Interactions between biosurfactant-producing *Pseudomonas* and *Phytophthora* species

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Chapter 1

General introduction and outline of the thesis

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Introduction

Phytophthora species form a diverse group of eukaryotic fungus-like microorganisms containing many economically important pathogens of plants (Kamoun, 2003). Although *Phytophthora infestans* is probably the most notorious plant pathogen, several other species also cause substantial yield losses in a variety of crops (van West *et al.*, 2003). Among the *Phytophthora* species causing root rot, *Phytophthora cinnamomi* is a major pathogen of over 900 plant species (van West *et al.*, 2003). Similarly, *Phytophthora capsici* infects various hosts, including peppers, tomato, cucumber, eggplant, pumpkin, squash, and melon (Erwin & Ribeiro, 1996; Hausbeck & Lamour, 2004). *Phytophthora* species reproduce asexually, forming mobile flagellate zoospores under moist conditions. Zoospores swim for a period of time, varying from minutes to hours, and locate healthy tissue by responding to chemo-attractants released by the plant (Deacon & Donaldson, 1993; Zhou & Paulitz, 1993) and by electrotaxis to root-generated electric fields (van West *et al.*, 2002). Zoospores accumulate on plant surfaces, encyst and germinate in response to various stimuli including calcium and isoflavones (reviewed in Deacon & Donaldson, 1993; van West *et al.*, 2003). Thus, zoospore taxis is an essential part of the pre-infection process and is a potential target for controlling diseases caused by *Phytophthora* species.

Phytophthora diseases in Vietnam

Plant pathogenic *Phytophthora* species cause substantial yield losses in a variety of crops in Vietnam, including fruits, vegetables, spices, and trees. To date, thirteen species of *Phytophthora* have been identified in Vietnam, including *Phytophthora infestans* on tomato and *Phytophthora capsici* on peppers (Dang *et al.*, 2004). Late blight caused by *P. infestans* is one of the major diseases of tomato in the Red River Delta in the North of Vietnam; it occurs annually from December to March when climatic conditions are favourable. Infection generally results in yield losses of 30-70% (Dang *et al.*, 2004) and in severe cases the crop is completely destroyed (Vu, 1973). All varieties are susceptible to the disease. Farmers control the disease mainly by application of Bordeaux mixture, Maneb, Zineb or Ridomil (Ha *et al.*, 2002; Dang *et al.*, 2004).

P. capsici was first described as the causal agent of blight of chilli pepper (*Capsicum annuum* L.) in New Mexico (Leonian, 1922). Although this pathogen was originally considered to be host specific (Tucker, 1931), host specificity can no longer be used as a taxonomic criterion since *P. capsici* has been reported as a pathogen on squash, pumpkin, tomato and other solanaceous and cucurbit hosts worldwide (Erwin & Ribeiro, 1996; Hausbeck & Lamour, 2004). It also has been reported to cause disease on a variety of tropical hosts, including cacao, rubber, macadamia, papaya, and black pepper (Erwin & Ribeiro, 1996). In Vietnam, *P. capsici* causes blight, root and crown rot of pepper (*Capsicum annuum* L.) and foot rot of black pepper (*Piper nigrum* L.). Foot rot of black pepper (Figure 1) is also known as

"quick wilt" and was first reported in Indonesia in 1985. *P. capsici* can attack all parts of black pepper plants at any stage of crop growth (Sarma, 2002). In Vietnam, *P. capsici* causes an estimated annual loss of 15-20% (Drenth & Sendall, 2004), but disease losses have not been accurately quantified for specific black pepper areas. *P. capsici* is a soil-borne heterothallic oomycete with two mating types, A1 and A2. Both mating types have been reported to coexist in several black pepper areas in Vietnam (Nguyen *et al.*, 2006). The pathogen can survive in soil as oospores for a long time under unfavourable conditions. Zoospores produced from germinating oospores or from sporangia formed on infected leaves play an important role in the epidemiology of this disease.

Figure 1: Pictures of black pepper and symptoms of Phytophthora foot rot
(a1) Overview of a healthy black pepper orchard
(a2) Black pepper plant infected by *P. capsici* showing typical symptoms of yellowing and defoliation
(b1**)** Black pepper berries.
(b2) Black, white and green black pepper grains

Current strategies to control *P. infestans* and *P. capsici*

Several methods are currently used to control *P. infestans* and *P. capsici*. Metalaxyl, a synthetic compound with activity against oomycetes, has been widely used for many years. Due to extensive use, however, metalaxyl-resistant strains have evolved and distributed rapidly, and also the efficacy of other chemical fungicides is hampered by the emergence of resistant strains of *Phytophthora* (Lee *et al.*, 2005; Ziogas *et al.*, 2006). Most organic crop production systems use copper-based fungicides to control diseases. The use of copper fungicides has, however, been restricted recently (Stephan *et al.*, 2005). Next to the emergence of fungicide-resistant strains of *Phytophthora*, the extensive use of pesticides also causes general concerns for environmental pollution and human health. Alternative measures for control are thus demanded (Lee *et al.*, 2005).

Physical-cultural methods can be used to control *Phytophthora* species, especially those that cause damping-off, root and foot rot diseases. The moisture of the soil plays an important role in the disease cycle of many *Phytophthora* species and by raising the beds of the crops and the use of drainage systems, the disease can be reduced (Ristaino & Johnston, 1999). Also the use of cultivar mixtures or crop rotations can decrease late blight on potatoes to some extent (Pilet *et al.*, 2006). Physical-cultural methods are, however, of limited efficacy and the costs and labour demands are usually high.

Breeding for resistance in crops against Phytophthora diseases is ongoing for many years. Numerous investigations are and have been undertaken on quantitative trait loci in order to obtain resistant cultivars (Bradshaw *et al.*, 2006). Besides classical breeding for resistance, genetic modification can also be a tool for introducing resistance in crops (Osusky *et al.*, 2005). At this moment, however, there are no commercial tomato cultivars with effective resistance to *P. infestans*. There are also no commercial black pepper varieties available with resistance to *P. capsici* (Anith et al., 2002), although some varieties with different degrees of tolerance to Phytophthora foot rot have been reported (Erwin & Ribeiro, 1996; Manohara *et al.*, 2003).

Biological control of plant diseases, including late blight of tomato and foot rot of black pepper, involves the use of beneficial micro-organisms to reduce the onset, development and spread of the disease. Several antagonistic microorganisms have been tested for their activity against *P. infestans*, including non-pathogenic *Phytophthora cryptogea* (Stromberg & Brishammar, 1991), *Cellulomonas flavigena*, *Candida*, and *Cryptococcus* spp. (Lourenço Júnior *et al.*, 2006). Although some effective fungal antagonists were identified, bacterial antagonists have shown by far the most promising results to date. Bacteria with antagonistic activities against *P. infestans* reported so far were mainly found in the genera of *Pseudomonas* and *Bacillus* (Sanchez, 1998; Yan *et al.*, 2002; Daayf *et al.*, 2003; Kloepper *et al.*, 2004). Also for the control of *P. capsici*, several antagonistic microorganisms, including *Trichoderma*, *Alcaligenes* and fluorescent *Pseudomonas* species, have shown promising results in the control of foot rot on black pepper (Anandaraj & Sarma, 1995; Jubina & Girija, 1998; Anith & Manomahandas, 2001; Anith *et al.*, 2002; Anith *et al.*, 2003; Diby *et al.*, 2005; Diby & Sarma, 2006). In hydroponic systems, control of *P. capsici* root rot of pepper (*Capsicum annuum* L.) was achieved by

addition of synthetic- or microbial biosurfactants to the recirculating nutrient solution (Stanghellini *et al.*, 1996; Stanghellini & Miller, 1997; Stanghellini *et al.*, 2000; De Jonghe, 2006; Nielsen *et al.*, 2006). This strategy of applying the (bio)active compounds instead of the producing bacteria may also be a practically feasible strategy for the initial protection of tomato leaves and black pepper stem cuttings against *P. infestans* and *P. capsici*, respectively. However, for the control of *P. capsici* foot rot of black pepper plants grown in field soils, application of the bioactive compounds will most likely be less effective due to the difficulty of delivering these compounds along the root system to the sites of pathogen infection. In this context, application of beneficial bacteria that are well-adapted to the rhizosphere of black pepper plants is expected to be more effective in reaching the sites of pathogen infection and to effectively deliver the bioactive metabolites. Moreover, bacteria may colonize and persist on the roots, thereby providing long-term protection against pathogens.

Biological control by *Pseudomonas* species

Among the bacterial genera used in biological control, *Pseudomonas* and *Bacillus* species are the most widely studied (Whipps, 2001; Raaijmakers *et al.*, 2002; Haas & Defago, 2005). *Pseudomonas* strains are highly amenable for research, because 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 wide range of plant pathogens, including fungi, oomycetes, nematodes and bacteria (Raaijmakers *et al.*, 2002; Haas & Defago, 2005). The mechanisms by which they protect plants against pathogens are diverse and include competition, antibiosis, parasitism, degradation of fungal pathogenicity factors, and induced systemic resistance (van Loon *et al.*, 1998; Bakker *et al.*, 2003; Haas & Defago, 2005). Currently, several biocontrol products with *Pseudomonas* strains as the active ingredient are available on the market (Table 1). Although the modes of action of some of these products are unknown, they are probably based on a combination of different mechanisms. These biocontrol products can be applied as dry product (granules or powders), cell suspensions or seed coatings, and are developed for the control of a wide range of pathogens on various crops (Table 1).

