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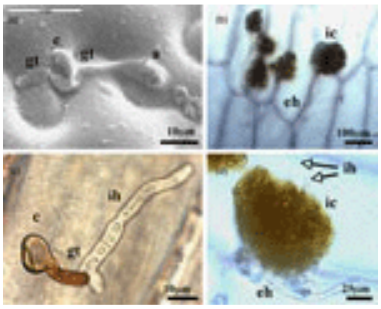
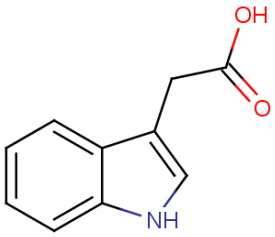
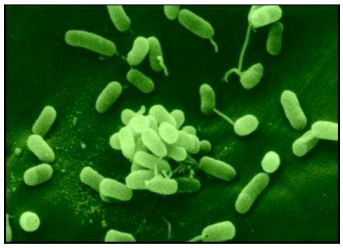
Phytopathology Laboratory

Master Thesis Plant sciences MPS

Efficacy of multiple strategies to control grey mould disease caused by *Botrytis cinerea*







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Efficacy of multiple strategies to control grey mould disease caused by *Botrytis cinerea*

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Summary

Botrytis cinerea is an air-borne plant pathogenic fungus attacking over 200 crops grown in the field and green house. It is currently controlled by chemical methods, cultural methods, biological methods and plant breeding for resistance. The problems associated in controlling *B. cinerea* are its development of resistance to synthetic fungicides and natural compounds, and the lack of genetic resistance in plants against *B. cinerea*. *B. cinerea* has 14 ABC transporters and among them BcatrB has been well studied in achieving resistance against antimicrobial compounds produced by microorganisms and plants. Arabidopsis shows a certain level of resistance to *B. cinerea* due to the production of camalexin. And also we know that Atrbpg1 is resistant to endopolygalacturonases, secreted by *B. cinerea*, which degrade pectin in the host cell wall and serve as important virulence factors.

In this study, I investigated (1) the growth inhibiting properties of massetolide A, a cyclic lipopeptide surfactant (CLPs) produced by the antagonistic bacterium *Pseudomonas fluorescens* against *B. cinerea* and the involvement of *Bcatr*B gene in resistance against massetolide A, (2) the ability of CLPs from *P. fluorescens* to prevent disease and induce systemic resistance in plants against *B. cinerea* and (3) the ability of the Atrbpg1 locus to confer partial resistance to *B. cinerea*. There was no significant difference among fungal strains in sensitivity to different concentrations of massetolide A. BcatrB was not involved in resistance against massetolide A. *P. fluorescens* SS101 was effective in reducing *B. cinerea* disease in tomato leaves. Massetolide A was not important for this activity. *A thaliana* genotypes with the Atrbpg1 locus showed a transient resistance against *B. cinerea*.

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Contents

1. Introduction

1.1 *Botrytis cinerea*

Botrytis cinerea is an airborne plant pathogenic fungus with a necrotropic lifestyle. It can attack over 200 crop hosts worldwide. Over 200 mainly dicotyledonous plant species including important protein, oil, fiber and horticultural crops get affected. It can cause soft rotting of all aerial plant parts, and rotting of vegetables, fruits and flowers. When we consider the pathogenicity of *B. cinerea* , it produces phytotoxic metabolites to kill its host and an array of enzymes, including cell wall-degrading enzymes, for the decomposition and consumption of plant biomass (van Kan, 2006).

1.2. Economic importance of *Botrytis cinerea*

B. cinerea causes serious losses in more than 200 crop species worldwide. Serious damages are caused after harvesting of apparently healthy crops and the subsequent transport to distant markets where the losses become evident. This is mainly because this pathogen can be destructive on mature or senescent tissues of dicotyledonous host and also it can gain entry to such tissues at early stage in crop development and remain for a considerable period when the environment is conducive and the host physiology changes (Williamson et al., 2007). *B. cinerea* can also cause massive losses in some field and green house grown horticultural crops. The fungus attacks different organs, such as shoots, leaves, flowers and fruits. Flowers are usually infected during blossoming and then the pathogen enters into young fruits at a very early stage of their development (Vellicce et al., 2006).

1.3. Current strategies to control *B. cinerea*

Several methods are currently used to control *B. cinerea* including chemical control, cultural practices, biological control and breeding for resistance. Different fungicides affecting fungal respiration have been used against *B.cinerea* over a long period without substantial resistance developing in field populations (Leroux, 1996). Fungicides

inhibiting β – tubulin formation as well as cyprodinil and fludioxonil can be used against *B.cinerea*. Fludioxonil inhibits germ tube elongation or initial mycelium growth (Forster and Staub, 1996). Dicarboximides also have been used extensively as botryticides because they show activity against both conidia and mycellium by affecting sensitivity to osmotic stress (Faretra and Pollastro, 1991) .

Cultural methods can be used to control *B. cinerea*. The main aim here is to reduce the environmental conditions which are favorable for the life cycle of this pathogen. Different cultural practices are used in crop management to create unfavorable conditions for the pathogen. For example, it is helpful to create an open canopy to provide adequate air movement and good light interception, resulting in reducing leaf wetness. It is important to pay attention on applying fertilizers, specially nitrogen fertilizers. Excessive addition of nitrogen fertilizer encourages rapid vegetative growth of crops and increases the risk of *B. cinerea*. (Xiao et al., 2001). Cultural methods are , however, of limited efficacy and the costs and the labour demands are usually high.

Breeding for resistance in crops against *B. cinerea* is ongoing for many years. Numerous investigations have been undertaken on quantitative trait loci in order to obtain resistant cultivars (Finkers et al., 2007). Besides classical breeding for resistance, genetic modification can also be a tool for introducing resistance in crops (Osusky et al., 2005). At this moment, there are no commercial crop cultivars with effective resistance to *B. cinerea.*

Biological control of grey mould disease involves the use of beneficial microorganisms to reduce the onset, development and spread of the disease. Several antagonistic microorganisms have been tested for their activity against *B. cinerea*. It has been reported that the fungal antagonist , *Trichoderma harzianum* T39 can control grey mould disease in strawberries in the field (Shafir et al., 2006). It was found that grey mould in tomato and bean plants can be controlled by using the fungus, *Gliocladium catenulatum* (Elead et al., 1994) . Although some effective fungal antagonists were identified against *B. cinerea* ,

bacterial antagonists have shown the most promising results to date. The antagonistic activity of these bacteria against many plant diseases has been mainly found in the bacterial genera of *Pseudomonas* and *Bacillus* (Yan et al., 2002).

1.4 Problems associated in controlling *B. cinerea*

For a long time, *B. cinerea* has been a threat for many growers in many crops all over the world. Although chemical control has been used as a standard practice for many years, sometimes the pathogen shows the ability to adapt to new chemicals and to develop tolerant or resistant strains. The other problem associated in controlling this fungus is that the lack of genetic resistance in plants. This is mainly because breeders focus to produce high yielding crop cultivars without considering traits involved in disease resistance. Genes involved in resistance against pathogens get lost. Therefore, it is very important to consider these two problems in detail.

