoresis gel.

2.6 Statistical analysis

Students t-test was used to assess if differences observed between control and *C. rosea* treatment were significant. A two-tailed t-test was performed in excel with variance set as equal. This test was applied for observed effects of *C. rosea* treatment on growth in *A. thaliana*; as well as for tolerance against *B. cinerea* and differences in gene expression in tomato and *A. thaliana*. Gene expression data was log-transformed before statistical analysis.

Table 1. *Primers used for RT-qPCR in A. thaliana (Arabidopsis) and tomato. The defence pathway that each gene represents is specified under defence pathway.*

Gene name	*Forward primer*	*Reverse primer*	*Defence pathway*	*Reference*
Arabidopsis				
ACTIN 8	GACTCAGATCATGTTTGAGACC	CATGTAACCTCTCTCGGTAAGG	Reference gene	Salas-Marina et al. 2011
PR-1a	ATCTAAGGGTTCACAACCAGGCAC	TGCCTCTTAGTTGTTCTGCGTAGC	SA signalling pathway	Salas-Marina et al. 2011
PAD3	CGATGGAGATGCTCTCAAGTTC	GTCTCCTTGACCACGAGC	Tepenoid phytoalexin pathway	Salas-Marina et al. 2011
PDF1.2	CACCCTTATCTTCGCTGCTC	GGAAGACATAGTTGCATGATCC	JA/ET pathway	Salas-Marina et al. 2011
Tomato				
actin	CACCACTGCTGAACGGGA	GGAGCTGCTCCTGGCAGTTT	Reference gene	Tucci et al. 2011
PR1a	CTGGTGCTGTGAAGATGTGG	TGACCCTAGCACAACCAAGA	SA pathway	Harel et al. 2014
PR-P2	GGAACAGGAACACAAGAAACAGTGA	CCCAATCCATTAGTGTCCAATCG	SA pathway	Tucci et al. 2011
CHI9	ATGTGGAAGAGCCATTGGAG	TGAGGAGTCATCCAGAACCA	SA pathway	Harel et al. 2014
TomLoxA	TGAACCATGGTGGGCTGAAA	CGTGGTACATCCGGTGGGATA	JA/ET pathway	Tucci et al. 2011
PINII	GGCCAAATGCTTGCACCTTT	CTGCCCGAAATTGACTGCTG	JA/ET pathway	Tucci et al. 2011

3 Results

3.1 Endophytic colonization by *C. rosea*

When seedlings were inoculated on MS medium *C. rosea* successfully colonized the roots of both tomato and *A. thaliana*. After four days on selective media *C. rosea* covered the water-washed and surface sterilized root samples of both species completely (Fig. 1 and 2). The recovery frequency was close to 100% (Table 2). There was also bacterial growth in the control samples of tomato roots and contamination of *Aspergillus*-like fungi in the control and *C. rosea* treated samples of *A. thaliana*.

On the other hand, endophytic colonization by *C. rosea* when inoculated in soil conditions was unclear. *C. rosea* was recovered once from selective media of a surface sterilized stem sample in tomato, while three times in water washed root samples (Table 3). Similarly, *C. rosea* was present in one surface sterilized stem sample in *A. thaliana* and in three water washed root samples. It was also present in one water washed stem sample and in all four water washed leave samples (Table 3). However, *C. rosea* was also identified in one leaf sample from a control replicate, both in water washed and surface sterilized treatment. All petri-dishes contained bacterial and other fungal growth.

From the PCR identification, the presence of *C. rosea* was confirmed in 6 of 10 inoculated biological replicates of *A. thaliana*. It was detected in above ground (leaf and stem) samples. In control samples no *C. rosea* was detected. DNA isolation of the root samples was unsuccessful, hence no data could be collected for these.

Table 2. *Endophytic growth of C. rosea in A. thaliana (Arabidopsis) and tomato. Root samples inoculated on petri-dishes were collected from control and C. rosea treatment. These were water-washed or surface sterilized. Samples where C. rosea was recovered are marked with +, while samples without C. rosea are marked with -*

	Control		C. rosea treatment		T. atroviride treatment	
Replicate	Water-wash	Surface sterilized	Water-wash	Surface sterilized	Water-wash	Surface sterilized
Arabidopsis						
1	-	-	missing data	-	+	+
2	-	-	+	+	+	+
3	-	-	+	+	+	+
4	-	-	+	+	+	+
5	-	-	+	+	+	+
6	-	-	+	+	+	+
Tomato						
1	-	-	+	-	+	+
2	-	-	+	+	+	+
3	-	-	+	+	+	+
4	-	-	+	+	+	+
5	-	-	+	+	+	+
6	-	-	+	+	+	+
7	-	-	+	+	+	+
8	-	-	+	+	+	+
9	-	-	+	+	+	+
10	-	-	+	+	+	+

Table 3. *Endophytic growth of C. rosea in A. thaliana (Arabidopsis) and tomato. Root, stem and leave samples inoculated in soil conditions were collected from control and C. rosea treatment. These were water-washed or surface sterilized. Samples where C. rosea was recovered are marked with +, while samples without C. rosea are marked with -*

	Control						C. rosea treatment					
Replicate	Water-wash			Surface sterilized			Water-wash			Surface sterilized		
Arabidopsis	*leaf*	*stem*	*root*	*leaf*	*stem*	*root*	*leaf*	*stem*	*root*	*leaf*	*stem*	*root*
1	-	-	-	-	-	-	+	+	-	-	-	-
2	+	-	-	+	-	-	+	-	+	-	+	-
3	-	-	-	-	-	-	+	-	+	-	-	-
4	-	-	-	-	-	-	+	-	+	-	-	-
Tomato												
1	-	-	-	-	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-	+	-	-	-
3	-	-	-	-	-	-	-	-	+	-	+	-
4	-	-	-	-	-	-	-	-	+	-	-	-

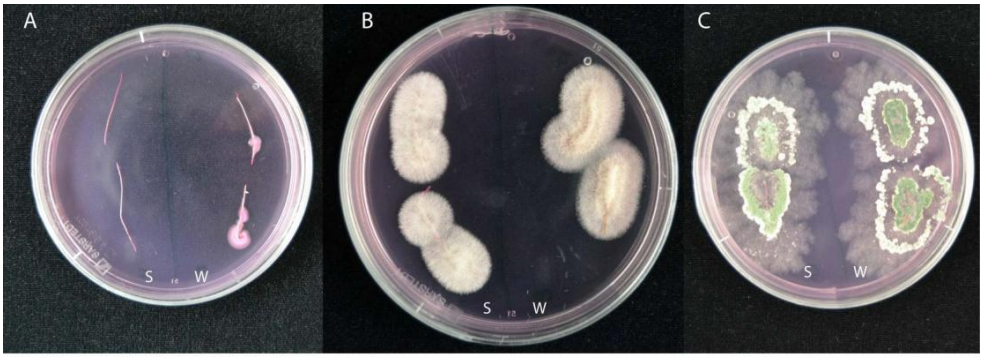

Figure 1. Root samples of tomato inoculated with *C. rosea* on MS media. These samples were surface sterilized (S) or water-washed (W). The plates correspond to control samples (A), *C. rosea* treated samples (B) and *T. atroviride* treated samples (C).