Cyclic lipopeptide surfactants

Cyclic lipopeptides (CLPs) are bioactive compounds produced by a wide range of *Pseudomonas* species and strains (Nybroe & Sørensen, 2004; Raaijmakers *et al.*, 2006; de Bruijn *et al.*, 2007). CLPs are biosurfactants composed of a fatty acid tail linked to a short cyclic oligopeptide (Figure 2). Based on the length and composition of the fatty acid tail as well as the number, type and configuration (L-D form) of the amino acids in the peptide moiety, CLPs produced by *Pseudomonas* species were classified into four major groups, i.e. the viscosin, amphisin, tolaasin and syringomycin groups (Nybroe & Sørensen, 2004). The CLPs in the syringomycin group harbour unusual amino acids, including 2,4-diaminobutyric acid (Dab) and the C-terminal 4-chlorothreonine (Figure 2). Most CLPs share similar functions for the producing bacteria, including a role in antimicrobial activity, biofilm formation and

motility (Raaijmakers *et al.*, 2006). In plant pathogenic *P. syringae* pv. *syringae,* CLPs also function as virulence factors (Bender et al., 1999). Plant-beneficial *Pseudomonas* strains produce CLPs with growth-inhibitory activities against a wide range of plant and human pathogens, including mycoplasmas, trypanosomes, gram-positive bacteria, fungi and oomycetes (Raaijmakers *et al.*, 2006).

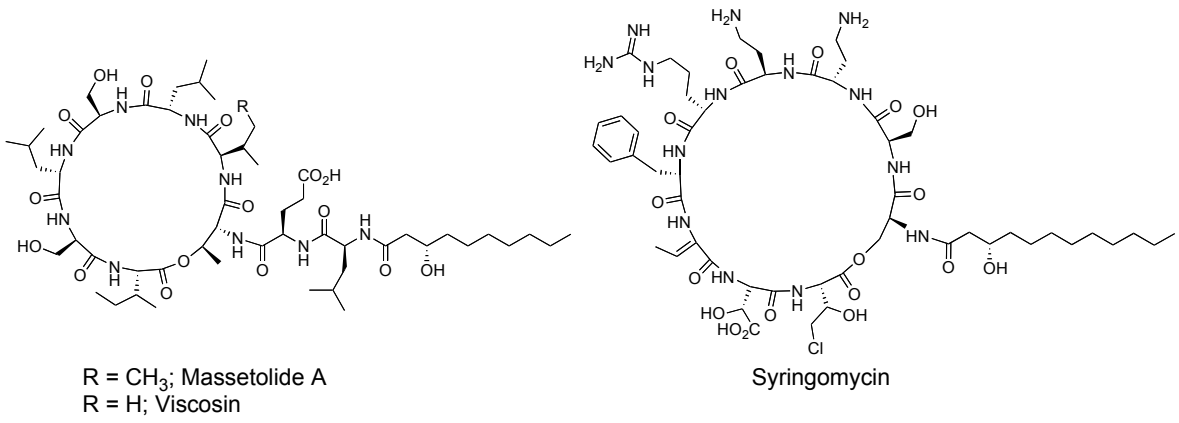

Figure 2: Structures of the cyclic lipopeptide surfactants massetolide A, viscosin and syringomycin

One of the main modes of action of CLPs involves the formation of ion channels in the plasma membrane of the target organism leading to cytolysis (Mott & Takemoto, 1989; Rainey *et al.*, 1991; Hutchison *et al.*, 1995; Hutchison & Gross, 1997; Dalla Serra *et al.*, 1999). Massetolide A produced by *P. fluorescens* strain SS101, one of the CLPs under study in this thesis project (Figure 2), solubilizes membranes of zoospores of oomycete pathogens, including *P. infestans* and *P. capsici*, leading to complete elimination of zoospore populations within 1 minute of exposure (de Souza *et al.*, 2003; de Bruijn *et al.*, 2007). Viscosinamide, produced by soil-inhabiting *Pseudomonas* strain DR54, was shown to induce encystment of *Pythium* zoospores, and adversely affected mycelium of *Rhizoctonia solani* and *Pythium ultimum* causing reduced growth and intracellular activity, hyphal swellings, increased branching and rosette formation (Thrane *et al.*, 1999; Hansen *et al.*, 2000; Thrane *et al.*, 2000). The CLP amphisin produced by *Pseudomonas* strain DSS73 appeared to play an important role in the surface motility of the producing strain, allowing efficient containment of root infecting plant pathogenic fungi (Andersen *et al.*, 2003). 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 Pseudomonads for protection of plants against a range of pathogens, including *Phytophthora* species.

Table 1: Biocontrol products with *Pseudomonas* strains as active ingredient

***Pseudomonas* strains**	**Biopesticide product**	**Target pathogen/disease**	**Crop**	**Ref.***
P. aureofaciens Tx-1	Spot-Less Biofungicide Bio-Ject spotless	Dollar spot, anthracnose, pink snow mold, *Sclerotinia homeocarpa*, *Colletotrichum graminicola*, *Pythium aphanidermatum*, *Michrodochium rivale*	Turfgrass	1/2/7
P. chlororaphis MA-342	Cedomon	Seed-borne diseases, *Drechslera* sp., *Ustilago* sp., *Tilletia caries*, *Microdochium nivale*, *Stagonospora nodorum*	Barley, oats, wheat, potential for other cereals	1/5
P. chlororaphis 63-28	AtEze	Wilt diseases, stem and root rots, *Pythium* spp., *Rhizoctonia solani*, *Fusarium oxysporum*	Ornamentals and vegetables grown in greenhouses	1/2/3/4/7
P. fluorescens	Conquer	Brown blotch disease, *Pseudomonas tolaasii*	Mushrooms	3/5
P. fluorescens 1629RS	Frostban A Frostban D	Frost-forming bacteria	Certain fruits, almond, potato, tomato	1/2
P. fluorescens A506	Blightban A506 Frostban A Frostban B	Frost damage, frost-forming bacteria, fire blight, bunch rot, *Erwinia amylovora*, russet-inducing bacteria	Almond, apple, blueberry, cherry, peach, pear, potato, strawberry, tomato	1/2/3/7
P. fluorescens BV5	Victus	Brown blotch disease, *Pseudomonas tolaasii*	Mushrooms	3
P. fluorescens NCIB12089	Victus	Brown blotch disease, *Pseudomonas tolaasii*	Mushrooms	3
P. fluorescens WCS374	Biocoat	*Fusarium* wilt	Radish	6/7
P. syringae 742RS	Frostban A, C	Frost-forming bacteria	Certain fruits, almond, potato, tomato	1/2
P. syringae ESC10	Bio-save 1000 Bio-save 10LP	Post harvest diseases, *Botrytis cinerea*, *Penicillium* spp., *Mucor pyroformis*, *Geotrichum candidum*	Pome fruits, apple, pear, citrus, cherry, potato	1/2/5/7
P. syringae ESC11	Bio-save 11LP	Post harvest diseases, *Botrytis cinerea*, *Penicillium* spp., *Mucor pyroformis*, *Geotrichum candidum*	Pome fruits, apple, pear, citrus, cherry, potato	1/2/5
Pseudomonas sp. plus *Azospirillum*	BioJet	Brown patch, dollar spot		3/5

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(1) APS**,** American Phytopathological Society, http://www.oardc.ohio-state.edu/apsbcc
(2) EPA, Environmental Protection Agency, http://www.epa.gov/pesticides/biopesticides
(3) IPPC**,** Intergrated Plant Protection Center, http://www.ippc.orst.edu/biocontrol/biopesticides
(4) LTO**,** Glastuinbouw & Productschat Tuinbouw, http://www.gewasbescherming.nl/index10i.html
(5) ATTRA**,** Appropriate Technology Transfer for Rural Areas, http://www.attra.ncat.org
(6) Bakker PAHM, Ran LX, Pieterse CMJ, van Loon LC. 2003. Understanding the involvement of rhizobacteria-mediated induction of systemic resistance in biocontrol of plant diseases. *Canadian Journal of Plant Pathology-Revue Canadienne De Phytopathologie* **25**: 5-9.
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Outline of the thesis

The appearance of highly aggressive and fungicide-insensitive strains of *Phytophthora* species (Kamoun, 2003), and the worldwide policy to enhance the sustainability of agriculture and horticulture have led to an increased demand for new measures to control these and other plant pathogens. An interesting group of antagonistic bacteria with significant potential for biocontrol of Phytophthora diseases are the biosurfactant-producing bacteria. Biosurfactants, including rhamnolipids and cyclic lipopeptides (CLPs), disrupt the membrane integrity of *Phytophthora* zoospores leading to complete elimination of infectious zoospore populations within minutes (Stanghellini & Miller, 1997; de Souza *et al.*, 2003; De Jonghe, 2006; de Bruijn *et al.*, 2007). Recent studies have shown that biosurfactant-producing Pseudomonads are very effective in controlling diseases caused by Oomycete pathogens, including *Pythium* and *Phytophthora* species (Stanghellini *et al.*, 1996; Stanghellini & Miller, 1997; Stanghellini *et al.*, 2000; De Jonghe, 2006; Nielsen *et al.*, 2006). This thesis further builds on these findings and focuses on the frequency, diversity and activity of CLP-producing *Pseudomonas* species. The activity of the CLPs and the CLP-producing Pseudomonads against *P. infestans* and *P. capsici* was studied both *in vitro* and *in vivo* with tomato and black pepper as host plants, respectively.