1.4.1 Development in fungi of resistance to synthetic and natural compounds

Growers tend to apply synthetic chemicals and natural compounds to their fields to protect their crops from plant pathogens. One of the problems associated with applying synthetic and natural compounds is the development of fungal resistance to these compounds. Therefore, it is important to know about the fungal resistance mechanisms to these compounds.

1.4.1.1 Fungal resistance mechanisms and the role of the ABC transporters

Plant pathogenic fungi have developed a range of defense mechanisms to cope with toxic compounds produced by plants and antagonistic microorganisms. These defenses include non-degradative mechanisms and enzymatic detoxification. Among the non-degradative resistance mechanisms, membranebound efflux pumps have received considerable attention. Transport by efflux pumps not only enables target organisms to tolerate exogenous toxic compounds, but also plays a crucial role in preventing self-intoxication in antibiotic-producing microorganisms (Schouten et al., 2008).

In fungi, active efflux by ATP binding cassette (ABC) and major facilitator (MFS) transporters provide resistance to endogenous and exogenous toxic compounds such as antibiotics, plant defense compounds and fungicides (Stefanato et al., 2009). Among the active efflux mechanisms, ABC transporters are well studied. The natural functions of ABC transporters include protection against plant defense compounds and synthetic fungicides. These phenomena have been described for *B. cinerea*, *Magnaporthe grisea* and *Mycosphaerella graminicola* (Schoonbeek et al., 2002). ABC transporters also may play an important role in protection against antimicrobial compounds produced by other microorganisms. It has been shown that the ABC transporters from *B. cinerea* provide protection against phenazine antibiotics produced by *Pseudomonas* spp (Schoonbeek et al., 2002), and against the phytoalexin camalexin produced by *Arabidopsis* plants (Stefanato et al., 2009) .

B. cinerea has 14 ABC transporters. Schoonbeek et al (2002) demonstrated that the efflux pump BcAtrB (*B. cinerea* ABC transporter B) plays an important role in defense against phenazine antibiotics. Mutants of *B. cinerea* disrupted in *BcatrB* could be controlled more effectively by phenazine-producing biocontrol agents. *B. cinerea* can protect itself against DAPG produced by antagonistic microorganisms (Schouten et al., 2008). The mechanism of tolerance of *B. cinerea* to camalexin produced by Arabidopsis has been studied. Camalexin can induce the expression of *Bcatr*B and mutants in the *Bcatr*B gene are more sensitive to camalexin (Stefanato et al., 2009). *Bcatr*B also provides protection against the grapevine phytoalexin, resveratrol and to phenylpyrrole

fungicides indicating that one ABC transporter can transport multiple and structurally diverse compounds (Schoonbeek et al., 2002).

1.4.2 Lack of genetic resistance in plants against *B. cinerea*

Besides the ability of *B. cinerea* to develop resistance to synthetic and natural compounds, the other problem in controlling the pathogen is the limited availability of natural genetic resistance to *B. cinerea* in plants. Plants activate a range of partially effective defense mechanisms in response to *B. cinerea* attack, including structural barriers and the production of antifungal proteins or secondary metabolites (Van Baarlen et al., 2007). *B. cinerea* can induce the expression of marker genes for SAR (ex. PR-1), however the induction of SAR does not result in enhanced resistance to *B. cinerea* (Govrin and Levine, 2002). So far, resistance genes that completely restrict the growth of the pathogen have not been reported in any crop species. However, a number of quantitative trait loci (QTL) has been identified that originate from a wild relative of tomato, *Solanum habrochaites* LYC 4, and confer partial resistance to *B. cinerea* in cultivated tomato, *Solanum lycopersicum* (Finkers et al., 2007). The genes and mechanisms underlying partial resistance conferred by these loci remain to be unraveled.

1.5 Biological control , induced systemic resistance (ISR) and genetic resistance against *B.cinerea*

1.5.1 Biological control by *Pseudomonas* species

Among the bacterial genera used in biological control, *Pseudomonas* and *Bacillus* species are the most widely studied (Raaijmakers et al., 2002). *Pseudomonas* strains are highly amenable for research, as they are fast growing, easy to culture, metabolically versatile and easy to manipulate genetically (Whipps, 2001). Different *Pseudomonas* strains have the ability to inhibit the growth and activity of a range of plant pathogens including fungi, oomycetes, nematodes and bacteria (Raaijmakers et al., 2002). The mechanisms by which they protect against pathogens include competition, antibiosis, parasitism, degradation of pathogenicity factors, induced systemic resistance (Bakker et al., 2007).

1.5.1.1 Cyclic lipopeptides (CLPs)

Cyclic lipopeptides (CLPs) are versatile molecules produced by a variety of bacterial and fungal genera. CLPs are produced by several plant associated *Pseudomonas* spp, including pathogenic *Pseudomonas syringae, P. tolaasii*, *P. fuscovaginae, P. corrugate*, and *P. fluorescens* (Bender et al., 1999). CLPs which are produced by *Pseudomonas* are composed of a fatty acid tail linked to a short oligopeptide , which is cyclized to form a lactone ring between two amino acids in the peptide chain. Based on the length and composition of the fatty acid tail as well as the number, type and configuration of the amino acids in the peptide moiety, CLPs of *Pseudomonas* spp were classified into four major groups, ie. Viscosin , amphisin, tolaasin and syringomycin groups (de Souza et al., 2003). CLPs have received considerable attention for their antimicrobial, cytotoxic and surfactant properties. For plant pathogenic *Pseudomonas* spp, CLPs constitute important virulence factors, and pore formation followed by cell lysis is their main mode of action (Bender et al., 1999) For the antagonistic *Pseudomonas* spp , CLPs play a key role in antimicrobial activity, motility and biofilm formation (Raaijmakers *et al.*, 2006).

1.5.1.2 Role of massetolide A in *Pseudomonas fluorescens*

The cyclic lipopeptide surfactant (CLP) massetolide A consists of a nine amino acid cyclic oligopeptide linked to 3- hydroxydecanoic acid. It was first identified in cultures of a marine *Pseudomonas* spp isolated from the surface of a leafy red algae collected in Masset Inlet, British Columbia, Canada. Massetolide A was subsequently identified in *P. fluorescens* SS101, a biocontrol strain isolated from the wheat rhizosphere (de Souza et al., 2003). Massetolide A has potent surfactant and broad- spectrum antimicrobial activities (Raaijmakers et al., 2006). It has destructive effects on zoospores of multiple Oomycete plant pathogens, including *Pythium* and *Phytophthora* spps (de Souza et al., 2003). Massetolide A is an important determinant of the activity of *P. fluorescens* SS101 against *Phytophthora infestans*, the causal organism of late blight disease of tomato and contributes to the rhizosphere competence of strain SS101 (Tran et al., 2007). The activity of massetolide A against the late blight pathogen was attributed at least in part to

its zoosporicidal activity and to the induction of systemic resistance response in tomato plants (Tran et al., 2007). Furthermore, in combination with cell wall- degrading enzymes of Trichoderma atroviride, CLPs acted synergistically in antagonism toward various plant pathogenic fungi (Fogliano et al., 2002). Collectively, these studies clearly indicate the potential of biosurfactants and biosurfactant producing *Pseudomonas* for protection of plants against a range of pathogens.