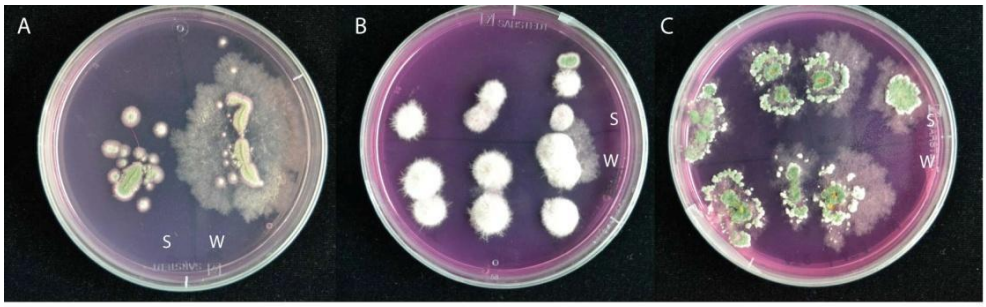

Figure 2. Root samples of *A. thaliana* inoculated with *C. rosea* on MS media. These samples were surface sterilized (S) or water-washed (W). The plates correspond to control samples (A), *C. rosea* treated samples (B) and *T. atroviride* treated samples (C).

3.2 Effects of *C. rosea* treatment on systemic resistance against *B. cinerea*

To test if root-inoculation with *C. rosea* induced systemic resistance in leaves against *B. cinerea,* the lesion area was used as measurement. The average lesion area in tomato was 32261 ± 12833 (mean ± standard deviation) mm^2 for control and 25098 ± 10856 mm^2 for *C. rosea* treatment, when inoculated in soil conditions. In *A. thaliana* the average lesion area was 4698 ± 1052 mm^2 for control and 5820 ± 1198 mm^2 for *C. rosea* treatment when inoculated in soil conditions. These lesion areas were not significantly different between treatment and control (Fig. 3 A, B). The P-values of these were P = 0.42 for tomato and P = 0.21 for *A. thaliana* inoculated in soil conditions.

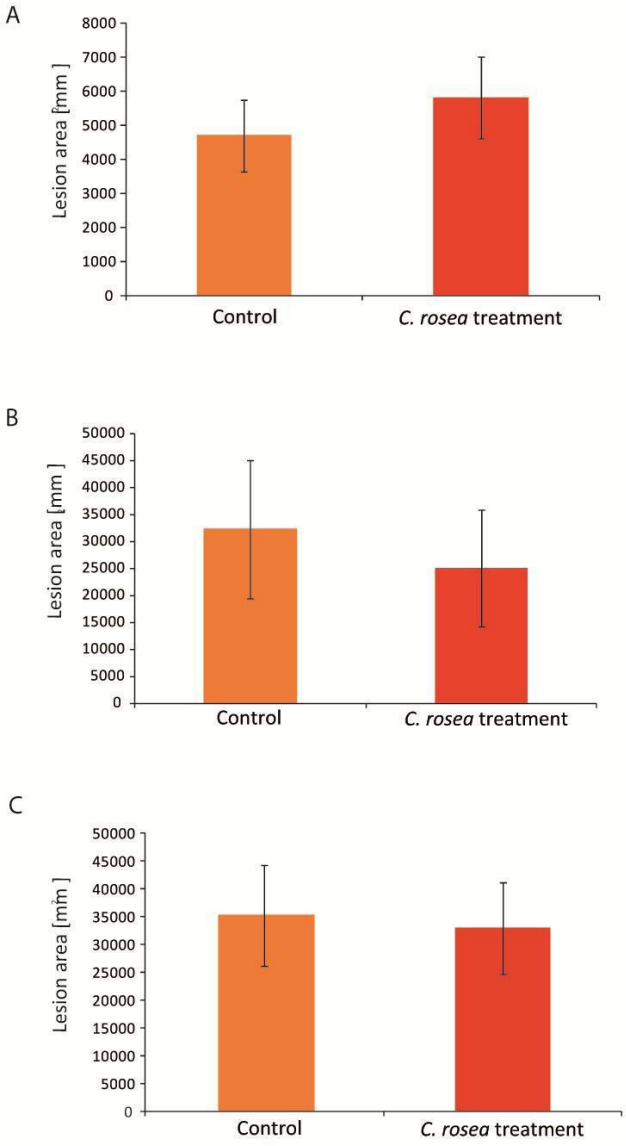

Figure 3. Lesion area caused by *B. cinerea* infection in *A. thaliana* and tomato leaves. The lesion area was measured in *A. thaliana* (A) and tomato (B) for control and *C. rosea* treated plants inoculated in soil conditions. As well as, for tomato when *C. rosea* treated plants were inoculated on MS medium (C). No significant differences were observed.

In addition, the lesion area in tomato when inoculated on MS medium was – 35286 ± 9136 mm^2 for control and 32992 ± 8186 mm^2 for *C. rosea*. Neither of these results showed significant differences between lesion areas (P = 0.72) (Fig. 3 C). *C. rosea* was recovered on selective media for all surface sterilised root sam-

ples of tomato. Although this was not quantified, *C. rosea* appears scarcely on root samples (Fig. 4). Several seedlings of *A. thaliana* (68%) inoculated on MS medium did not survive when transferred into soil. Hence, this experiment was excluded.

3.3 Effects of plant growth promotion from *C. rosea* treatment

A. thaliana showed no significant difference in fresh or air-dried biomass between treatment and control plants. The average of above ground fresh biomass was 0.612 ± 0.359 g for control and 0.664 ± 0.247 g for *C. rosea* treatment. Corresponding dry biomass was 0.056 ± 0.031 g for control and 0.060 ± 0.027 g for *C. rosea* treatment. Furthermore, the below ground biomass of fresh weight was 0.008 ± 0.005 g for control and 0.009 ± 0.003 g for *C. rosea* treatment. The dry weight was 0.003 ± 0.002 g in control and 0.003 ± 0.001 g in *C. rosea* treatment (Table 4). The P-values for above ground were P = 0.662 in fresh biomass and P = 0.685 in dry biomass, and for below ground the P-values were P = 0.573 in fresh biomass and P = 0.863 in dry biomass.