In **chapter 2**, the effects of the CLP massetolide A produced by *P. fluorescens* SS101 on various stages of the life-cycle of *P. infestans* were investigated. Dose-response experiments were performed to determine the effects of massetolide A on mycelial growth, sporangia formation, cyst germination, and zoospore behaviour, including chemotaxis, autoaggregation and encystment. The role of massetolide A in the biocontrol activity of *P. fluorescens* SS101 against late blight disease of tomato is addressed in **chapter 3**. More specifically, the ability of strain SS101 to prevent infection and to control the development of existing infections of *P. infestans* was investigated by application of strain SS101 to tomato leaves, roots and seeds. The role of massetolide A in control of *P. infestans* was studied by comparing the activity of wild type strain SS101 with the activity of its massetolide A-deficient mutant and with purified massetolide A. Induction of systemic resistance in tomato plants against late blight was investigated by using both strain SS101 and massetolide A as inducers, physically separated from the pathogen. To determine the role of salicylic acid as a signal in the induced resistance response, the level of protection in wild-type and in *nahG* tomato plants were compared (**chapter 3**).

In **chapter 4**, an extensive survey was conducted in 2005 and 2006 in Quang Tri province, one of the main black pepper areas in North-Central Vietnam, to determine the distribution and frequency of Phytophthora foot rot and to examine the current strategies employed by farmers to control this disease. In the same area used for this survey, indigenous biosurfactant-producing *Pseudomonas* species were isolated from roots of black pepper plants and their genotypic diversity was determined (**Chapter 5**). The biosurfactants produced by selected bacterial isolates were classified biochemically. The activity of the biosurfactants against zoospores of *P. capsici* as well as the ability of the biosurfactant-producing Pseudomonads to control foot rot and to promote growth of black pepper stem cuttings was evaluated in

in vitro and in greenhouse experiments in Vietnam. The biosurfactants produced by *P. putida* 267, one of the strains isolated from the roots of black pepper, were further characterized by mass spectrometry and their contribution to the biocontrol activity of strain 267 was evaluated in bioassays with cucumber seedlings. Mutants of *P. putida* strain 267 deficient in biosurfactant production were generated by random mutagenesis and the genes involved in biosurfactant production were partially sequenced and characterized (**chapter 6**).

In the general discussion (**chapter 7**), a summary of the most important findings of this thesis is given as well as an outlook to the future of biological control of Phytophthora diseases.

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Chapter 2

Responses of the late blight pathogen *Phytophthora infestans* to the cyclic lipopeptide surfactant massetolide A

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Responses of the late blight pathogen *Phytophthora infestans* to the cyclic lipopeptide surfactant massetolide A

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Abstract

Aims. *Phytophthora infestans* is a notorious oomycete pathogen of potato and tomato. In this study, we investigated the response of *P. infestans* to massetolide A, a cyclic lipopeptide surfactant produced by the antagonistic bacterium *Pseudomonas fluorescens* SS101.

Methods. Dose-response experiments were performed to determine the effects of massetolide A on mycelial growth, sporangia formation, cyst germination, and zoospore behaviour, including chemotaxis, autoaggregation and encystment.

Results. Swimming zoospores were the most sensitive to massetolide A followed by mycelium and zoospore cysts. Massetolide A reduced sporangia formation by *P. infestans* and caused increased branching of hyphae. At concentrations below the critical micelle concentration, massetolide A induced encystment of zoospores. Massetolide A had no effect on chemotaxis but did interfere with autoaggregation of swimming zoospores. Autoaggregation of zoospores of a transformant of *P. infestans* that constitutively produces an active form of the α subunit of the heterotrimeric G-protein, was less affected by massetolide A.

Conclusions. These results suggest that G-protein signalling is involved in the response of *P. infestans* to the cyclic lipopeptide massetolide A. We postulate that the Gα protein interferes, to some extent, with ion channel formation by massetolide A thereby reducing the influx of Ca^{2+} and/or K^{+}, two ions involved in encystment of zoospores

Keywords: *Phytophthora infestans*, sporangia, zoospores, cyclic lipopeptides, G-proteins

Introduction

Cyclic lipopeptide surfactants (CLPs) are produced by a variety of bacterial genera, including *Bacillus* and *Pseudomonas* (Finking & Marahiel, 2004; Nybroe & Sørensen, 2004; Raaijmakers *et al.*, 2006; Romero *et al.*, 2007). CLPs are composed of a fatty acid tail linked to a short oligopeptide, which is cyclized by 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 (L-D form) of the amino acids in the peptide moiety, CLPs of *Pseudomonas* species were classified into four major groups, i.e. the viscosin, amphisin, tolaasin and syringomycin groups (Nybroe & Sørensen, 2004). Most CLPs share similar functions for the producing bacteria, including a role in i) antimicrobial activity, ii) biofilm formation, and iii) motility (reviewed in Raaijmakers *et al.*, 2006). For plant pathogenic *Pseudomonas fluorescens* and *Pseudomonas syringae* pv. *syringae*, the CLPs viscosin and syringomycin also function as virulence factors (Bender *et al.*, 1999).

One of the main modes of action of natural and synthetic CLPs involves the formation of ion channels in the plasma membrane of the target organism leading to cytolysis (Mott & Takemoto, 1989; Rainey *et al.*, 1991; Hutchison *et al.*, 1995; Hutchison & Gross, 1997; Dalla Serra *et al.*, 1999). For example, the CLP massetolide A produced by beneficial *Pseudomonas fluorescens* strain SS101 acts on membranes of zoospores of plant pathogenic oomycetes, including *Pythium* and *Phytophthora* species, leading to complete elimination of infectious zoospore populations within 1 minute of exposure (de Souza *et al.*, 2003; de Boer *et al.*, 2006). The destructive effects of massetolide A on zoospores may explain, at least in part, the biocontrol activity of *P. fluorescens* SS101 against *Pythium* root rot of flower bulb crops (de Souza *et al.*, 2003; de Boer *et al.*, 2006) and tomato late blight caused by *Phytophthora infestans* (Tran *et al.*, 2007). However, CLPs not only act on zoospores but also inhibit mycelial growth of oomycetes and fungi, including the plant pathogens *Alternaria brassicae*, *Sclerotinia sclerotiorum, Pythium ultimum, Fusarium* species, *Rhizoctonia solani*, and *Botrytis cinerea* (reviewed in Nybroe & Sørensen, 2004; Raaijmakers *et al.*, 2006). The observations that several CLPs, i.e. surfactin, fengycin and massetolide A, induce systemic resistance in bean against *B. cinerea* (Ongena *et al.*, 2007) or in tomato against the late blight pathogen *P. infestans* (Tran *et al.*, 2007), further emphasizes the versatile activities of CLPs and their potential for crop protection. To explore and exploit CLPs as a novel supplementary strategy for late blight control, more knowledge is required on their mode(s) of action and the cellular responses of *P. infestans* to CLPs

In this study, we investigated the effects of the CLP massetolide A produced by *P. fluorescens* SS101 on various propagules of *P. infestans* that play important roles in the life cycle of this oomycete pathogen. More specifically, we performed dose-response experiments to determine the effects of massetolide A on mycelial growth, sporangia formation, cyst germination, and zoospore behaviour, including chemotaxis, autoaggregation and encystment, and we examined the involvement of the heterotrimeric G-protein in the response of zoospores of *P. infestans* to massetolide A. The results show

that swimming zoospores are most sensitive to massetolide A followed by mycelium and zoospore cysts. At concentrations below the critical micelle concentration, massetolide A induces encystment of zoospores and prevents autoaggregation of swimming zoospores. Moreover, autoaggregation of zoospores of a transformant of *P. infestans* that constitutively produces an active form of the G-protein α subunit PiGPA1, was less affected by massetolide A suggesting that the mechanism by which *P. infestans* senses the cyclic lipopeptide is, in part, dependent on the Gα subunit .