1.5.2 Induced systemic resistance in plants by *Pseudomonas fluorescens*

Induced resistance (ISR) is a state of enhanced defensive capacity developed by a plant reacting to specific biotic or chemical stimuli (van Loon et al., 1998). In 1990, it has been described that induced systemic resistance is the mode of action of disease suppression by non-pathogenic rhizosphere bacteria (Van Peer et al., 1990). The involvement of ISR in disease suppression has been studied for a wide range of biological control microorganisms and in many cases, ISR was found to be involved. The ability to induce ISR is a common phenomenon among multiple strains of antagonistic bacteria representing various genera, including *Pseudomonas* and *Bacillus* (Kloepper et al., 2004). *Pseudomonas* and *Bacillus* strains that induce resistance in plants can release molecules including lipopolysaccharides (Leeman et al., 1995), flagellin (Meziane et al., 2005), siderophores (Leeman et al., 1995), pyocyanin (De Vleesschauwer et al., 2006). According to the study of Tran et al, (2007) massetolide A is a bacterial determinant of induced systemic resistance in tomato by a saprophytic *P. fluorescens* strain.

1.5.3 Resistance in Arabidopsis against *B. cinerea*

1.5.3.1 Natural variation in *Arabidopsis thaliana* for resistance to *B. cinerea*

B. cinerea causes disease symptoms on *Arabidopsis thaliana* (Hammond-Kosack and Parker, 2003; Thomma et al., 1998), the major model in plant biology including plantmicrobe interactions. *A. thaliana* is a self-fertilizing annual species with a broad geographical distribution throughout the Northern hemisphere (Hoffmann, 2002). Accessions representing natural variation have been collected from natural populations.

Most of the variation is of quantitative nature, exhibiting a continuous range of phenotypic variation. Variation in susceptibility to *B. cinerea* among sixteen *A. thaliana* accessions was reported and multiple QTL that govern susceptibility were identified, most of which were specific for an individual *B. cinerea* isolate (Denby et al., 2004). Also, the effects of defined mutations on susceptibility to *B. cinerea* are usually quantitative (Glazebrook, 2001; Thomma et al., 1999; Van Baarlen et al., 2007), illustrating the complex nature of the interaction between *B. cinerea* and its hosts.

1.5.3.2 Role of camalexin in resistance

Camalexin is a phytoalexin that can contribute to the innate immune response of the plant. This plant compound is absent in healthy *Arabidopsis thaliana* plants but it is synthesized in response to abiotic stresses or upon inoculation with pathogens (Stefanato et al., 2009). Several mutants have been found in which the production of camalexin is attenuated and some of the genes corresponding to these phytoalexin-deficient (pad) mutants have been characterized (Stefanato et al., 2009). By using these mutants in experimental studies, the involvement of camalexin in *Arabidopsis* local resistance to *B. cinerea* was demonstrated.

1.5.3.3 Resistance to *Botrytis cinerea* endopolygalacturonase in *Arabidopsis thaliana*

A variety of *B. cinerea* pathogenicity factors has been identified (van Kan, 2006), including endopolygalacturonases (Elad et al., 2007). BcPGs are important in the maceration of plant cell walls and decomposition of host tissue (Kars, 2007) and it is plausible to predict that genetic determinants of a plant that contribute to delimiting maceration and tissue decomposition may confer (partial) disease resistance to *B. cinerea*. Plants possess a family of defense proteins known as polygalacturonase-inhibiting proteins (PGIPs) that can interact with and inhibit PGs of several sources (Juge, 2006).

The constitutive expression of PGIPs in transgenic plants has indeed been shown to reduce *B. cinerea* disease symptoms (AgÜEro et al., 2005; Ferrari et al., 2003; Joubert et al., 2006; Powell et al., 2000). To identify genetic factors of plants that contribute to reduction of the damage inflicted by BcPGs, a study has been conducted on the natural

variation in *A. thaliana* accessions in their response to infiltration of pure BcPGs, produced in the yeast *Pichia pastoris* (Kars, 2007). QTL analyses have been performed in order to investigate the genetic nature of the observed variation in responses to BcPGs in segregating progenies from crosses between parents that differed in sensitivity to BcPGs .

The locus RBPG1 (resistance to *Botrytis* polygalacturonases) controls the response to BcPGs and was identified in a Col-0 x Br-0 F2 population (Kars, 2007). The Br-0 allele at this QTL is recessive and significantly diminishes the response to the BcPGs. The resistant allele originated from Br-0 and showed a recessive mode of inheritance (Kars, 2007). According to this study we can hypothesize that resistance to BcPGs may confer (partial) disease resistance to *B. cinerea*.

Objectives of the study

The appearance of highly aggressive and fungicide- insensitive strains of *B. cinerea* and the worldwide policy to enhance the sustainability of agriculture and horticulture have led to an increased demand for new measures to control this pathogen. Since biosurfactant producing *Pseudomonas* are very effective in controlling plant diseases caused by Oomycete pathogens (de Souza et al., 2003), it was interesting to explore whether these bacteria can also confer protection against *B. cinerea*. Secondly, in order to contribute to breeding for resistance against *B. cinerea*, it was interesting to explore whether the locus RBPG1, which confers insensitivity to *Botrytis cinerea* endopolygalacturonases (Kars, 2007), can confer (partial) disease resistance in *Arabidopsis* plants. This thesis further builds on the earlier findings and focuses on

1. The growth inhibiting properties of the CLP massetolide A from *Pseudomonas fluorescens* strain SS 101 against *Botrytis cinerea* by determining the variation in sensitivity of *B. cinerea* isolates to massetolide A and the involvement of the ATRB gene in resistance.

2.The ability of *P. fluorescens* strain SS 101 to prevent infection and induce resistance in plants towards *Botrytis cinerea*, and the role of massetolide A in biocontrol.

3. The ability of the Atrbpg1 locus to confer resistance against *B.cinerea*.

2. Materials and Methods

2.1 *In- vitro* assay

2.1.1 Fungal cultures and growth conditions

Botrytis cinerea strains	Phenotype
B05.10	Wild type strain (Büttner et al., 1994)
BcatrB	BcatrB gene- replacement mutant derived from B05.10 (Schoonbeek et al., 2002)
CH1.7	Field isolate showing a constitutive expression of BcatrB
SAS56	Field isolate, sensitive to fungicides
SAS405	Field isolate resistant to fungicides (benomyl and dicarboximides)

Table 1: Characteristics of *Botrytis cinerea* strains

The fungal strains and the mutant were grown on malt extract agar plates. Agar plates completely covered with mycelium were placed under near-UV light to induce sporulation. Conidia were harvested from the sporulating cultures with sterilized distilled water. The conidial suspension was filtered through glass wool to remove mycelium, washed once by centrifugation (800rpm for 5min) and resuspended in sterile distilled water.

2.1.2 Plate assay

The effect of massetolide A on mycelial growth of *Botrytis cinerea* was studied on maltose extract agar (MEA). Sterilized growth media were cooled down to 55 ^{0}c and amended with massetolide A to final concentrations of 0,1,3,10,30 and 100 µg/ml, each plate contained 20 ml of growth medium. A plug of *Botrytis cinerea* mycelium , excised from full-grown MEA plates was placed in the centre of the massetolide A amended growth media and kept in the incubators. Radial mycelium growth was measured with an electronic ruler after 3 and 4 days. For each treatment, four replicates were used and the assay was performed twice.