There was neither a significant difference in fresh or oven-dried biomass between treatment and control plants in *A. thaliana*. Since much of the root biomass was lost in the earlier experiment only the above ground biomass was measured. The average fresh biomass was 0.621 ± 0.231 g for control and 0.673 ± 0.297 g for *C. rosea* treatment, and dry biomass 0.069 ± 0.028 g for control and 0.076 ± 0.042 g for *C. rosea* treatment (Table 4). The P- value was 0.630 for fresh biomass and 0.651 for dry biomass.

Table 4. *Fresh and dry weight [g] of above ground and below ground biomass of A. thaliana (Arabidopsis). Plants in experiment 1 were dried at room temperature, while plants in experiment 2 were dried at 70°C in oven. Amount spores inoculated and days of inoculation also varied between experiments. Still, there were no significant differences in weight between the control and treatment in neither experiment.*

	Control 1	*C. rosea treatment 1*	*Control 2*	*C. rosea treatment 2*
Fresh weight				
Above ground biomass	0,612 ± 0,359	0,664 ± 0,247	0,621 ± 0,213	0,673 ± 0,297
Below ground biomass	0,008 ± 0,005	0,009± 0,003	-	-
Dry weight				
Above ground biomass	0,056 ± 0,031	0,06 ± 0,027	0,069 ± 0,028	0,076 ± 0,042
Below ground biomass	0,003 ± 0,002	0,003 ± 0,001	-	-

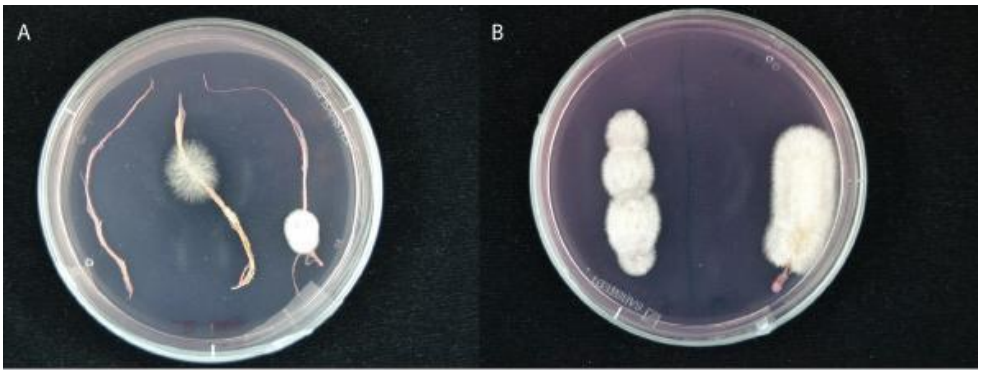

Figure 4. Endophytic growth of *C. rosea* in root samples of tomato. Samples in plate A were inoculated in soil conditions and used for *B. cinerea* assay. While, samples in plate B were inoculated on MS medium for endophytic root colonization assay.

3.4 Results from gene expression

Biocontrol agents can initially induce defence responses in plants. To test if *C. rosea* activates a defence response in tomato and *A. thaliana*, expression of SA- and JA/ET-related genes were measured in root samples. To compare the expression levels of these defence genes the relative expression ratio was estimated and normalized with expression of the reference gene *Actin* according to the Pffafl method.

In order to calculate relative expression the primer efficiency (E) was established from a standard curve. In tomato 7 dilution points were used. These ranged from 300000000 to 300 copies. However, 4 dilutions (300000000 – 300000 copies) were used for *TomLoxA* and 5 dilutions (300000000 – 300000 copies) for *PINII*. The R^2 values in all standard curves were >0.950. Primer efficiency was calculated to 1.91 for *Actin*, 1.72 for *PR1a*, 1.71 for *PR-P2*, 1.87 for *Chi9*, 1.71 for *TomLoxA* and 1.71 for *PINII*. In *A. thaliana* the primer efficiency was previously calculated from 8 dilutions that ranged from 300000000 to 30 copies. Primer efficiencies were 1.85 for *ACTIN8*, 1.91 for *PR-1a*, for 1.85 *PDF1.2* and 1.94 for *PAD3*.

In tomato, mean transcript levels of SA-related defence genes *PR1a*, *PR-P2* and *CHI9* were higher after *C. rosea* treatment (Fig. 5), although only the induction of *CHI9* was significant (P = 0.025). In contrast, mean transcript levels of the JA/ET defence related genes *TomLoxA* and *PINII* were lower after *C. rosea* treatment (Fig. 5), although only the repression of *TomLoxA* was significant (P = 0.016). The results in *A. thaliana* showed high mean transcript levels of JA/ET-related defence gene *PDF1.2* and camalexin-related defence gene *PAD3* after 50 h and 72 h of *C.*

rosea treatment (Fig. 6). Only induction of *PAD3* after 72 h was significant (P = 0.001). Yet mean transcript levels of *PDF1.2* (P = 0.054) and *PAD3* (P = 0.008) were significantly increased from 50 h to 72 h after *C. rosea* treatment.

In *A. thaliana*, the mean transcript levels of SA-related defence gene *PR-1a* were below detection limit at both time points and treatments. Overall, CT-values obtained in *A. thaliana* were high and ranged between 29-38 for all genes.

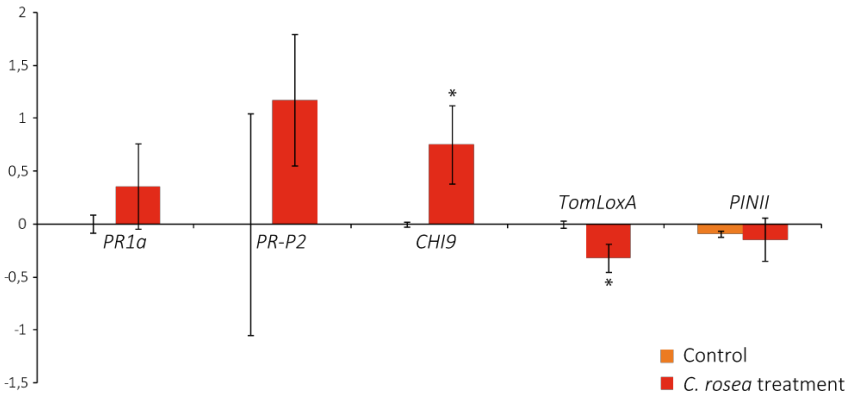

Figure 5. Gene expression ratio in tomato roots. The P – values for different expression between control samples (orange) and *C. rosea* treatment (red) were in SA related defence genes: P = 0.207 in *PR1a*, P = 0.171 in *PR-P2* and P = 0.025 in *CHI9*. In JA/ET related genes the P-values were P = 0.016 in *TomLoxA* and P = 0.740 in *PINII*. Asteriks indicate statistically significant differences ($P \leq 0.05$) obtained with a t-test.