Results

Effect of massetolide A on mycelial growth and sporangia formation of *P. infestans*

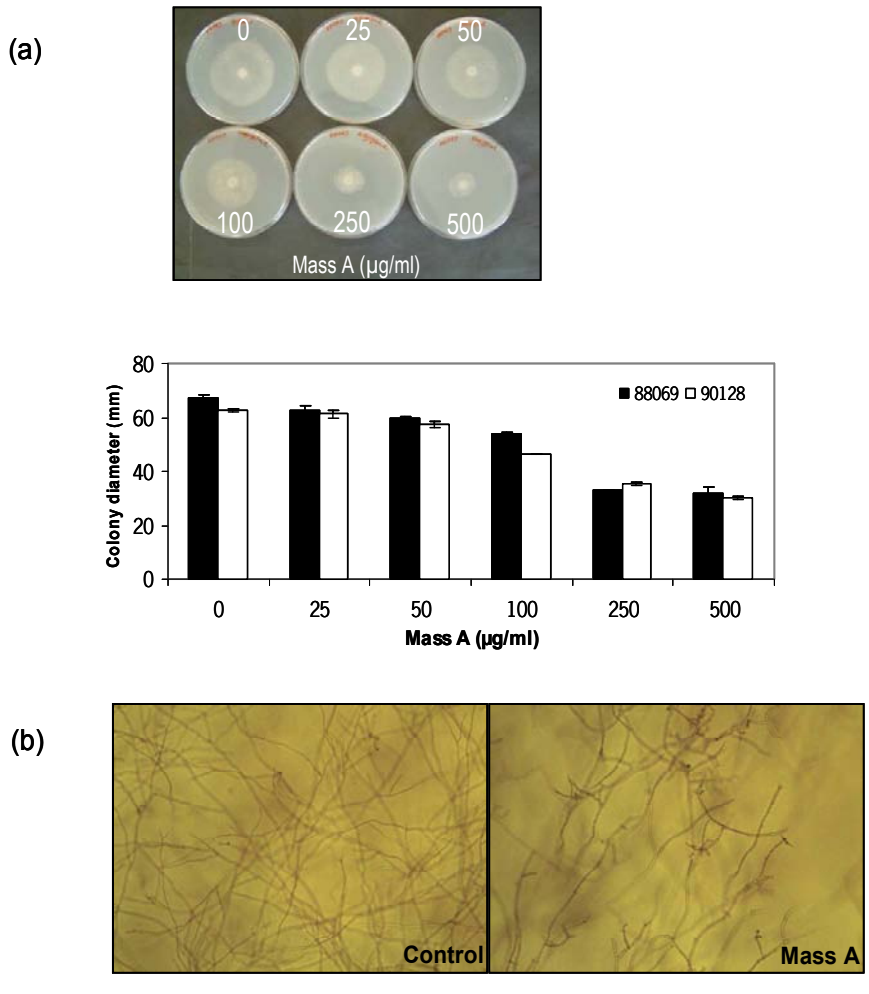

Figure 1:
(a) Effect of the cyclic lipopeptide massetolide A (MassA) on mycelial growth of *Phytophthora infestans* strains 90128 and 88068. Strains were grown on clarified rye sucrose (CRSA) agar medium amended with different concentrations of MassA. Radial mycelial growth was determined after 11 days. Mean values of 4 replicates are given; error bars represent the standard error of the mean.
(b) Representative microscopic pictures (100X magnification) of mycelium of *P. infestans* strain 90128 grown for 11 days on CRSA supplemented with massetolide A (MassA) at a concentration of 500 µg/ml. Medium without massetolide A served as a control.

The effect of massetolide A on mycelial growth of *P. infestans* was studied for the two strains 90128 and 88069. On CRSA medium, mycelial growth of the *P. infestans* strains was significantly ($p < 0.05$) inhibited at massetolide A concentrations of 50 µg ml^{-1} and higher (Fig. 1a). Similar results were obtained in experiments performed with $1/5^{th}$-strength PDA (data not shown). Microscopic analysis of mycelium obtained from the agar plates revealed that massetolide A, at concentrations of 100 µg ml^{-1} and higher, caused increased branching of hyphae (Fig. 1b). Also in liquid medium, growth of both *P. infestans* strains was significantly inhibited at massetolide A concentrations of 10 µg ml^{-1} and higher; fresh and dry weights of *P. infestans* decreased logarithmically with increasing concentrations of massetolide A reaching the maximum reduction in biomass at massetolide A concentrations of 50 µg ml^{-1} and higher (Fig. 2).

The effect of massetolide A on sporangia formation by *P. infestans* strains 90128 and 88069 was determined after 11 days of incubation. On CRSA medium, the number of sporangia formed per mm^2 mycelium of both *P. infestans* strains significantly decreased with increasing concentrations of massetolide A (Fig. 3a). Also when the strains were grown in liquid medium, sporangia formation per mg of dry weight decreased significantly with increasing concentrations of massetolide A (Fig. 3b). Similar results were obtained when sporangia formation was expressed per biomass fresh weight (data not shown).

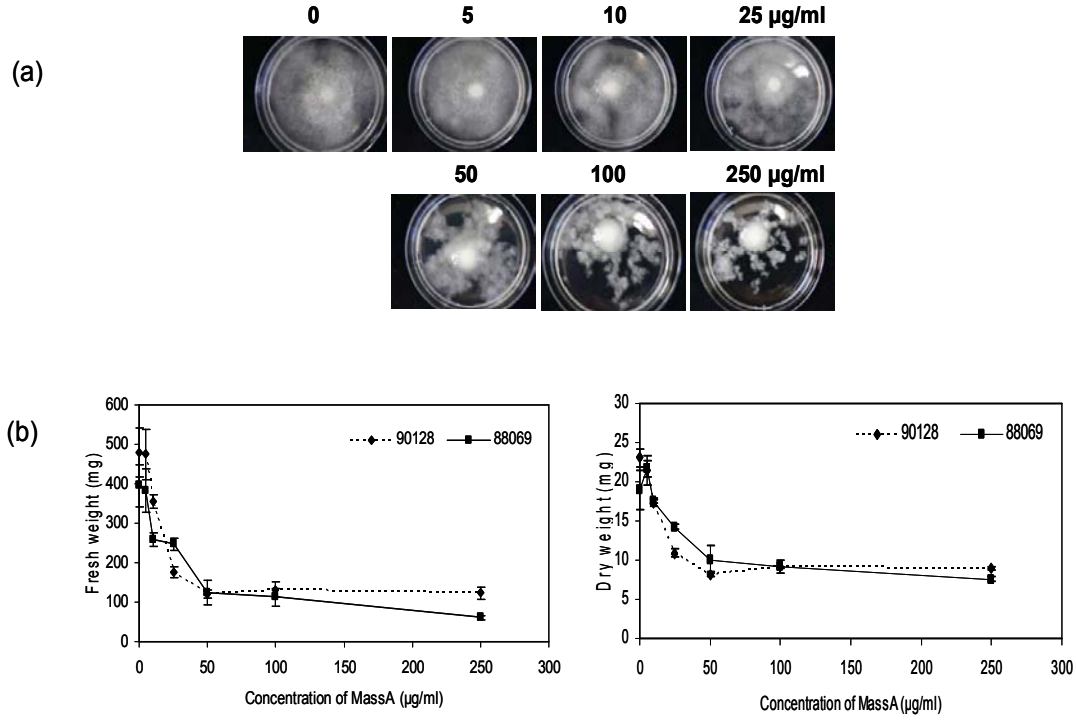

Figure 2:
(a) Representative effects of massetolide A on growth of *P. infestans* strains 90128 and 88068. The strains were grown for 11 days in clarified rye sucrose (CRS) liquid medium amended with different concentrations of massetolide A (indicated on top of each of the individual picture frames).
(b) Effect of massetolide A on biomass (fresh and dry weights) of *P. infestans* 90128 and 88069. For each concentration, means of 4 replicates are shown. Error bars represent the standard error of the mean.

Effect of massetolide A on encysted zoospores of *P. infestans*

Germination of encysted zoospores was not adversely affected by massetolide A, except at a relatively high concentration of 500 µg ml^{-1} showing a small but statistically significant ($p < 0.05$) reduction (Fig. 4a). Massetolide A significantly reduced subsequent outgrowth of the germ tube from the encysted zoospores: germ tube length decreased with increasing concentrations of massetolide A and was significantly ($p < 0.05$) reduced at massetolide A concentrations of 100 µg ml^{-1} and higher (Fig. 4b).

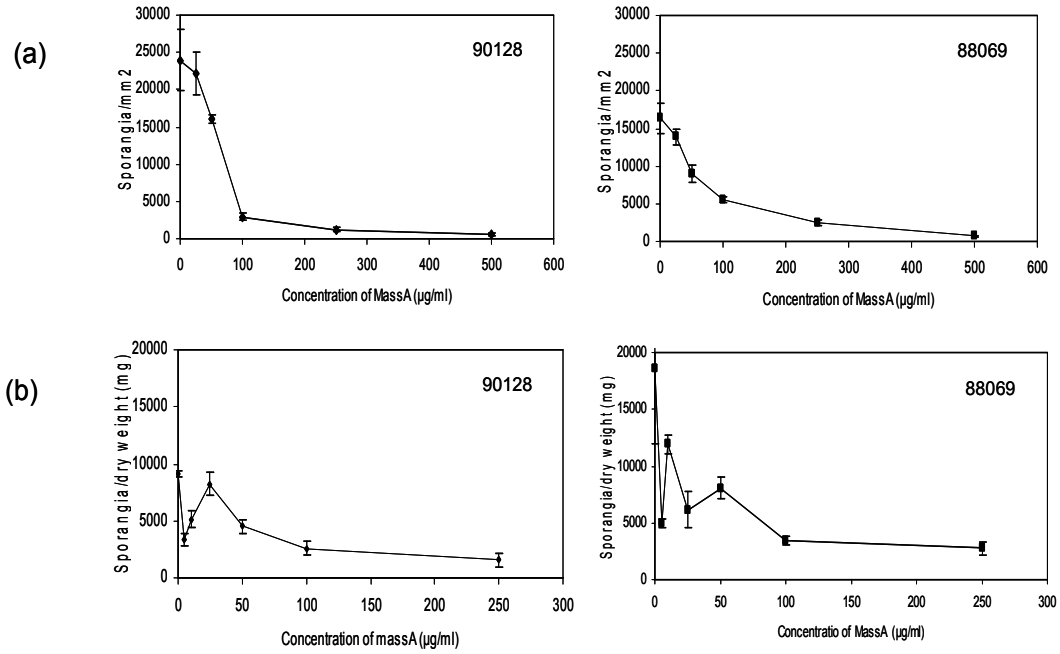

Figure 3:
(a) Effect of massetolide A on sporangia formation by *P. infestans* strains 90128 and 88068. The strains were grown for 11 days on clarified rye sucrose (CRSA) agar medium amended with different concentrations of massetolide A (MassA). After 11 days of growth, the number of sporangia formed per mm^2 of mycelium was determined. Mean values of 4 replicates are shown. Error bars represent the standard error of the mean.