2.2 Plant assay

2.2.1Bacterial strains and growth conditions

In this study, a spontaneous rifampicin-resistant derivative of SS101 was used. Mutant 10.24 was derived from the rifampicin-resistant derivative of SS101 by mutagenesis and has a single Tn*5* insertion in *massA*, the first nonribosomal peptide synthetase (NRPS) gene required for the biosynthesis of massetolide A (de Bruijn and Raaijmakers, 2009). Mutant 10.24 does not produce massetolide A, nor any of the other massetolide A derivatives produced by wild-type strain SS101. Mutant 10.24 is resistant to rifampicin (100 µg ml^{-1}) and kanamycin (100 µg ml^{-1}). For the bacterial inoculum used in the plant assays, strain SS101 and mutant 10.24 were grown on *Pseudomonas* agar (PSA) plates (Difco, Le Pont de Claix, France) at 25°C for 48 h. Bacterial cells were washed in sterile demineralized water before use. For treatment of tomato leaves and Arabidopsis seeds, washed cell suspensions of SS101 or 10.24 were diluted in sterile demineralized water to a final concentration of 10^9 CFU ml^{-1} (OD 600_{nm} = 1).

2.2.2 Disease prevention by *Pseudomonas fluorescens* SS101 and massetolide A in tomato leaves

The effect of massetolide A and *Pseudomonas fluorescens* SS101 on infection of tomato leaves by B. cinerea was tested using a standardized assay that allows comparison of three B. cinerea strains. Leaves were cut from 7 week-old tomato plants, placed in wet florist foam and kept in plastic boxes. Leaves were dipped in suspensions of *P. fluorescens* SS101 strain (10^9 CFU ml^{-1}) for 1min or its mutant lacking the mass A gene (10.24) or purified massetolide A (50µg/ml). Leaves immersed in sterile demineralized water for 1 min served as a control. Leaves were incubated in boxes with closed lids for 24 hours. For each treatment, leaflets were inoculated on the upper side with droplets (2µl) of a conidial suspension (10^{6} ml^{-1}) in PDA medium. Each leaflet was inoculated with six droplets of three fungal strains separately. Boxes were closed and incubated and the size of the lesions determined after 4 days. For each treatment four replicates were used and the assay was performed twice.

2.3. In-vitro plant assay for role of Atrbpg1 locus against *B. cinerea*

2.3.1 Plant lines and growth conditions

Arabidopsis seeds from each ecotype and backcross lines were grown in in the climate chamber at21^{0}C with a photoperiod of 10 hours and 70% relative humidity. Plants of five to six week old were used to conduct the *in-vitro* assay.

2.3.2 Fungal culture

Botrytis cinerea strain B05.10 was used for plant infection.

2.3.3 *In-vitro* bioassay for scoring the phenotypic changes and measuring fungal biomass in *Arabidopsis* ecotypes inoculated with *B. cinerea*

Leaves from six week old *Arabidopsis* plants were placed in square petri dishes containing 1.5% agar, with the petiole embedded in the medium. Inoculation was done by placing 2µl of a suspension of 10^6/ml of spores in 1.2% potato dextrose broth (PDB) on one side of the middle vein. Disease symptoms were scored at 2 and 3 days post inoculation. For measuring the fungal biomass, weight of each sample (6 leaves were pooled as one sample) was measured. Each sample was ground by adding liquid nitrogen. 2ml of extraction buffer was added to make a mixture and 1ml from the mixture was taken into a 1.5ml tube. Each tube was centrifuged for 5 minutes at 13000rpm and 500 µl of supernatant was taken to measure the signal intensity (SI) value. By using the SI values, fungal biomass (µg/ml) in mg of fresh leaves was determined. Experiment was repeated twice.

2.4 Statistical Analysis

Data were analyzed by ANOVA followed by Duncan's multiple range test (α =0.05). All the assays described in this study were performed at least twice and representative data are shown.

3. Results

3.1.1 Sensitivity of *Botrytis* strains to massetolide A

The effect of massetolide A on mycelial growth of *Botrytis cinerea* was studied for the three strains B05.10, SAS 56 and SAS 405 (Figure 1). There was no significant difference among fungal strains in sensitivity to different concentrations of massetolide A.

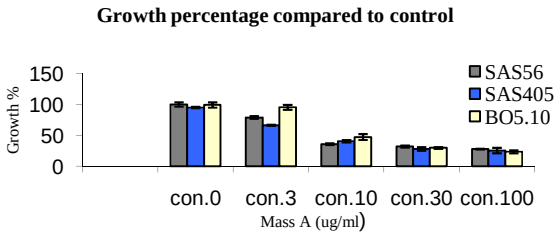


Figure 1 : Effect of cyclic lipopeptide massetolide A (Mass A) on mycelial growth of *Botrytis cinerea* strains B05.10, SAS 56 and SAS 405. Mean values of 4 replicates are given; error bars represent the standard deviation of the mean.

3.1.2 Role of the ABC transporter gene *Bcatr*B in resistance against massetolide A

To study the involvement of *Bcatr*B gene in resistance against massetolide A, an experiment was conducted with *B. cinerea* wild type strain B05.10, a *Bcatr*B deletion mutant (made in wild type strain B05.10) and a *Bcatr*B overexpressing, fungicide resistant strain CH 1.7 (Figure 2). There was no significant difference among these three strains in sensitivity to different concentrations of massetolide A.

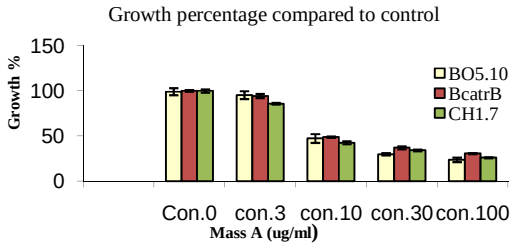


Figure 2 : Effect of cyclic lipopeptide massetolide A on mycelium growth of wild type strain B05.10, BcatrB deletion mutant and BcatrB overexpressing strain CH1.7. Mean values of 4 replicates are given; error bars represent the standard deviation of the mean.

3.2. Plant assay

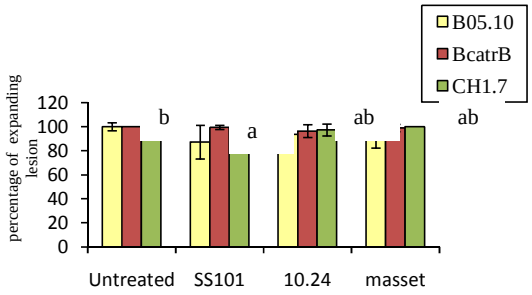
3.2.1 Preventing *Botrytis* infection of tomato leaves by *P. fluorescens* SS101 and massetolide A

To study the effect of *P. fluorescens* strain SS101 on the infection of *B. cinerea* on tomato leaves, an experiment was conducted with three *B. cinerea* strains. To investigate the role of massetolide A in plant protection, I also studied the massetolide A- deficient mutant 10.24 and the application of purified massetolide A (Figure 3). Application of suspensions of *P. fluorescens* SS101 to tomato leaves one day prior to inoculation with spores of *B. cinerea* strains B05.10 significantly reduced the percentage of expanding lesions compared to the untreated tomato leaves (Figure 3a). There was no significant effect of SS101 on the percentage of expanding lesions caused by *B. cinerea* strains, *Bcatr*B and CH1.7 (Figure 3a). Pretreatment of tomato leaves with *P. fluorescens* mutant 10.24 or with pure massetolide A did not cause any reduction of the percentage of expanding lesions with any of the three *B. cinerea* strains (Figure 3a).