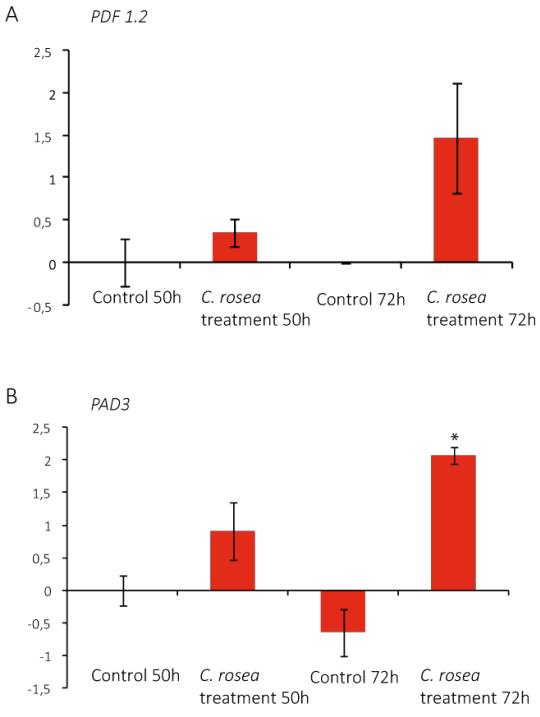

Figure 6. Gene expression in *A. thaliana* roots measured at two time points, 50 and 72 hours after inoculation. Expression of both *PAD3* and *PDF 1.2* in samples treated with *C. rosea* (red) increased significantly from 50h to 72h after inoculation. The P – values were P = 0.054 in *PDF1.2* (A) and P = 0.008 in *PAD3* (B). *PAD3* was also significantly expressed in *C. rosea* treated samples (red) 72h after inoculation, its P – value was P = 0.001 (*).

4 Discussion

4.1 Endophytic growth of *C. rosea* in tomato and *Arabidopsis*

Endophytic colonization by BCAs may induce systemic resistance in plants (Yedidia et al. 1999; Yedidia et al. 2003; Salas-Marina et al. 2011). This resistance allows the BCAs to indirectly reduce pathogen infection (Shoresh et al. 2005; Chatterton et al. 2008; Korolev et al. 2008; Salas-Marina et al. 2011). The initial step to determine if *C. rosea* could induce resistance in tomato and *A. thaliana,* was to verify that it could grow endophytically in these plants.

Results showed that roots of tomato and *A. thaliana* were widely colonized by *C. rosea* both on the surface and endophytically. These results are also consistent with a study by Lahlali and Peng (2013), which reported endophytic growth in canola roots. Similarly, Chatterton et al. (2008) described high endophytic growth in cucumber roots, in shoots and to a lesser extent in stems. Although *C. rosea* was recovered in surface sterilized stems when inoculated in soil conditions it is still only weakly supported from this study that *C. rosea* is able to endophytically colonize stems since it was only observed in one biological replicate in tomato and *A. thaliana.*

In contrast, several biological replicates of leaves of *A. thaliana* were colonized on the surface by *C. rosea*. Even though *C. rosea* is a soil fungus, it has been previously documented to colonize geranium foliage as well as deleafed stems of tomato (Sutton et al. 2002; Chatterton & Punja 2011). Yet identification of *C. rosea* in *A. thaliana* was only assessed visually. If a morphologically similar fungus was present, as was the case with tomato, it was not distinguished.

In addition, there is a clear variation of results between inoculation methods. Samples inoculated on MS media clearly showed colonization of *C. rosea* in both tomato and *A. thaliana,* while results from inoculation in soil were more difficult to interpret. These may have resulted from poor establishment of *C. rosea* in the

soil, which was mainly composed by peat and is not optimal for colonization by *C. rosea* (Chatterton & Punja 2010).

Furthermore, *C. rosea* neither increased nor decreased plant growth. This may also reflect a poor establishment of *C. rosea* in the rhizosphere. However increase of plant growth by BCAs might only be visible in the plants under abiotic stress (Mastouri et al. 2010). The lack of growth promotion may also be because of optimal growing conditions during the experiment.

4.2 Molecular dialogue between antagonist and plant

The interaction of the BCA with the plant could initially be perceived as pathogenic, and trigger expression of defence proteins in the plant (Yedidia et al. 1999, Yedidia et al. 2003; Salas-Marina et al. 2011; Alonso-Ramírez et al. 2014). In tomato, root colonization by *C. rosea* triggered activation of SA-related genes that are often related to defence against biotrophic fungi, while in *A. thaliana* it triggered expression of JA-related and camalexin-biosynthesis genes. The JA signalling pathway is mostly associated with necrotrophic pathogens, and the synthesis of camalexin with the plant's basal defence response (Glazebrook 2005, Ahuja et al. 2010). Additionally, both these markers were significantly up-regulated between 50 to 72h after inoculation. Although interactions with *C. rosea* activated different signalling pathways, a defence response was elicited in both tomato and *A. thaliana*. Similar defence responses in these plants are also triggered by interactions with *Trichoderma spp.* For example, BCA strains of *T. harzianum* elicited either SA- or JA-dependent genes in tomato (Tucci et al. 2011; Harel et al. 2014), as well as SA signalling pathway in *A. thaliana* (Alonso-Ramirez et al 2014).

Which signalling pathway is activated by the BCA varies. Contreras-Cornejo et al. (2011) observed that the amount of inoculated conidia determined if JA or SA hormone was synthesized when *A. thaliana* was inoculated with *T. virens* or *T. atroviride*. Lower concentrations would trigger synthesis of SA, whereas higher concentration of inoculum would trigger JA accumulation. In contrast, Salas-Marina et al. (2011) observed that inoculation of *T. atroviride* in *A. thaliana* activated both SA and JA/ET signalling pathways. This was also the case for *T. hamatum*, where SA signalling in part overlapped with JA signalling (Mathys et al. 2012). In addition, Tucci et al. (2011) observed that expression of defence genes elicited by SA or JA/ET synthesis varied with plant genotype.

In tomato and *A. thaliana* no overlap in expression of these pathways were observed, although additional time points are needed to explore this better. Alternatively, the activation of different pathways by *C. rosea* might vary with amount of conidia inoculated or with difference of root morphology between species.