(b) Effect of massetolide A on sporangia formation by *P. infestans* strains 90128 and 88068 grown for 11 days in CRS liquid medium amended with different concentrations of massetolide A. Sporangia numbers are expressed per unit of *P. infestans* biomass (dry weight). For each concentration, means of 3 replicates are shown. Error bars represent the standard error of the mean.

Effects of massetolide A on autoaggregation, encystment and chemotaxis of zoospores

Dose-response experiments performed previously (de Souza *et al.*, 2003) with partially purified massetolide A showed that lysis of *P. infestans* zoospores occured within 1 min at concentrations of 25 µg ml^{-1} or higher. The same result was obtained in this study with HPLC-purified massetolide A: lysis of zoospores of *P. infestans* strain 88069 occurred within 1 min at massetolide A concentrations of 25 µg ml^{-1} and higher (Fig. 5a). Also for *Pigpa1*-silenced strain gs2 and the PiGPA1 gain-of-function mutant R2 zoospore lysis occurred at massetolide A concentrations of 25 µg ml^{-1} and higher. The results from the surface tension measurements indicated that the critical micelle concentration (CMC) of massetolide A is approximately 25 µg ml^{-1} (Fig. 5a). When zoospores were exposed to massetolide A for 15 min

instead of 2 min, 8-10% and 33-47% of the zoospores disintegrated at massetolide A concentrations of 5 and 10 µg ml^{-1}, respectively (Fig. 5a). This result shows that massetolide A also has zoosporicidal activity at concentrations below the CMC, although more time is required to evoke lysis.

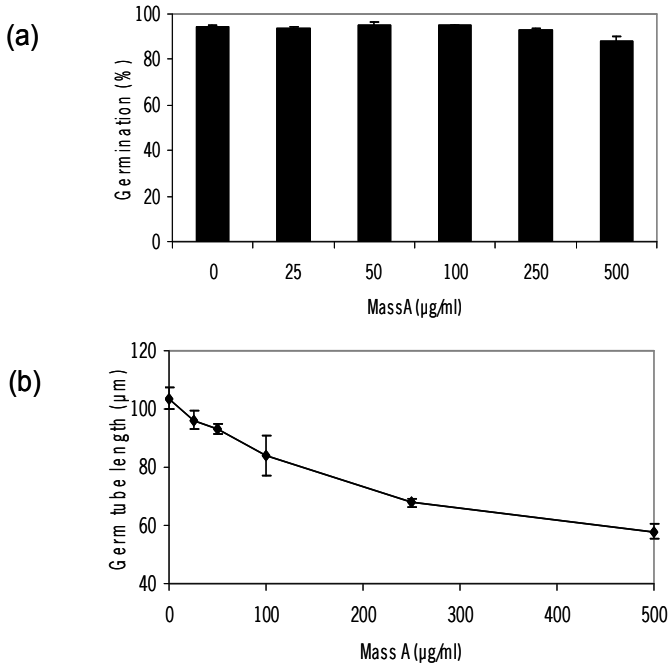

Figure 4:
(a) Effect of massetolide A (MassA) on germination of zoospore cysts of *P. infestans* strain 90128. Cysts were exposed to different concentrations of massetolide A for 3 hours at 25 oC after which germination was determined microscopically. Mean values of 3 replicates are shown. Error bars represent the standard error of the mean.

(b) Effect of massetolide A on germ tube length of zoospore cysts of *P. infestans* strain 90128. Germ tube length was determined microscopically. For each MassA concentration, means of 3 replicates are shown. Error bars represent the standard error of the mean.

When a suspension with high concentrations of *P. infestans* zoospores (equal or higher than 2x10^5 per ml) is transferred to a Petri dish, aggregates of swimming zoospores were visible within 2 min as white spots (Fig. 5b). When zoospores of wildtype strain 88069 were exposed to different massetolide A concentrations, autoaggregation of the zoospores did not occur at 5 and 10 µg ml^{-1} (Fig. 5b). The *Pigpa1*-silenced mutant gs2 was impaired in autoaggregation, as expected (Latijnhouwers *et al.*, 2004), and addition of massetolide A did not influence this impairment (Fig. 5b). Zoospores of R2, the *Pipga1* gain-of-function mutant, showed the typical autoaggregation behaviour of its parental strain. The interference of massetolide A with autoaggregation of R2 zoospores, however, was only observed at a concentration of 10 µg ml^{-1}, but not at 5 µg ml^{-1} as was shown for parental strain 88069 (Fig. 5b). This two-fold difference in response between zoospores of R2 and its parental strain 88069 was confirmed in three independent experiments.

The interference of massetolide A with autoaggregation of zoospores was further investigated microscopically by determining the percentage of swimming and encysted zoospores. With increasing concentrations of massetolide A, the number of swimming and encysted zoospores of parental strain 88069 significantly decreased and increased, respectively (Fig. 5c). Especially at massetolide A

concentrations of 2.5, 5 and 10 µg ml^{-1}, there was a significant induction of zoospore encystment for parental strain 88069 (Fig. 5c). Also for its *Pigpa1*-silenced mutant gs2, similar effects of massetolide A on zoospore encystment were observed; however, at a massetolide A concentration of 2.5 µg ml^{-1} the percentage encysted zoospores was significantly less in mutant gs2 than in the parental strain (Fig. 5c). Massetolide A also induced encystment of zoospores of R2, the *Pipga1*gain-of-function mutant, but to a lesser extent than for zoospores of the parental strain; at a massetolide A concentration of 5 µg ml^{-1}, 35-40 % of the R2 zoospores were swimming compared to only 5-10% of the zoospores of the parental strain (Fig. 5c). This relatively higher number of swimming R2 zoospores may explain why autoaggregation still occurred at a massetolide A concentration of 5 µg ml^{-1} for mutant R2 and not for parental strain 88069 (Fig. 5b).