The area of expanding lesions observed on tomato leaves treated with SS101 against *B. cinerea* strain B05.10 was significantly smaller than that of the lesions in the untreated leaves (Figure 3b). There was no significant effect of SS101 pretreatment on the lesion expansion rate of *B. cinerea* strains *Bcatr*B and CH1.7 (Figure 3b).

The application of massetolide A-deficient mutant strain 10.24 and purified massetolide A significantly reduced the lesion expansion rate of *B. cinerea* strain B05.10, but not of the strains *Bcatr*B and CH1.7 (Figure 3b).

a. Percentage of expanding lesions



b. Average of expanding lesion sizes (mm)

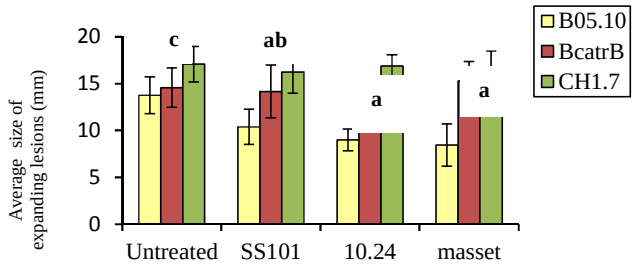


Figure 3 : Effect of *P. fluorescens* , massetolide A- deficient mutant 10.24 and purified massetolide A on *B. cinerea* disease development. (a) percentage of expanding lesions; (b) average of expanding lesion size (mm) at 4 days after inoculation with spores of *B. cinerea* strains B05.10 (wild type), BcatrB (mutant) and CH1.7 (BcatrB overexpressing isolate). Mean values of 4 replicates are given; error bars represent the standard deviation of the mean.

3.3. *In-vitro* plant assay for role of Atrbpg1 locus against *B.cinerea*

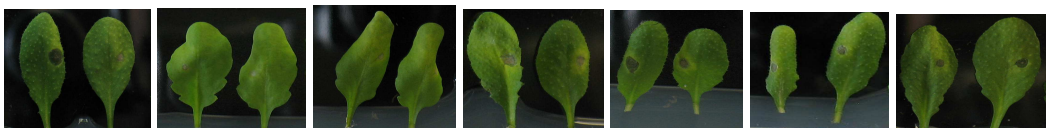
To study the ability of the Atrbpg1 locus to confer partial resistance against *B. cinerea*, an experiment was conducted with seven genotypes of *Arabidopsis thaliana* (Table 2).
It was important to test in a camalexin- deficient background, because the effect of camalexin on resistance to *B. cinerea* is so pronounced. We made use of the pad3 mutant, which is defective in the last step of camalexin biosynthesis. Three back cross lines were used that were homozygous for the rbpg1 locus and for the pad3 mutation (Table 2).

Table 2: Arabidopsis ecotypes and back cross lines

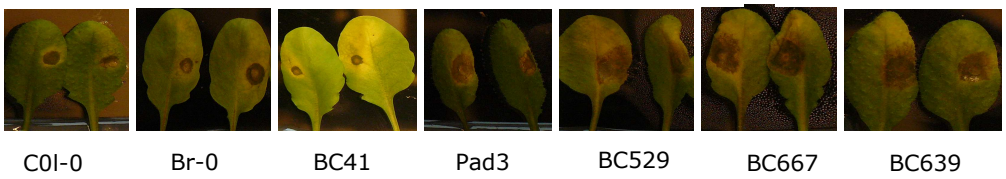
Genotype	**Camalexin**	**Rbpg1**	
Col-0	+	-	Wild type accession
Br-0	+	+	Wild type accession
BC41	+	+	Recombinant inbred progeny of Col-0 × Br-0
Pad3	-	-	Mutant of Col-0
BC529	-	+	Backcross progeny of BC41 × pad3 (F2)
BC667	-	+	Backcross progeny of BC41 × pad3 (F2)
BC639	-	+	Backcross progeny of BC41 × pad3 (F3)

Plants were inoculated with *B. cinerea* wild type strain B05.10. Disease development was followed visually and fungal biomass was quantified in extracts from inoculated leaves by an immunological quantification method.
At 2 days post inoculation,BC529, BC667 and BC639 showed less severe disease symptoms compared to their parental line pad3 but more severe disease symptoms compared to parental line BC41 (Figure 4a). At 3 days post inoculation, however, these three lines showed more severe disease symptoms compared to both parental line pad3 and BC41 (Figure 4b) . The fungal biomass in BC529, BC667 and BC639 at 2 days post inoculation was lower than in the parental line pad3 and higher than in parental line BC41 (Figure 4c). It means that these three lines, which have the RBPG1 locus but lack camalexin production display partial resistance towards *B.cinerea*. It is, however, evident from Fig.4d that the fungal biomass in these three backcross lines is higher than in the parental line BC41. This observation is probably caused by the effect of camalexin.

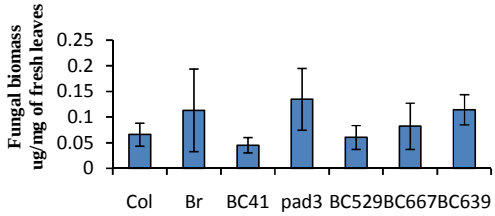
(a)



(b)



(c)



(d)

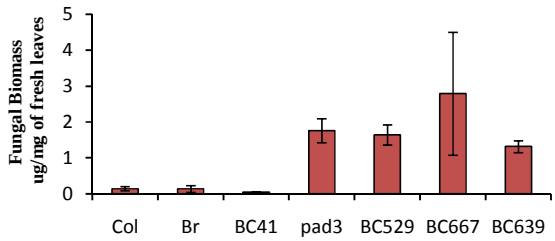


Figure 4: The function of Atrbpg1 locus against *B.cinerea*. (a) disease symptoms in Col-0, Br-0 , BC41, pad3, BC 529, BC639 and BC667 at 2 days post inoculation with *B.cinerea*. (b) disease symptoms in Col-0, Br-0 , BC41, pad3, BC 529, BC639 and BC667 at 3 days post inoculation with *B.cinerea*. (c) fungal biomass in Col-0, Br-0 , BC41, pad3, BC 529, BC639 and BC667 at 2 days post inoculation with *B.cinerea*. (d) fungal biomass in Col-0, Br-0, BC41, pad3, BC 529, BC639 and BC667 (back cross lines) at 3 days post inoculation with *B.cinerea*.

4. Discussion

4.1 Sensitivity of *Botrytis* strains to massetolide A

Most studies focus on sensitivity of plant pathogenic fungi and oomycetes to antimicrobial compounds produced by antagonistic microorganisms (Raaijmakers et al., 2002). The cyclic lipopeptide massetolide A inhibits the growth of oomycete, *Phytophthora infestans*, of potato and tomato. Massetolide A had an effect on mycelium growth, sporangia formation, cyst germination and zoospore behavior in *P. infestans* (Tran et al., 2007). In this study , I investigated the sensitivity of a plant pathogenic fungus, *B. cinerea* to cyclic lipopeptides massetolide A produced by antagonistic *Pseudomonas fluorescens* SS101. There was no difference in sensitivity among these three *Botrytis* strains to massetolide A (Figure 1).