Moreover, BCA capacity to induce resistance against plant pathogens has been studied in both *A. thaliana* and tomato. Root colonization of *A. thaliana* by biocontrol species of *Trichoderma* induced both local and systemic responses, and decreased *B. cinerea* infection in leaves (Korolev et al. 2008; Contreras-Cornejo et al. 2011; Salas-Marina et al. 2011). If *C. rosea* elicits similar responses during interaction with the plants' roots is still unknown. Despite that *C. rosea* elicited plant defence responses, systemic acquired resistance or induce systemic resistance were not observed in tomato or *A. thaliana*. Leaves of plants previously inoculated with *C. rosea* and infected with *B. cinerea* showed no significant reduction of symptoms compared to leaves in untreated control plants. Yet this result may occur due to poor endophytic colonization of *C. rosea*. Recovery of *C. rosea* from plants inoculated on MS plates and subsequently transplanted into the soil showed a scarce endophytic colonization. In addition, *C. rosea* is previously described to induce systemic resistance in wheat and canola (Roberti et al. 2008; Lahlali & Peng 2013). Therefore these results might not be representative.

In conclusion, these results show that *C. rosea* is able to colonize tomato and *A. thaliana* endophytically, and consequently elicit defence responses in the plant. However, whether systemic resistance is induced is still unclear, as well as whether signalling pathways are activated during induced resistance against pathogens.

References

Achuo, E., Audenaert, K., Meziane, H. & Höfte, M., 2004. The salicylic acid-dependent defence pathway is effective against different pathogens in tomato and tobacco. *Plant Pathology*, 53, pp.65–72.

Ahuja, I., Kissen, R. & Bones, A.M., 2012. Phytoalexins in defense against pathogens. *Trends in Plant Science*, 17(2), pp.73–90.

Alabouvette, C., Olivain, C. & Steinberg, C., 2006. Biological control of plant diseases: The european situation. *European Journal of Plant Pathology*, 114(3), pp.329–341.

Alonso-Ramírez, A., Poveda, J., Martín, I., Hermosa, R., Monte, E. & Nicolás, C., 2014. Salicylic acid prevents *Trichoderma harzianum* from entering the vascular system of roots. *Molecular Plant Pathology*, doi: 10.1111/mpp.12141.

Bardoel, B.W., van der Ent, S., Pel, M.J.C, Tommassen, J.,Pieterse, C.M.J., van Kesel, K.P.M. & van Strijp, J.A.G., 2011. *Pseudomonas* evades immune recognition of flagellin in both mammals and plants. *PLoS Pathogens*, 7(8), p.e1002206.

Bari, R. & Jones, J.D.G., 2009. Role of plant hormones in plant defence responses. *Plant Molecular Biology*, 69(4), pp.473–488.

Beed, F.D., 2014. Managing the biological environment to promote and sustain crop productivity and quality. *Food Security*, 6(2), pp.169–186.

Benhamou, N., Rey, P., Picard, K. & Tirilly, Y., 1999. Ultrastructural and cytochemical aspects of the interaction between the mycoparasite *Pythium oligandrum* and soilborne plant pathogens. *Phytopathology*, 89(6), pp.506–517.

Beyers, T., Vos, C., Aerts, R., Heyens, K., Vogels, L., Seels, B., Höfte, M., Cammue, B.P.A. & De Coninck, B., 2014. Resistance against *Botrytis cinerea* in smooth leaf pruning wounds of tomato does not depend on major disease signalling pathways. *Plant Pathology*, 63(1), pp.165–173.

Birch, N.A., Begg, G.S. & Squire, G.R., 2011. How agro-ecological research helps to address food security issues under new IPM and pesticide reduction

policies for global crop production systems. *Journal of Experimental Botany*, 62(10), pp.3251–3261.

Cai, F., Yu, G., Wang, P., Wei, Z., Fu, L., Shen, Q. & Chen, W., 2013. Harzianolide, a novel plant growth regulator and systemic resistance elicitor from *Trichoderma harzianum*. *Plant Physiology and Biochemistry*, 73, pp.106–113.

Chatterton, S., Jayaraman, J. & Punja, Z.K., 2008. Colonization of cucumber plants by the biocontrol fungus *Clonostachys rosea* f. *catenulata*. *Biological Control*, 46(2), pp.267–278.

Chatterton, S. & Punja, Z., 2011. Colonization of geranium foliage by *Clonostachys rosea* f. *catenulata*, a biological control agent of botrytis grey mould. *Botany*, 90, pp.1–10.

Chatterton, S. & Punja, Z.K., 2009. Chitinase and b-1,3-glucanase enzyme production by the mycoparasite *Clonostachys rosea* f. *catenulata* against fungal plant pathogens. *Canadian Journal of Microbiology*, 55, pp.356–367.

Chatterton, S. & Punja, Z.K., 2010. Factors influencing colonization of cucumber roots by *Clonostachys rosea* f. *catenulata*, a biological disease control agent. *Biocontrol Science and Technology*, 20(1), pp.37–55.

Contreras-Cornejo, H.A., Macías-Rodríguez, L., Cortés-Penagos, C. & López-Bucio, J., 2009. *Trichoderma virens*, a plant beneficial fungus, enhances biomass production and promotes lateral root growth through an auxin-dependent mechanism in Arabidopsis. *Plant Physiology*, 149(3), pp.1579–1592.

Contreras-Cornejo, H.A., Macías-Rodríguez, L., Beltrán-Peña, E., Herrera-Estrella, A. & López-Bucio, J., 2011. Trichoderma-induced plant immunity likely involves both hormonal- and camalexin-dependent mechanisms in *Arabidopsis thaliana* and confers resistance against necrotrophic fungi *Botrytis cinerea*. *Plant Signaling & Behavior*, 6(10), pp.1554–1563.

Cota, L. V., Maffia, L.A., Mizubuti, E.S.J., Mazedo, P.E.F. & Antunes, R.F., 2008. Biological control of strawberry gray mold by *Clonostachys rosea* under field conditions. *Biological Control*, 46(3), pp.515–522.

Druzhinina, I.S., Seidl-Seiboth, V., Herrera-Estrella, A., Horwitz, B.A., Kenerley, C.M., Monte, E., Mukherjee, P.K., Zeilinger, S., Grigoriev, I.V. & Kubicek, C.P., 2011. Trichoderma: the genomics of opportunistic success. *Nature Reviews. Microbiology*, 9(10), pp.749–759.

Dubey, M.K., Jensen, D.F. & Karlsson, M., 2014. Hydrophobins are required for conidial hydrophobicity and plant root colonization in the fungal biocontrol agent *Clonostachys rosea*. *BMC Microbiology*, 14, p.18.