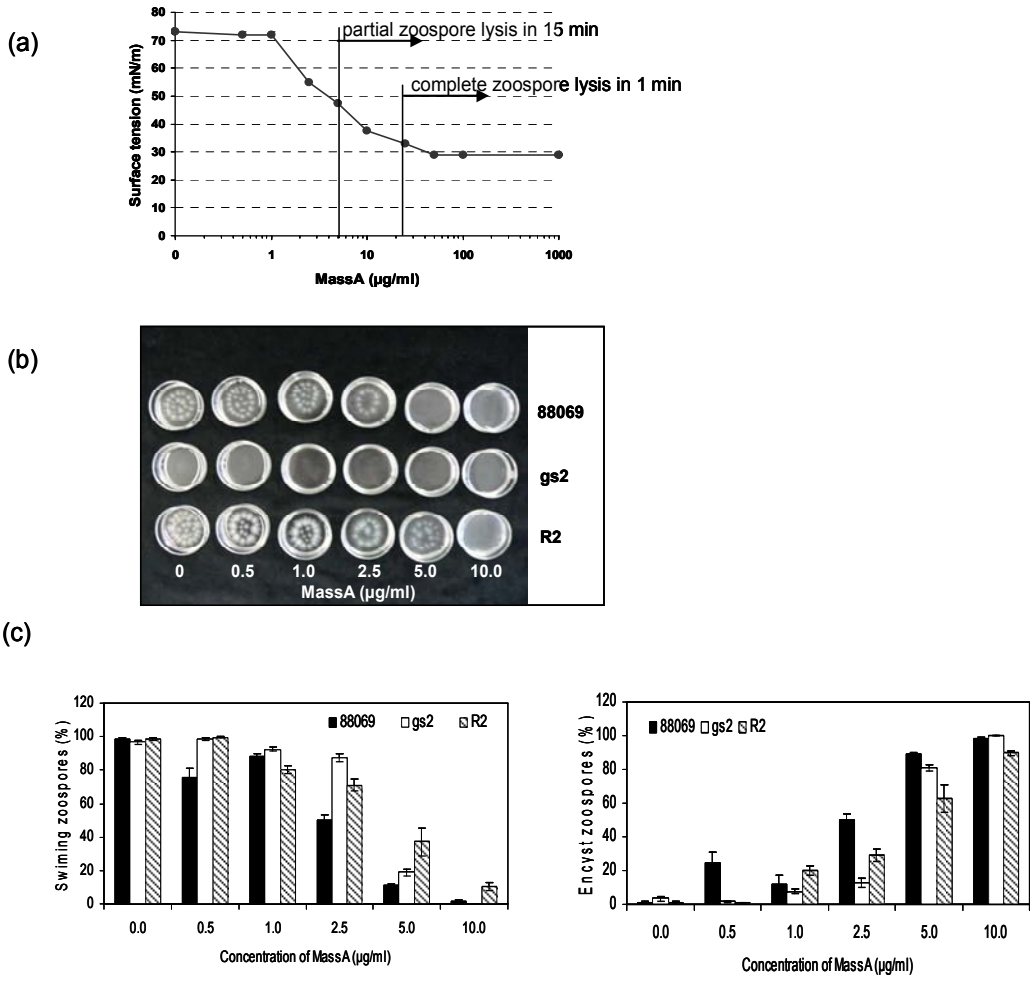

Figure 5:
(a) Dose-response relationship between massetolide A (MassA), surface tension (mN/m) and zoospore lysis. Zoospores of *P. infestans* strain 88069 were exposed to different concentrations of massetolide A for 2 to 15 minutes, during which lysis was determined microscopically. Mean values of 2 replicates are shown. Error bars represent the standard error of the mean.
(b) Effect of massetolide A on autoaggregation of zoospores of *P. infestans* strain 88069, *Pipga1*-mutant gs2, and *Pipga*1-gain-of-function mutant R2. White aggregates of zoospores are visible within 2-3 min.
(c) Effect of massetolide A on the ratio of swimming and encysted zoospores of *P. infestans* strain 88069, *Pipga1*-mutant gs2, and *Pipga*1-gain-of-function mutant R2.

Sublethal concentrations of massetolide A did not affect chemotaxis of zoospores toward the attractant glutamic acid: both wildtype strain 88069 and its *Pipga1* gain-of-function mutant R2 showed chemotaxis toward glutamic acid, whereas zoospores of the *Pigpa1*-silenced mutant gs2 did not show this response (Table 1).

Table 1: Effect of massetolide A on chemotaxis of zoospores of *Phytophthora infestans* strain 88069 toward water agar plugs supplemented or not with the chemoattractant glutamic acid. The role of G-protein signalling in chemotaxis was studied by including mutants gs2 and R2. Mutant gs2 is mutated in *Pigpa1*, the gene encoding the G-protein α subunit; R2 is a *Pipga1* gain-of-function mutant exhibiting constitutive expression of *Pigpa1* (Latijnhouwers et al., 2004).

Strain/mutant	Taxis in absence of massetolide A		Taxis in presence of massetolide A [(a)]	
	Without glutamic acid [(b)]	With glutamic acid	Without glutamic acid	With glutamic acid
88069	-	+	-	+
	-	+	-	+
gs2	-	-	-	-
	-	-	-	-
R2	-	+	-	+
	-	+	-	+

[(a)]: concentration of massetolide A was 2.5 µg ml^{-1}
[(b)]: concentration of glutamic acid was 50 mM
+: chemotaxis toward agar plug
-: no chemotaxis

Effect of osmotic protectants on massetolide A-induced zoospore lysis

Several cyclic lipopeptide surfactants, including tolaasin, syringomycin and WLIP, partition into membranes and form pores that lead to cell lysis (Rainey *et al.*, 1991; Hutchison *et al.*, 1995; Lo Cantore *et al.*, 2006). Similar to the experiments performed with erythrocytes in these previous studies, osmotic protection assays were conducted in this study with *P. infestans* zoospores to determine whether massetolide A-induced lysis can be prevented by compounds with a specific molecular size. The results showed that none of the osmoprotectants, except polyethylene glycol (P and Q), were able to prevent lysis of zoospores by massetolide A (Fig. 6). Although these observations may suggest that massetolide A forms pores in zoospore membranes of approximately 0.6 - 1.0 nm, subsequent microscopic analysis revealed that the osmoprotectants P and Q induced encystment of *P. infestans* zoospores rendering them insensitive to lysis by massetolide A. Therefore, these assays provided no conclusive indications on the potential ability of massetolide A to form pores in zoospore membranes.

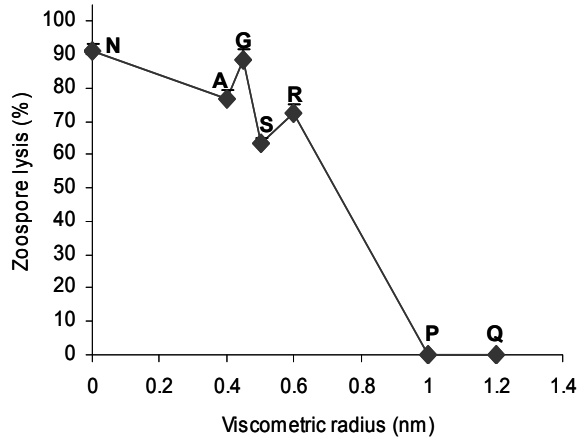

Figure 6: Effect of osmotic protectants on massetolide A-induced lysis of zoospores of *P. infestans*. Massetolide A was used at a final concentration of 25 µg/ml. The following osmotic protectants, representing different viscometric radii, were tested: arabinose (A), glucose (G), sucrose (S), raffinose (R), polyethylene glycol (MW 1,000; P) and polyethylene glycol (MW 1,500; Q). N is a negative control containing only massetolide A. Data are the mean of 3 replicates. Error bars represent the standard error of the mean.

Discussion

Most studies focusing on sensitivity of plant pathogenic fungi and oomycetes to antibiotic metabolites produced by antagonistic microorganisms take into account only one particular stage in the life cycle of the pathogen, usually hyphal growth (Duffy *et al.*, 2003). Life cycles of pathogens, however, are often more complex and may comprise numerous infectious propagules. Oomycete pathogens, including *Pythium* and *Phytophthora* species, can infect plant tissue by means of mycelium, sporangia, zoospores and oospores. Understanding the variation in sensitivity of different infectious propagules to a specific biocontrol trait will give more insight into the potential efficacy (or limitation) of biocontrol agents harboring that trait. In this study, we investigated the response of two *P. infestans* strains to the cyclic lipopeptide massetolide A produced by antagonistic *Pseudomonas fluorescens* SS101. The results showed that zoospore cysts are the most tolerant to massetolide A followed by mycelium and swimming zoospores. Combined with the zoosporicidal activities of massetolide A, the reduction in sporangia formation may have a significant impact on the onset and epidemic progress of late blight disease.

Microscopic analysis further revealed that massetolide A caused increased branching of hyphae of *P. infestans* and induced encystment of zoospores at concentrations below the critical micellar concentration (CMC). Induction of zoospore encystment and increased branching were also described by Thrane et al. (1999, 2000) for *Pythium ultimum* exposed to the CLP viscosinamide. Other typical cellular responses of *P. ultimum* exposed to viscosinamide included increased hyphal septation and swelling, altered mitochondrial activity and structure, decreased hydrophobicity of cell walls and membranes, decreased intracellular esterase activity and decreased intracellular pH. Thrane et al. (1999), who found similar responses in the fungus *Rhizoctonia solani,* hypothesized that the increased branching may have been the result of an increased influx of Ca^{2+} and H^{+} due to ion channel formation by viscosinamide. An increase in the cytosolic Ca^{2+} concentration was also proposed as an explanation for increased branching in *Neurospora crassa* exposed to the ionophore A23187 (Reissig & Kinney, 1983). In the present study though, no conclusive evidence was obtained for ion channel formation in zoospores by massetolide A. The experiment was hampered by the observation that some of the osmoprotectants (polyethylene glycol), induced zoospore encystment thereby rendering the zoospores insensitive to lysis by massetolide A. However, given that massetolide A is structurally highly similar to the pore-forming WLIP, a CLP produced by *Pseudomonas reactans* (Lo Cantore *et al.*, 2006; Raaijmakers *et al.*, 2006), we expect that also massetolide A forms transmembrane pores with a radius of approximately 1.5-1.7 nm as was shown for WLIP (Lo Cantore *et al.*, 2006). Assays with erythrocytes or artificial membranes could provide evidence for the potential of massetolide A to form ion channels in membranes. Alternatively, fluorescence microscopy with specific stains (e.g. ethidium bromide) could be used to monitor the ability

of massetolide A to cause structural disorders or to form pores in membranes of zoospores or hyphae of *P. infestans*.