The *Bcatr*B gene plays an important role in resistance to antimicrobial compounds produced by antagonistic microorganisms and plants (Schoonbeek et al., 2002; Schouten et al., 2008). In this study, I also investigated the involvement of the *Bcatr*B gene in resistance against massetolide A. There was no difference in sensitivity among *Botrytis* wild type strain B05.10, the *Bcatr*B deletion mutant derived from B05.10 and a *Bcatr*B overexpressing field strain CH1.7. The result suggests that BcatrB is not involved in resistance to massetolide A (Figure 2).

4.2 Prevention and induction of systemic resistance against *B. cinerea* by Pseudomonas fluorescens SS101

This study also focused on disease prevention ability and induction of systemic resistance by *P.fluorescens* SS101 in plants towards *B. cinerea*. In previous studies, *P.fluorescens* strain SS101 has shown promising results in biological control of late blight disease caused by *Phytophthora infestans*, both in preventing infection of tomato leaves and in reducing the expansion of existing lesions (Tran et al., 2007). The CLP massetolide A was an important component of the biocontrol activity since the massetolide A-deficient mutant 10.24 was significantly less effective in biocontrol (Tran et al., 2007). In this study, I examined the disease prevention ability of *P.fluorescens* SS101 in tomato leaves against three *B.cinerea* strains. *P.fluorescens* SS101 caused significant reduction in

disease only against B05.10 but it did not show any effect on other two fungal strains, ATRB and CH1.7 (Figure 3).

Induced systemic resistance (ISR) is a common phenomenon among multiple strains of antagonistic bacterial genera including *Pseudomonas* and *Bacillus* (van Loon et al., 1998). Bacterial determinants of *Pseudomonas* and *Bacillus* strains shown to be involved in induction of resistance in plants (Leeman et al., 1995). I intended to test the ability of *P. fluorescens* SS101 strain to induce systemic resistance in Arabidopsis against *B. cinerea*. However, the experiment could not be conducted due to growth problems, stress and high variability among Arabidopsis plants. In these situations, it is impossible to show the effect of induced systemic resistance. Further studies should be carried out to determine the ability of *P. fluorescens* SS101 strain to induce systemic resistance in plants against *B. cinerea*.

4.3 Role of the Atrbpg1 locus against *B.cinerea*

Arabidopsis are highly resistant to *B. cinerea* due to the production of camalexin (Stefanato et al., 2009). Arabidopsis plants that are homozygous for the recessive locus Atrbpg1 are resistant to BcPGs. This means that infiltration with purified BcPGs does not cause any visible cell wall degradation and tissue collapse (Kars, 2007). In this study, I wanted to investigate whether Atrbpg1 not only confers resistance to purified enzymes, but also confers (partial) resistant to *B. cinerea.* To study the ability of the Atrbpg1 locus to confer partial resistance to the pathogen *B. cinerea*, itself. In order to obtain reliable quantitative data, it was important to test in a camalexin- deficient background, because the effect of camalexin on resistance to *B. cinerea* is so pronounced. We made use of the pad3 mutant which is defective in the last step of camalexin biosynthesis. Three back cross lines were used that were homozygous for the rpbg1 locus and for the pad3 mutation (Table 2). The susceptibility of these lines was compared to the parental lines of Arabidopsis ecotypes Col-0 and Br-0, to the Recombinant Inbred line BC41 and to the Col-0 mutant with the mutation in the pad3 gene (Table 2). First I observed the disease symptoms in each Arabidopsis genotype after inoculating with *B. cinerea* strain B05.10 and did an immunological test to measure the fungal biomass. The results showed that

three back cross lines BC529, BC667 and BC639 showed a certain resistance against *B. cinerea* at 2 days post inoculation but they were fully susceptible to *B. cinerea* at 3 days post inoculation (Figure 4). Fungal pectinases are mainly involved in early stage of plant infection from plant surface penetration to growth into middle-lamella. The data presented here suggest that plants having the Atrbpg1 locus can achieve a partial resistance against *B. cinerea* for a short period of time because it can prevent damage caused by pectinase enzyme activity. *B. cinerea* may however contain other non-pectinolytic cell wall degrading (CWDEs) enzymes. These enzymes might enable the fungus to proceed the infection process and the Atrbpg1 locus cannot provide protection against these enzyme, making such plants susceptible.

5. Conclusions

- The results showed that five different B. cinerea strains are equally sensitive to massetolide A. There is a strong growth inhibiting effect of massetolide A on *B. cinerea* with an EC50 of 10µg/ml of massetolide A.
- The efflux pump BcAtrB does not provide tolerance to massetolide A. We can conclude that massetolide A does not act as a substrate for BcAtrB.
- *P.fluorescens* SS101 causes a significant reduction in the percentage of expanding lesions of *B. cinerea* strain B05.10, but not for two other fungal strains, ATRB and CH1.7.
- The Atrbpg1 locus can confer a certain level of resistance against *B. cinerea*, but only for a short period of time.

References

Agüero, C.B., Uratsu, S.L., Greve, C., Powell, A.L.T., Labavitch, J.M., Meredith, C.P. and Dandekar, A.M. (2005) Evaluation of tolerance to Pierce's disease and Botrytis in transgenic plants of Vitis vinifera L. expressing the pear PGIP gene. *Molecular Plant Pathology* **6**, 43-51.

Bakker, P.A.H.M., Pieterse, C.M.J. and van Loon, L.C. (2007) Induced Systemic Resistance by Fluorescent Pseudomonas spp. *Phytopathology* **97**, 239-243.

Bender, C.L., Alarcon-Chaidez, F. and Gross, D.C. (1999) Pseudomonas syringae Phytotoxins: Mode of Action, Regulation, and Biosynthesis by Peptide and Polyketide Synthetases. *Microbiol. Mol. Biol. Rev.* **63**, 266-292.

Büttner, P., Koch, F., Voigt, K., Quidde, T., Risch, S., Blaich, R., Brückner, B. and Tudzynski, P. (1994) Variations in ploidy among isolates of *Botrytis cinerea*: implications for genetic and molecular analyses. *Current Genetics* **25**, 445-450.

de Bruijn, I. and Raaijmakers, J.M. (2009) Regulation of cyclic lipopeptide biosynthesis in *Pseudomonas fluorescens* by ClpP protease.*J. Bacteriol.***191**,1910-23.

de Souza, J.T., de Boer, M., de Waard, P., van Beek, T.A. and Raaijmakers, J.M. (2003) Biochemical, genetic and zoosporicidal properties of cyclic lipopeptide surfactants produced by *Pseudomonas fluorescens*. *Appl. Environ. Microbiol.* **69**, 7161-7172.

De Vleesschauwer, D., Cornelis, P. and Höfte, M. (2006) Redox-active pyocyanin secreted by *Pseudomonas aeruginosa* 7NSK2 triggers systemic resistance to *Magnaporthe grisea* but enhances *Rhizoctonia solani* susceptibility in rice. *Molecular Plant-Microbe Interactions* **19**, 1406-1419.