Ferrari, S., Plotnikova, J.M., De Lorenzo, G. & Ausubel, F.M., 2003. Arabidopsis local resistance to *Botrytis cinerea* involves salicylic acid and camalexin and requires *EDS4* and *PAD2*, but not *SID2*, *EDS5* or *PAD4*. *The Plant Journal*, 35(2), pp.193–205.

Le Floch, G., Rey, P., Déniel, F., Benhamou, N., Picard, K. & Tirilly, Y., 2003b. Enhancement of development and induction of resistance in tomato plants by the antagonist, *Pythium oligandrum*. *Agronomie*, 23, pp.455–460.

Le Floch, G., Rey, P., Benizri, E., Benhamou, N. & Tirilly, Y., 2003a. Impact of auxin-compounds produced by the antagonistic fungus *Pythium oligandrum* or the minor pathogen *Pythium* group F on plant growth. *Plant and Soil*, 257(2), pp.459–470.

Flood, J., 2010. The importance of plant health to food security. *Food Security*, 2(3), pp.215–231.

Fonseca, S., Chico, J.M. & Solano, R., 2009. The jasmonate pathway: the ligand, the receptor and the core signalling module. *Current Opinion in Plant Biology*, 12(5), pp.539–547.

Fravel, D., Olivain, C. & Alabouvette, C., 2003. *Fusarium oxysporum* and its biocontrol. *New Phytologist*, 157, pp.493–502.

Fu, Z.Q. & Dong, X., 2013. Systemic acquired resistance: turning local infection into global defense. *Annual Review of Plant Biology*, 64, pp.839–863.

Geraldine, A.M., Cardoso Lopez, F.A., Costa Carvahlo, D.D., barbosa, E.T., Rodrigues, A.R., Brandão, R.S., Ulhoa, C.J. & Lobo Junior, M., 2013. Cell wall-degrading enzymes and parasitism of sclerotia are key factors on field biocontrol of white mold by *Trichoderma spp*. *Biological Control*, 67(3), pp.308–316.

Gerbore, J., Benhamou, N., Vallance, J., Le Floch, G., Grizard, D., Regnault-Roger, C. & Rey, P., 2014. Biological control of plant pathogens: advantages and limitations seen through the case study of *Pythium oligandrum*. *Environmental Science and Pollution Research International*, 21(7), pp.4847–4860.

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

Godfray, H.C.J., Beddington, J.R., Crute, I.R., Haddad, L., Lawrence, D., Pretty, J., Robinson, S., Thomas, S.N. & Toulmin, C., 2010. Food security: the challenge of feeding 9 billion people. *Science*, 327(5967), pp.812–818.

Görlach, J. Volrath, S., Knauf-Beiter, G., Hengy, G., beckhove, U., Kogel, K.H., Oostendorp, M., Staub, T., Ward, E., Kessmann, H. & Ryals, J., 1996. Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. *The Plant Cell*, 8(4), pp.629–643.

Harel, Y.M., Mehari, Z.H., Rav-David, D. & Elad, Y., 2014. Systemic resistance to gray mold induced in tomato by benzothiadiazole and *Trichoderma harzianum* T39. *Phytopathology*, 104(2), pp.150–157.

Harman, G.E., Howell, C.R., Viterbo, A., Chet, I. & Lorito, M., 2004. *Trichoderma* species--opportunistic, avirulent plant symbionts. *Nature Reviews. Microbiology*, 2(1), pp.43–56.

Hase, S., Takahashi, S., Takenaka, S., Nakaho, K., Arie, T., Seo, S., Ohashi, Y. & Takahashi, H., 2008. Involvement of jasmonic acid signalling in bacterial wilt disease resistance induced by biocontrol agent *Pythium oligandrum* in tomato. *Plant Pathology*, 57(5), pp.870–876.

Howell, C., 2003. Mechanisms employed by *Trichoderma* species in the biological control of plant diseases: the history and evolution of current concepts. *Plant Disease*, 87(1), pp.4–10.

Howell, C.R., 2002. Cotton seedling preemergence damping-off Incited by *Rhizopus oryzae* and *Pythium* spp. and its biological control with *Trichoderma* spp. *Phytopathology*, 92(2), pp.177–180.

Hu, X., Webster, G., Xie, L., Yu, C., Li, Y. & Liao, X., 2013. A new mycoparasite, *Aspergillus* sp. ASP-4, parasitizes the sclerotia of *Sclerotinia sclerotiorum*. *Crop Protection*, 54, pp.15–22.

Ikeda, S., Shimisu, A., Shimizu, M., Takahashi, H. & Takenaka, S., 2012. Biocontrol of black scurf on potato by seed tuber treatment with *Pythium oligandrum*. *Biological Control*, 60(3), pp.297–304.

Jabaji-Hare, S. & Neate, S.M., 2005. Nonpathogenic binucleate *Rhizoctonia* spp. and benzothiadiazole protect cotton seedlings against rhizoctonia damping-off and alternaria leaf spot in cotton. *Phytopathology*, 95(9), pp.1030–1036.

Jensen, D.F., Knudsen, I.M.B., Lübeck, M., Mamarabadi, M., Hockenhull, J. & Jensen, B., 2007. Development of a biocontrol agent for plant disease control with special emphasis on the near commercial fungal antagonist *Clonostachys rosea* strain "IK726." *Australasian Plant Pathology*, 36, pp.95–101.

Jones, J.D.G. & Dangl, J.L., 2006. The plant immune system. *Nature*, 444(7117), pp.323–329.

De Jonge, R., van Esse, P.H., Kombrink, A., Shynia, T., Desaki, Y., Bours, R., van der Krol, S., Shibuya, N., Joosten, M.H.A. & Thomma, B.P.H.J., 2010. Conserved fungal LysM effector Ecp6 prevents chitin-triggered immunity in plants. *Science*, 329(5994), pp.953–955.

Jung, H.W., Tschaplinski, T.J., Wang, L., Glazebrook, J. & Greenberg, J.T., 2009. Priming in systemic plant immunity. *Science*, 324(5923), pp.89–91.

Kojima, H., Hossain, Md.M., Kubota, M. & Hyakumachi, M., 2013. Involvement of the salicylic acid signaling pathway in the systemic resistance induced in Arabidopsis by plant growth-promoting fungus *Fusarium equiseti* GF19-1. *Journal of Oleo Science*, 62(6), pp.415–426.

Korolev, N., Rav David, D. & Elad, Y., 2008. The role of phytohormones in basal resistance and *Trichoderma*-induced systemic resistance to *Botrytis cinerea* in *Arabidopsis thaliana*. *Biocontrol*, 53(4), pp.667–683.

Lahlali, R. & Peng, G., 2013. Suppression of clubroot by *Clonostachys rosea* via antibiosis and induced host resistance. *Plant Pathology*, 63(2), pp.447–455.