In addition to the zoosporicidal activity, massetolide A was shown to adversely affect autoaggregation of swimming zoospores at relatively low concentrations. Aggregation of zoospores of oomycetes occurs rapidly and leads to accumulation of large numbers of zoospores (Ko & Chase, 1973; Porter & Shaw, 1978; Reid *et al.*, 1995). Autoaggregation appears to be a spontaneous event requiring no apparent exogenous stimulus. This intriguing phenomenon has been reported for *Phytophthora palmivora* and *Pythium* species (Reid *et al.*, 1995), *Phytophthora drechsleri* (Porter & Shaw, 1978), *Phytophthora palmivora* (Ko & Chase, 1973) and *Achlya* species (Thomas & Peterson, 1990). Aggregation has been proposed to enhance the inoculum potential at the infection site and to provide protection against other harmful microorganisms or metabolites thereby enhancing the success of host penetration and infection. Carlile (1983) demonstrated that zoospores do not utilize external nutrients until germination but internal energy reserves for motility until a suitable host is encountered. Reid et al. (1995) then suggested that autoaggregation is a survival mechanism when internal energy reserves become low, permitting the regermination of some zoospores in the population by remobilization of resources of other zoospores in the aggregate. Thomas and Peterson (1990) showed that autoaggregation of *Achlya* zoospores occurred only in the absence of exogenous chemotactic signals; they also noted that, in contrast to single spores, spores within aggregates showed germ tubes with a larger diameter and some developed both zoosporangia and oospores. The results of this study show that massetolide A interferes with autoaggregation of zoospores and may therefore have an effect on survival or plant colonization by *P. infestans* and other oomycete pathogens. How massetolide A interferes with autoaggregation is still unclear. The observation that 2-fold higher concentrations of massetolide A are required to prevent autoaggregation of zoospores from *P. infestans* R2, a mutant that constitutively produces an active form of the α subunit of the heterotrimeric G-protein, suggests that G-protein signalling plays a role in the response of zoospores to massetolide A. Also for mammalian cells it has been reported that CLPs can interfere with canonical signalling pathways by inhibiting the activity of a G-protein $\alpha_{q/11}$ subunit (Takasaki *et al.*, 2004). Given that G proteins are key regulators of ion channels in animal (Wickman & Clapham, 1995) and plant cells (Wang *et al.*, 2001), in particular K^+ and Ca^{2+} channels, we postulate that constitutive expression of the Gα protein interferes, to some extent, with ion channel formation by massetolide A thereby reducing the influx of Ca^{2+} and/or K^+, two ions that have been shown to induce encystment of zoospores (Deacon & Donaldson, 1993; Warburton & Deacon, 1998; Connolly *et al.*, 1999; Appiah *et al.*, 2005) and to affect autoaggregation (Reid *et al.*, 1995).

Materials and methods

Microorganisms and growth conditions:

Pseudomonas fluorescens strain SS101 was isolated from the rhizosphere of wheat grown in a soil suppressive to take-all disease (de Souza *et al.*, 2003). Biochemical analysis revealed that SS101 produces at least five cyclic lipopeptide surfactants. Massetolide A is the main cyclic lipopeptide produced by SS101 (de Souza *et al.*, 2003). The other four are derivatives of massetolide A differing in amino acid composition of the peptide ring (I. de Bruijn and J.M. Raaijmakers, unpublished data). Strain SS101 was grown on Pseudomonas Agar (PSA) plates (Difco, France) at 25 ^{o}C for 48 h, unless otherwise indicated. *Phytophthora infestans* strains 88069 (A1 mating type, race 1.3.4.7) and 90128 (A2 mating type, race 1.3.4.6.7.8.10.11) are Dutch field isolates obtained in 1988 and 1990 from infected tomato and potato plants, respectively. R2 and gs2 are transformed derivatives of strain 88069 that carry extra copies of a mutated form (R177H) of the G-protein α subunit gene *Pigpa1* (Latijnhouwers *et al.*, 2004). R2 is a gain-of-function mutant in which the transgene is expressed constitutively thus leading to the production of an active form of PiGPA1. In gs2 the extra copies caused silencing of *Pigpa1* resulting in a strain that lacks the G-protein α subunit (Latijnhouwers *et al.*, 2004). *P. infestans* wild type strains and transformants were grown on rye sucrose agar for 7-9 days in the dark at 18 ^{o}C (Latijnhouwers *et al.*, 2004). To obtain zoospores, full-grown plates (9-cm-diameter) were flooded with 20 ml of sterile distilled water and hyphae were fully submerged with a sterile glass spreader. Flooded plates were placed in the cold (4 ^{o}C) for 1-2 h after which the supernatant was gently filtered over a 22-µm-mesh nylon membrane to remove sporangia. Zoospore density was determined microscopically at 100X magnification and adjusted to a final concentration of 10^5 swimming zoospores ml^{-1}. Encysted zoospores were obtained by vigorously vortexing the zoospore suspensions for 1 min; encystment was checked microscopically at 100X magnification.

Extraction and purification of the cyclic lipopeptide surfactant massetolide A

The cyclic lipopeptide surfactant massetolide A (molecular mass 1,139 Da) was extracted as described previously (de Souza *et al.*, 2003). In summary, *P. fluorescens* SS101 was grown on PSA agar plates for 48-72 h at 25 ^{o}C. Bacterial lawns were suspended in sterile demineralised water and cells were pelleted by centrifugation at 6000 rpm for 20 minutes. The cell-free culture supernatant was collected, acidified with HCl to pH 2.0 and incubated for 1 h on ice to precipitate massetolide A. The precipitate was obtained by centrifugation (6000 rpm, 30 min) and washed twice with acidified (pH 2.0) demineralised water. The precipitate was dissolved in sterile demineralised water by adjusting the pH to 8.0 with 0.5 M NaOH. Finally, the precipitate was lyophilized and stored at -20 ^{o}C. RP-HPLC analysis was performed as described by Souza et al. (2003) and De Bruijn et al. (2007) and showed that Massetolide A makes up 65-70% of the partially purified extract based on peak areas at 206 nm; the other 30-35% consists of massetolide A derivatives (Tran et al. 2007; I de Bruijn et al., unpublished data). To obtain pure massetolide A, the partially purified extract was fractionated by RP-HPLC. The eluent was removed with a rotary evaporator (Büchi) *in vacuo* and the identity and purity of massetolide A was confirmed by LC-MS and NMR analyses as described previously (de Souza *et al.*, 2003; de ruijn *et al.*, 2007). Purified massetolide A was used in all experiments, except in the assays to determine the effect on mycelial growth and sporangia formation; in these assays that required high amounts of massetolide A, we used the partially purified (65-70%) massetolide A.

Effect of massetolide A on mycelial growth, biomass and sporangia formation

The effect of massetolide A on mycelial growth of *P. infestans* was studied on clarified rye-sucrose agar (CRSA, pH 7.0) or on 1/5th-strength potato dextrose agar (PDA, pH 7.0). Sterilised growth media were cooled down to 55 ^{o}C and amended with massetolide A to final concentrations of 0, 25, 50, 100, 250 and 500 µg ml^{-1}; each plate contained 20 ml of growth medium. A plug (0.8 cm^2) of *P. infestans* mycelium, excised from full-grown CRSA plates, was placed in the centre of the massetolide A amended growth media and incubated in the dark at 18 ^{o}C. Radial mycelium growth was measured with an electronic ruler after 4, 7, 9 and 11 days. For each treatment, five replicates were used and the assays were performed twice. The effect of massetolide A on the morphology of the hyphae of *P. infestans* was determined microscopically at the different time points with

an inverted microscope (Zeiss, Axiovert10). Effects of massetolide A on biomass of *P. infestans* was studied in clarified rye-sucrose (CRS) liquid medium; final concentrations of massetolide A were 0, 5, 10, 25 50 100 and 250 µg ml^{-1}. A mycelial plug (0.8 cm^2) was transferred to 5-cm-diameter Petri dishes containing 5 ml of the growth medium. After incubating the still cultures at 18 ^oC for 11 days, mycelium was collected by centrifugation and blotted dry on a Whatmann filter prior to determining the fresh weight; after drying at 65 ^oC for 12 h, mycelium dry weight was determined; obtained weights were corrected for the weight (fresh and dry) of the agar plugs used to inoculate *P. infestans*.

To evaluate the effect of massetolide A on sporangia formation, four mycelial plugs (0.8 cm^2 each) were taken from the edge of 11-day-old cultures of *P. infestans* grown on CRSA or 1/5th-strength PDA containing different concentration of massetolide A (same experiment as described above). Each mycelial plug was transferred to a sterile 1.5 ml eppendorf tubes containing 1 ml of isotone II electrolytic buffer (Coulter Electronic, Inc.) and vortexed vigorously for 2 min to release the sporangia. The numbers of sporangia were determined microscopically in 3-µl-droplets at a 100X magnification. Also in liquid medium, the effect of massetolide A on sporangia formation was determined. After 11 days of incubation, the cultures were transferred to 50-ml tubes. Mycelium was harvested by centrifugation and resuspended in 5 ml of isotone II electrolytic buffer. The mycelial suspension was cooled on ice and homogenized by a Polytron mixer (Kinematica, Gmbh Luzern, Switzerland) for 2 min with the speed set at 3. The density of the sporangia was determined microscopically as described above. Combined with the fresh and dry weights of the harvested mycelium, sporangia formation per mg fresh and dry weight was calculated.

Effect of massetolide A on germination and germ tube length of cysts of *P. infestans*

Encysted zoospores were exposed to massetolide A concentrations of 0, 25, 50, 100, 250 and 500 µg ml^{-1} of sterile demineralised water. The experiment was performed in 96-well plates (Greiner). One hundred µl of a suspension of encysted zoospores was transferred to each well and massetolide A was added to obtain the final concentrations mentioned above. Plates were incubated at 25 ^oC and samples (20 µl) were taken after 60, 90 and 120 min and kept on ice prior to microscopic analysis. The percentage germinated cysts was determined in 3-µl-droplets at 100X magnification. Germ tube length was determined at 180 min after incubation at 25 ^oC. Therefore, five 3-µl-droplets were taken and for 10-15 randomly selected zoospore cysts the germ tube length was measured at 100X magnification with an ocular micrometer.