Denby, K.J., Kumar, P. and Kliebenstein, D.J. (2004) Identification of *Botrytis cinerea* susceptibility loci in *Arabidopsis thaliana*. *The Plant Journal* **38**, 473-486.

Elad, Y., Williamson, B., Tudzynski, P., Delen, N., Kars, I. and Kan, J.A.L. (2007) Extracellular enzymes and metabolites involved in pathogenesis of *Botrytis*. In *Botrytis: Biology, Pathology and Control*: Springer Netherlands, pp. 99-118.

Elad, Y., Köhl, J. and Fokkema, N.J. (1994) Control of infection and sporulation of *Botrytis cinerea* on bean and tomato by saprophytic bacteria and fungi. *European Journal of Plant Pathology* **100**, 315-336.

Faretra, F. and Pollastro, S. (1991) Genetic basis of resistance to benzimidazole and dicarboximide fungicides in *Botryotinia fuckeliana* (*Botrytis cinerea*). *Mycological Research* **95**, 943-951.

Ferrari, S., Vairo, D., Ausubel, F.M., Cervone, F. and De Lorenzo, G. (2003) Tandemly duplicated Arabidopsis genes that encode Polygalacturonase-Inhibiting Proteins are regulated coordinately by different signal transduction pathways in response to fungal infection. *Plant Cell* **15**, 93-106.

Finkers, R., van Heusden, A., Meijer-Dekens, F., van Kan, J., Maris, P. and Lindhout, P. (2007) The construction of a *Solanum habrochaites* LYC4 introgression line population and the identification of QTLs for resistance to *Botrytis cinerea*. *Theoretical and Applied Genetics* **114**, 1071-1080.

Fogliano, V., Ballio, A., Gallo, M., Woo, S., Scala, F. and Lorito, M. (2002) Pseudomonas lipodepsipeptides and fungal cell wall-degrading enzymes act synergistically in bological cntrol. *Molecular Plant-Microbe Interactions* **15**, 323-333.

Forster, B. and Staub, T. (1996) Basis for use strategies of anilinopyrimidine and

phenylpyrrole fungicides against *Botrytis cinerea*. *Crop Protection* **15**, 529-537.
Glazebrook, J. (2001) Genes controlling expression of defense responses in Arabidopsis -- 2001 status. *Current Opinion in Plant Biology* **4**, 301-308.
Govrin, E.M. and Levine, A. (2002) Infection of Arabidopsis with a necrotrophic pathogen, *Botrytis cinerea*, elicits various defense responses but does not induce systemic acquired resistance (SAR). *Plant Molecular Biology* **48**, 267-276.
Hammond-Kosack, K.E. and Parker, J.E. (2003) Deciphering plant-pathogen communication: fresh perspectives for molecular resistance breeding. *Current Opinion in Biotechnology* **14**, 177-193.
Hoffmann, M.H. (2002) Biogeography of *Arabidopsis thaliana* (L.) Heynh. (Brassicaceae). *Journal of Biogeography* **29**, 125-134.
Joubert, D., Slaughter, A., Kemp, G., Becker, J., Krooshof, G., Bergmann, C., Benen, J., Pretorius, I. and Vivier, M. (2006) The grapevine polygalacturonase-inhibiting protein (VvPGIP1) reduces *Botrytis cinerea* susceptibility in transgenic tobacco and differentially inhibits fungal polygalacturonases. *Transgenic Research* **15**, 687-702.
Juge, N. (2006) Plant protein inhibitors of cell wall degrading enzymes. *Trends in Plant Science* **11**, 359-367.
Kars, I. (2007) *The role of pectin degradation in pathogenesis of Botrytis cinerea.* PhD thesis. Wageningen: s.n.
Kloepper, J.W., Ryu, C.-M. and Zhang, S. (2004) Induced Systemic Resistance and Promotion of Plant Growth by Bacillus spp. *Phytopathology* **94**, 1259-1266.
Leeman, M., van Pelt, J.A., den Ouden, F.M., Heinsbroek, M., Bakker, P.A.H.M. and Schippers, B. (1995) Induction of systemic resistance by *Pseudomonas fluorescens* in radish cultivars differing in susceptibility to fusarium wilt, using a novel bioassay. *European Journal of Plant Pathology* **101**, 655-664.
Leroux, P. (1996) Recent developments in the mode of action of fungicides. *Pestic. Sci.* **47**, 191-197.
Meziane, H., Van Der Sluis, I., Van Loon, L.C., HÖFte, M. and Bakker, P.A.H.M. (2005) Determinants of Pseudomonas putida WCS358 involved in inducing systemic resistance in plants. *Molecular Plant Pathology* **6**, 177-185.
Osusky, M., Osuska, L., Kay, W. and Misra, S. (2005) Genetic modification of potato against microbial diseases: in vitro and in planta activity of a dermaseptin B1 derivative, MsrA2. *Theoretical and Applied Genetics* **111**, 711-722.
Powell, A.L.T., van Kan, J., ten Have, A., Visser, J., Greve, L.C., Bennett, A.B. and Labavitch, J.M. (2000) Transgenic Expression of Pear PGIP in Tomato Limits Fungal Colonization. *Molecular Plant-Microbe Interactions* **13**, 942-950.
Raaijmakers, J., Vlami, M. and de Souza, J. (2002) Antibiotic production by bacterial biocontrol agents. *Antonie van Leeuwenhoek* **81**, 537-547.
Raaijmakers, J.M., de Bruijn, I. and de Kock, M.J.D. (2006) Cyclic lipopeptide production by plant-associated *Pseudomonas* spp.: diversity, activity, biosynthesis, and regulation. *Molecular Plant-Microbe Interactions* **19**, 699-710.
Schoonbeek, H.-j., Raaijmakers, J.M. and De Waard, M.A. (2002) Fungal ABC transporters and microbial interactions in natural environments. *Molecular Plant-Microbe Interactions* **15**, 1165-1172.
Schouten, A., Maksimova, O., Cuesta-Arenas, Y., Van Den Berg, G. and Raaijmakers, J.M. (2008) Involvement of the ABC transporter BcAtrB and the

laccase BcLCC2 in defence of Botrytis cinerea against the broad-spectrum antibiotic 2,4-diacetylphloroglucinol. *Environmental Microbiology* **10**, 1145-1157.

Shafir, S., Dag, A., Bilu, A., Abu-Toamy, M. and Elad, Y. (2006) Honey bee dispersal of the biocontrol agent *Trichoderma harzianum* T39: effectiveness in suppressing *Botrytis cinerea* on strawberry under field conditions. *European Journal of Plant Pathology* **116**, 119-128.

Stefanato, F.L., Abou-Mansour, E., Buchala, A., Kretschmer, M., Mosbach, A., Hahn, M., Bochet, C.G., Métraux, J.-P. and Schoonbeek, H.-j. (2009) The ABC transporter BcatrB from *Botrytis cinerea* exports camalexin and is a virulence factor on Arabidopsis thaliana. *The Plant Journal* **58**, 499-510.

Thomma, B.P.H.J., Eggermont, K., Penninckx, I.A.M.A., Mauch-Mani, B., Vogelsang, R., Cammue, B.P.A. and 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**, 15107-15111.