Lorenzo, O. & Chico, J., 2004. *JASMONATE-INSENSITIVE1* encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in Arabidopsis. *The Plant Cell*, 16, pp.1938–1950.

Maldonado, A., Doerner, P. & Dixon, R., 2002. A putative lipid transfer protein involved in systemic resistance signalling in Arabidopsis. *Nature*, 419, pp.399–403.

Martínez-Medina, A., Fernández, I., Sánchez-Gúzman, M., Jung, S.C., Pascual, J.A. & Pozo, M.J., 2013. Deciphering the hormonal signalling network behind the systemic resistance induced by *Trichoderma harzianum* in tomato. *Frontiers in Plant Science*, 4, p.206.

Mastouri, F., Björkman, T. & Harman, G.E., 2010. Seed treatment with *Trichoderma harzianum* alleviates biotic, abiotic, and physiological stresses in germinating seeds and seedlings. *Biological Control*, 100, pp.1213-1221.

Mathys, J., De Cemer, K., Timmermans, P., Van Kerckhove, S., Lievens, B., Vanhaecke, M., Cammue, B.P.A. & De Coninck, B., 2012. Genome-wide characterization of ISR induced in *Arabidopsis thaliana* by *Trichoderma hamatum* T382 against *Botrytis cinerea* infection. *Frontiers in Plant Science*, 3, doi: 10.3389/fpls.2012.00108.

Métraux, J., AhlGoy, P. & Staub, T., 1991. Induced systemic resistance in cucumber in response to 2, 6-dichloro-isonicotinic acid and pathogens. In *Advances in Molecular Genetics of Plant-Microbe Interactions, Vol. 1*. pp. 432–439.

Moller, K., Jensen, B., Paludan Andersen, H., Stryhn, H. & Hockenhull, J., 2003. Biocontrol of *Pythium tracheiphilum* in chinese cabbage by *Clonostachys rosea* under field conditions. *Biocontrol Science and Technology*, 13(2), pp.171–182.

Mukherjee, P.K., Horwitz, B. a & Kenerley, C.M., 2012. Secondary metabolism in *Trichoderma*-a genomic perspective. *Microbiology*, 158, pp.35–45.

Mur, L.A.J., Kenton, P., Miersch, O. & Wasternack, C., 2006. The outcomes of concentration-specific interactions between salicylate and jasmonate signaling include synergy, antagonism, and oxidative stress leading to cell death. *Plant Physiology*, 140, pp.249–262.

Murashige, T. & Skoog, F., 1962. A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia Plantarum*, 15, pp.473–497.

Nobre, S. a. M., Maffia, L.A., Mizubuti, E.S.G., Cota, V. & Dias, A.P.S., 2005. Selection of *Clonostachys rosea* isolates from Brazilian ecosystems effective in controlling *Botrytis cinerea*. *Biological Control*, 34(2), pp.132–143.

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

Oerke, E.-C., 2006. Crop losses to pests. *The Journal of Agricultural Science*, 144(1), pp.31–43.

El Oirdi, M., El Rahman, T.A., Rigano, L., El Hadrami, A., Rodriguez, M.C., Daayf, F., Vojnov, A. & Bouarab, K., 2011. *Botrytis cinerea* manipulates the antagonistic effects between immune pathways to promote disease development in tomato. *The Plant Cell*, 23(6), pp.2405–2421.

Park, S.-W., Kaimoyo, E., Kumar, D., Mosher, S.& Klessig, D.F., 2007. Methyl salicylate is a critical mobile signal for plant systemic acquired resistance. *Science*, 318(5847), pp.113–116.

Pauwels, L. & Goossens, A., 2011. The JAZ proteins: a crucial interface in the jasmonate signaling cascade. *The Plant Cell*, 23(9), pp.3089–3100.

Pfaffl, M., 2001. A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Research*, 29(9), p.e45.

Pieterse, C.M.J., Van Der Does, Zamioudis, C., Leon-Reyes, A. & Van Wees, S.C.M., 2012. Hormonal modulation of plant immunity. *Annual Review of Cell and Developmental Biology*, 28, pp.489–521.

Pisi, A., Cesari, A. & Zakrisson, E., 2006. SEM investigation about hyphal relationships between some antagonistic fungi against *Fusarium* spp. foot rot pathogen of wheat. *Phytopathologia Mediterranea*, 40, pp.37–44.

Ravnskov, S., Jensen, B., Knudsen, I.M.B., Bodker, L., Jensen, D.F., Karlinski, L. & Larsen, J., 2006. Soil inoculation with the biocontrol agent *Clonostachys rosea* and the mycorrhizal fungus *Glomus intraradices* results in mutual inhibition, plant growth promotion and alteration of soil microbial communities. *Soil Biology and Biochemistry*, 38(12), pp.3453–3462.

Dos Reis Almeida, F.B., Menezez Cerqueira, F., do Nacimiento Silva, R., Ulhoa, C.J. & Lopes Lima, A., 2007. Mycoparasitism studies of *Trichoderma harzianum* strains against *Rhizoctonia solani*: evaluation of coiling and hydrolytic enzyme production. *Biotechnology Letters*, 29(8), pp.1189–1193.

Roberti, R., Veronesi, AR., Cesari, A., Cascone, A., Di Berardina, I., Bertini, L. & Caruso, C., 2008. Induction of PR proteins and resistance by the biocontrol agent *Clonostachys rosea* in wheat plants infected with *Fusarium culmorum*. *Plant Science*, 175(3), pp.339–347.

Rodríguez, M. A., Cabrera, G., Gozzo, F.C., Eberlin, M.N. & Godeas, A., 2011. *Clonostachys rosea* BAFC3874 as a *Sclerotinia sclerotiorum* antagonist: mechanisms involved and potential as a biocontrol agent. *Journal of Applied Microbiology*, 110(5), pp.1177–1186.

Salas-Marina, M.A., Silva-Flores, M.A., Uresti-Rivera, E.E:, Castro-Longoria, E., Herrera-Estrella, A. & Casas-Flores, S., 2011. Colonization of Arabidopsis roots by *Trichoderma atroviride* promotes growth and enhances systemic disease resistance through jasmonic acid/ethylene and salicylic acid pathways. *European Journal of Plant Pathology*, 131(1), pp.15–26.

Schroers, H.-J., Samuels, G., Seifert, K., Gams, W., 1999. Classification of the mycoparasite *Gliocladium roseum* in *Clonostachys* as *C. rosea*, its relationship to *Bionectria ochroleuca*, and notes on other *Gliocladium*-like fungi. *Mycologia*, 91(2), pp.365–385.