Thomma, B.P.H.J., Eggermont, K., Tierens, K.F.M.-J. and Broekaert, W.F. (1999) Requirement of Functional Ethylene-Insensitive 2 Gene for Efficient Resistance of Arabidopsis to Infection by Botrytis cinerea. *Plant Physiol.* **121**, 1093-1101.

Tran, H., Ficke, A., Asiimwe, T., Höfte, M. and Raaijmakers, J.M. (2007) Role of the cyclic lipopeptide massetolide A in biological control of Phytophthora infestans and in colonization of tomato by Pseudomonas fluorescens. *New Phytologist* **175**, 731-742.

Van Baarlen, P., Woltering, E.J., Staats, M. and Van Kan, J.A.L. (2007) Histochemical and genetic analysis of host and non-host interactions of Arabidopsis with three Botrytis species: an important role for cell death control. *Molecular Plant Pathology* **8**, 41-54.

van Kan, J.A.L. (2006) Licensed to kill: the lifestyle of a necrotrophic plant pathogen. *Trends in Plant Science* **11**, 247-253.

van Loon, L.C., Bakker, P.A.H.M. and Pieterse, C.M.J. (1998) Systemic resistance induced by rhizopshere bacteria. *Annual Review of Phytopathology* **36**, 453-483.

Van Peer, R., Van Kuik, A.J., Rattink, H. and Schippers, B. (1990) Control of Fusarium wilt in carnation grown on rockwool by Pseudomonas sp. strain WCS417r and by Fe-EDDHA. *European Journal of Plant Pathology* **96**, 119-132.

Vellicce, G., Ricci, J., Hernández, L. and Castagnaro, A. (2006) Enhanced Resistance to *Botrytis cinerea* mediated by the transgenic expression of the chitinase gene ch5B in Strawberry. *Transgenic Research* **15**, 57-68.

Whipps, J.M. (2001) Microbial interactions and biocontrol in the rhizosphere. *Journal of Experimental Botany* **52**, 487-511.

Williamson, B., Tudzynski, B., Tudzynski, P. and Van Kan, J.A.L. (2007) *Botrytis cinerea*: the cause of grey mould disease. *Molecular Plant Pathology* **8**, 561-580.

Xiao, C.L., Chandler, C.K., Price, J.F., Duval, J.R., Mertely, J.C. and Legard, D.E. (2001) Comparison of epidemics of Botrytis fruit rot and powdery mildew of strawberry in plastic tunnel and field production systems. *Plant Disease* **85**, 901-909.

Yan, Z., Reddy, M.S., Ryu, C.-M., McInroy, J.A., Wilson, M. and Kloepper, J.W. (2002) Induced systemic protection against tomato late blight elicited by plant growth-promoting rhizobacteria. *Phytopathology* **92**, 1329-1333.

Appendix A

Mycelium diameter (mm) of different Botrytis strains at different concentration levels of massetolide A

Massetolide A		**0**	**1**	**3**	**10**	**30**	**100**
B05.10	R1	53	51	55	19	14	9
	R2	54	57	55	27	17	15
	R3	54	58	49	31	17	12
	R4	53	60	48	25	16	13
SAS 56	R1	54	49	44	20	15	15
	R2	49	52	40	19	16	15
	R3	52	45	40	17	17	14
	R4	55	46	42	18	18	13
SAS 405	R1	52	51	35	22	17	8
	R2	49	50	34	19	16	11
	R3	49	49	36	20	10	16
	R4	49	48	34	24	15	18
BcatrB	R1	55	53	49	25	21	17
	R2	55	51	54	26	19	16
	R3	55	54	51	27	18	17
	R4	52	54	50	26	21	16
CH1.7	R1	59	56	52	23	20	15
	R2	61	56	53	26	21	15
	R3	59	60	53	27	22	16
	R4	64	58	51	27	20	16

Appendix B

Percentage of expanding lesions in treated tomato leaves after inoculating with *Botrytis* strains

Fungal Strain		Untreated	SS101	Untreated	10.24	Untreated	Massetolide A
B05.10	R1	100	82	100	65	100	100
	R2	100	95	100	100	100	95
	R3	100	91	100	91	96	65
	R4	100	66	82	91	100	100
	R5	100	100	100	100	100	100
	R6	100	96	100	100	100	100
	R7	100	66	100	100	100	100
	R8	100	100	100	100	100	96
BcatrB	R1	100	100	100	95	100	100
	R2	100	100	100	100	100	100
	R3	100	100	100	96	100	100
	R4	100	100	100	95	100	100
	R5	100	100	100	84	100	100
	R6	100	95	100	100	100	100
	R7	100	100	100	100	100	92
	R8	100	100	100	100	100	100
CH1.7	R1	100	96	100	100	100	100
	R2	100	100	100	100	100	100
	R3	100	100	100	100	100	100
	R4	100	100	100	100	100	100
	R5	100	100	100	88	100	100
	R6	100	100	100	90	100	100
	R7	100	100	100	100	100	100
	R8	100	95	100	100	100	100

Appendix C

Lesion size (mm) in treated tomato leaves after inoculating with *Botrytis* strains

Fungal Strain		Untreated	SS101	Untreated	10.24	Untreated	Massetolide A
B05.10	R1	17	11	15	8	13	9
	R2	16	13	16	10	11	6
	R3	17	8	13	7	6	5
	R4	16	13	12	9	14	11
	R5	13	10	15	10	15	10
	R6	13	9	13	10	13	10
	R7	14	8	14	8	14	8
	R8	14	11	13	9	13	8
BcatrB	R1	20	16	13	11	12	13
	R2	17	17	12	11	16	15
	R3	17	14	15	10	12	14
	R4	19	17	13	13	12	14
	R5	14	10	12	10	15	13
	R6	13	12	14	12	14	19
	R7	14	12	15	12	17	17
	R8	12	13	11	11	18	16
CH1.7	R1	18	16	17	17	15	14
	R2	18	14	18	16	14	13
	R3	17	13	16	16	15	13
	R4	17	18	18	15	13	13
	R5	12	18	17	15	18	19
	R6	14	13	20	18	17	17
	R7	16	18	17	18	18	18
	R8	20	18	18	18	18	18

Appendix D

Fungal biomass µg/mg of fresh leaves at 2day and 3 day post inoculation

		Fungal biomass µg/mg of fresh leaves at 2dpi	**Fungal biomass µg/mg of fresh leaves at 3dpi**
Col-0	R1	0.05	0.09
	R2	0.04	0.09
	R3	0.08	0.17
	R4	0.09	0.21
Br-0	R1	0.05	0.05
	R2	0.04	0.05
	R3	0.18	0.22
	R4	0.18	0.22
BC41	R1	0.03	0.03
	R2	0.03	0.04
	R3	0.06	0.06
	R4	0.05	0.05
Pad3	R1	0.08	2.15
	R2	0.08	1.57
	R3	0.19	1.41
	R4	0.19	1.93
BC529	R1	0.03	1.38
	R2	0.05	1.52
	R3	0.08	2.03
	R4	0.08	1.62
BC667	R1	0.06	4.74
	R2	0.04	3.72
	R3	0.14	1.27
	R4	0.09	1.42
BC639	R1	0.14	1.26
	R2	0.08	1.56
	R3	0.14	1.22
	R4	0.10	1.23