Shoresh, M., Yedidia, I. & Chet, I., 2005. Involvement of jasmonic acid/ethylene signaling pathway in the systemic resistance induced in cucumber by *Trichoderma asperellum* T203. *Phytopathology*, 95(1), pp.76–84.

Steindorff, A.S., Ramada, M.H.S., Coelho, A.S.G., Miller, R.N.G., Júnior, G.J.P., Ulhoa, J.C. & Noronha, E.F., 2014. Identification of mycoparasitism-related genes against the phytopathogen *Sclerotinia sclerotiorum* through transcriptome and expression profile analysis in *Trichoderma harzianum*. *BMC Genomics*, 15(1), p.204.

Stout, M.J., Thaler, J.S. & Thomma, B.P.H.J., 2006. Plant-mediated interactions between pathogenic microorganisms and herbivorous arthropods. *Annual Review of Entomology*, 51, pp.663–689.

Sutton, J., Li, D.-W., Peng, G., Yu, H. & Zhang, P., 1997. *Gliocladium roseum* a versatile adversary of *Botrytis cinerea* in crops. *Plant Disease*, 81(4), pp.316–328.

Sutton, J.C., Liu, W., Huang, R. & Owen-Going, N., 2002. Ability of *Clonostachys rosea* to Establish and Suppress Sporulation Potential of *Botrytis cinerea* in Deleafed Stems of Hydroponic Greenhouse Tomatoes. *Biocontrol Science and Technology*, 12(4), pp.413–425.

Tanwar, A., Aggarwal, A. & Panwar, V., 2013. Arbuscular mycorrhizal fungi and *Trichoderma viride* mediated *Fusarium* wilt control in tomato. *Biocontrol Science and Technology*, 23(5), pp.485–498.

Thomma, B.P.H., Eggermont, K., Penninckx, I.A.M.A., Mauch-Mani, B., Vogelsang, R., Cammue, B.P.A. & Broekaert, W.F., 1998. Separate jasmonate-dependent and salicylate-dependent defense-response pathways in *Arabidopsis* are essential for resistance to distinct microbial pathogens. *Proceedings of the National Academy of Sciences of the United States of America*, 95(25), pp.15107–15111.

Thomma, B.P.H., Eggermont, K., Broekaert, W.F. & Cammue, B.P.A., 2001. Different micro-organisms differentially induce Arabidopsis disease response pathways. *Plant Physiology and Biochemistry*, 39(7-8), pp.673–680.

Tilman, D. Balzer, C., Hill, J. & Befort, B.L., 2011. Global food demand and the sustainable intensification of agriculture. *Proceedings of the National Academy of Sciences of the United States of America*, 108(50), pp.20260–20264.

Tucci, M., Ruocco, M., De Masi, L., De Palma, M. & Lorito, M., 2011. The beneficial effect of *Trichoderma* spp. on tomato is modulated by the plant genotype. *Molecular Plant Pathology*, 12(4), pp.341–354.

Vargas Gil, S., Pastor, S. & March, G.J., 2009. Quantitative isolation of biocontrol agents *Trichoderma* spp., *Gliocladium* spp. and actinomycetes from soil with culture media. *Microbiological Research*, 164(2), pp.196–205.

Vinale, F., Sivasithamparam, K., Ghisalberti, E.L., Marra, R., Barbetti, M.J., Li, H., Woo, S.L. & Lorito, M., 2008. A novel role for *Trichoderma* secondary metabolites in the interactions with plants. *Physiological and Molecular Plant Pathology*, 72(1-3), pp.80–86.

De Vos, M., Van Oosten, V.R., Van Poecke, R.M.P., Van Pelt, J.A., pozo, M.J., Mueller, M.J., Buchala, A.J., Métraux, J.-P., Van Loon, L.C., Dicke, M. & Pieterse, C.M.J., 2005. Signal signature and transcriptome changes of *Arabidopsis* during pathogen and insect attack. *Molecular Plant-Microbe Interactions:MPMI*, 18(9), pp.923–937.

Van Wees, S.C., de Swart, E.A.M., van Pelt, J.A., van Loon, L.C., Pieterse, C.M.J., 2000. Enhancement of induced disease resistance by simultaneous activation of salicylate- and jasmonate-dependent defense pathways in *Arabidopsis thaliana*. *Proceedings of the National Academy of Sciences of the United States of America*, 97(15), pp.8711–8716.

Wang, J. (2012) The effect of combining two biological control microbes on seed and root colonization. MSc-thesis, Swedish University of Agricultural Sciences, Uppsala, Sweden.

White, R., 1979. Acetylsalycylic acid (asperin) induces resistance to tobacco mosaic virus in tobacco. *Virology*, 9(2), pp.410–412.

Williamson, B., Tudzynski, B., Tudzynski, P. & van Kan, A.L., 2007. *Botrytis cinerea*: the cause of grey mould disease. *Molecular Plant Pathology*, 8(5), pp.561–580.

Xue, A.G., 2003. Biological control of pathogens causing root rot complex in field pea using *Clonostachys rosea* strain ACM941. *Phytopathology*, 93(3), pp.329–335.

Yedidia, I., Shoresh, M., Kerem, Z., Benhamou, N., Kapulnik, Y. & Chet, I., 2003. Concomitant induction of systemic resistance to *Pseudomonas syringae* pv. *lachrymans in* cucumber by *Trichoderma asperellum* (T-203) and accumulation of phytoalexins. *Applied and Environmental Microbiology*, 69(12), pp.7343–7353.

Yedidia, I., Benhamou, N. & Chet, I., 1999. Induction of defense responses in cucumber plants (*Cucumis sativus* L.) by the biocontrol agent *Trichoderma harzianum*. *Applied and Environmental Microbiology*, 65(3), pp.1061–1070.

Zhang, F., Zhu, Z., Yang, X., Ran, W. & Shen, Q., 2013. *Trichoderma harzianum* T-E5 significantly affects cucumber root exudates and fungal community in the cucumber rhizosphere. *Applied Soil Ecology*, 72, pp.41–48.

Zimmerli, L., Métraux, J.-P. & Mauch-Mani, B., 2001. beta-Aminobutyric acid-induced protection of Arabidopsis against the necrotrophic fungus *Botrytis cinerea*. *Plant Physiology*, 126(2), pp.517–523.

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Appendix 1

Recipes

Bleach solution
20% household bleach
0.1% Tween 20

Water agar 1% (WA)
10 g Bacto agar (Saveen Werner AB)
1L distilled water

Rose Bengal plates (0.02 g/L)
39 g PDA (potato dextrose agar)
1 L distilled water
1 ml rose Bengal

Soil mix

S-jord
45 volume % low humified sphagnum peat (h2-4)
25 volume % high humified sphagnum peat (h6-8)
25 volume % perlite
5 volume % sand