Effect of massetolide A on chemotaxis, autoaggregation and encystment of swimming zoospores

The effect of massetolide A on chemotaxis of zoospores was studied as described by Latijnhouwers et al. (2004). However, instead of glass capillaries filled with water agar with and without the chemo-attractant we used two small agar plugs (0.78 cm^2), one consisting of 1% (w/v) water agar and the other of 1% water agar supplemented with glutamic acid (50 mM). The plugs were transferred to the bottom of a 3-cm-diameter Petri dish and subsequently submersed with 5 ml of a suspension containing 10^5 swimming zoospores per ml. Massetolide A was added to the zoospore suspension to obtain final concentrations of 0, 0.5, 1.0, 2.5, 5.0 and 10.0 µg ml^{-1}. Movement toward and accumulation of the zoospores on the water agar plugs was scored microscopically at 50X magnification over a time course of 5-10 min. Autoaggregation assays were performed in 3-cm-diameter Petri dishes as decribed by Latijnhouwers et al. (2004). The massetolide A concentrations used in these assays were the same as those used in the chemotaxis assays. Five to ten minutes after incubation at room temperature, zoospore autoaggregation was visually observed and recorded with a digital camera. For each of the zoospore suspensions exposed to different concentrations of massetolide A, the percentage of swimming and encysted zoospores was determined microscopically after 30 minutes of incubation at room temperature (~ 20 ^oC). In all of these assays, zoospores of *P. infestans* strain 88069 and its transformants gs2 and R2 were included.

Effects of osmotic protectants on massetolide A-induced zoospore lysis

To examine if pore-formation in zoospore membranes by massetolide A is the mode of action by which zoospore lysis occurs, experiments were conducted to determine if lysis can be blocked by osmoprotectants of specific molecular size. The osmotic protectants used were arabinose (A), glucose (G), sucrose (S), raffinose (R), polyethylene 1000 (P) and polyethylene 1500 (Q), representing viscometric radii of 0.4 (A), 0.45 (G), 0.54 (S), 0.66 (R), 1.0 (P) and 1.2 nm (Q), respectively. The

method used in these experiments was a modification of Knowles and Ellar (1987) and Weiner et al. (1985). One hundred µl of a suspension of swimming zoospores (10^5 ml^{-1}) was mixed with 100 µl 10 mM Tris-HCL (pH 8.0) supplemented with osmoprotectants at a concentration of 100 mM. Then, this mixture was divided in two equal volumes and transferred to sterile 1.5-ml centrifuge tubes; massetolide A was added to the first tube to a final concentration of 25 µg ml^{-1}; sterile demineralised water was added to the second tube and served as a control to determine possible direct effects of the osmoprotectants on zoospores. Lysis of zoospores was determined microscopically. For each osmoprotectant, four replicates were used. The assay was performed twice.

Statistical analysis

After certifying normal distribution and homogeneity of variances, data were analysed by ANOVA followed by Tukey's studentized range test (SAS Institute, Inc., Cary, NC). All the assays described in this study were performed at least twice and representative data are shown. In all assays, treatments were replicated 3-4 times. Percentages of swimming and encysted zoospores, cyst germination and zoospore lysis were arcsin-transformed prior to statistical analysis.

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Chapter 3

Role of the cyclic lipopeptide massetolide A in biological control of *Phytophthora infestans* and in colonization of tomato plants by *Pseudomonas fluorescens*

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Role of the cyclic lipopeptide massetolide A in biological control of *Phytophthora infestans* and in colonization of tomato plants by *Pseudomonas fluorescens*

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Abstract

- *Pseudomonas* strains have shown promising results in biological control of late blight caused by *Phytophthora infestans*. However, the mechanism(s) and metabolites involved are in many cases poorly understood. Here we examined the role of the cyclic lipopeptide massetolide A of *Pseudomonas fluorescens* SS101 in biocontrol of tomato late blight.

- *P. fluorescens* SS101 was effective in preventing infection of tomato leaves by *P. infestans* and significantly reduced the expansion of existing late blight lesions. Massetolide A is an important component of the activity of *P. fluorescens* SS101, since the *massA*-mutant was significantly less effective in biocontrol, and purified massetolide A provided significant control of *P. infestans*, both locally and systemically via induced resistance.

- Assays with *nahG* transgenic plants indicated that the systemic resistance response induced by SS101 or massetolide A is independent of salicylic acid signalling. Strain SS101 colonized the roots of tomato seedlings significantly better than its *massA*-mutant, indicating that massetolide A is an important trait in plant colonization.

- This study shows that the cyclic lipopeptide surfactant massetolide A is a metabolite with versatile functions in the ecology of *P. fluorescens* SS101 and in interactions with tomato plants and the late blight pathogen *P. infestans*.

Keywords: *Pseudomonas*, *Phytophthora infestans*, biocontrol, surfactants, induced systemic resistance, colonization.

Introduction

Oomycetes form a diverse group of eukaryotic, fungus-like microorganisms containing a wide range of economically important pathogens of plants, insects, fish and animals (Kamoun, 2003). Among the plant pathogenic Oomycetes, *Phytophthora infestans* is the most notorious causing late blight of potato and tomato. In the disease cycle, zoospores are essential propagules in the pre-infection process and a potential target to control *P. infestans* and other Oomycete pathogens (Erwin & Robeiro, 1996; Donaldson & Deacon, 1993; van West *et al.*, 2002). Late blight is traditionally controlled by a combination of cultural practices and chemical applications. To biologically control late blight, several antagonistic microorganisms have been tested for their activity against *P. infestans*, including non-pathogenic *Phytophthora cryptogea* (Stromberg & Brishammar, 1991) and endophytic microorganisms like *Cellulomonas flavigena*, *Candida* sp., and *Cryptococcus* sp. (Lourenço Júnior *et al.*, 2006). Although some effective fungal antagonists were identified, bacterial antagonists have shown by far the most promising results to date. Bacteria with antagonistic activities against *P. infestans* are mainly found in the genera of *Pseudomonas* and *Bacillus* (Sanchez, 1998; Yan *et al.*, 2002; Daayf *et al.*, 2003; Kloepper *et al.*, 2004). In most of these studies, however, the mechanisms and metabolites involved in the biocontrol activity were not investigated in detail.

Several strains of *Pseudomonas fluorescens* were recently described that produce surface-active compounds, designated biosurfactants, with destructive effects on zoospores of *P. infestans* and other Oomycetes, including *Pythium* species (de Souza *et al.*, 2003). For *P. fluorescens* strain SS101, the biosurfactant was identified as massetolide A, a cyclic lipopeptide with a nine amino acid peptide ring linked to 3-hydroxydecanoic acid (de Souza *et al.*, 2003). Application of *P. fluorescens* SS101 to soil or bulbs effectively controls Pythium root rot of flowerbulb crops in both bioassays and small-scale field experiments (de Boer *et al.*, 2006). The role of massetolide A in the biocontrol activity of *P. fluorescens* SS101 against Oomycete pathogens, however, has not been resolved to date.

The present study aims at a comprehensive investigation of the potential of *P. fluorescens* SS101 to control late blight of tomato. The ability of strain SS101 to prevent infection and to control the development of existing infections of *P. infestans* was investigated in plant assays. The role of massetolide A in biocontrol of *P. infestans* was studied by comparing the activity of strain SS101 with that of its massetolide A-deficient mutant and purified massetolide A. The role of systemic resistance in tomato plants induced by strain SS101 or massetolide A was determined by physically separating the inducing agents from the late blight pathogen. Transgenic *nahG* tomato plants, which are unable to accumulate salicylic acid, were included to assess whether salicylic acid acts as a signal in the induced systemic resistance response. Finally, the role of massetolide A in plant colonization by *P. fluorescens* SS101 was investigated by comparing the population dynamics of wild-type strain SS101 on the surface and in the interior of tomato plants with that of its massetolide A-deficient mutant.

Results

Preventing late blight infections of tomato leaves by *P. fluorescens* SS101

Application of cell suspensions of *P. fluorescens* SS101 to leaves of tomato plants one day prior to inoculation with zoospores of *P. infestans* substantially reduced disease incidence (Fig 1a). Also *massA*-mutant 10.24 reduced disease incidence significantly but to a lesser extent than wild-type strain SS101. The area of the few lesions observed on leaves treated with strain SS101 was significantly smaller than that of the late blight lesions in the control treatment (Fig. 1b). The effect of mutant 10.24 on lesion area was intermediate. In the control treatment, disease severity (lesion area) increased exponentially over a period of nine days after zoospore inoculation, whereas lesion area remained very low for leaves treated with strain SS101 (Fig. 1c). For mutant 10.24, disease progress was intermediate between the control and the treatment with strain SS101. The population densities of wild-type strain SS101 and mutant 10.24 on treated leaves at ten days after zoospore inoculation were 8.3 and 8.4 Log CFU/g leaf, respectively.

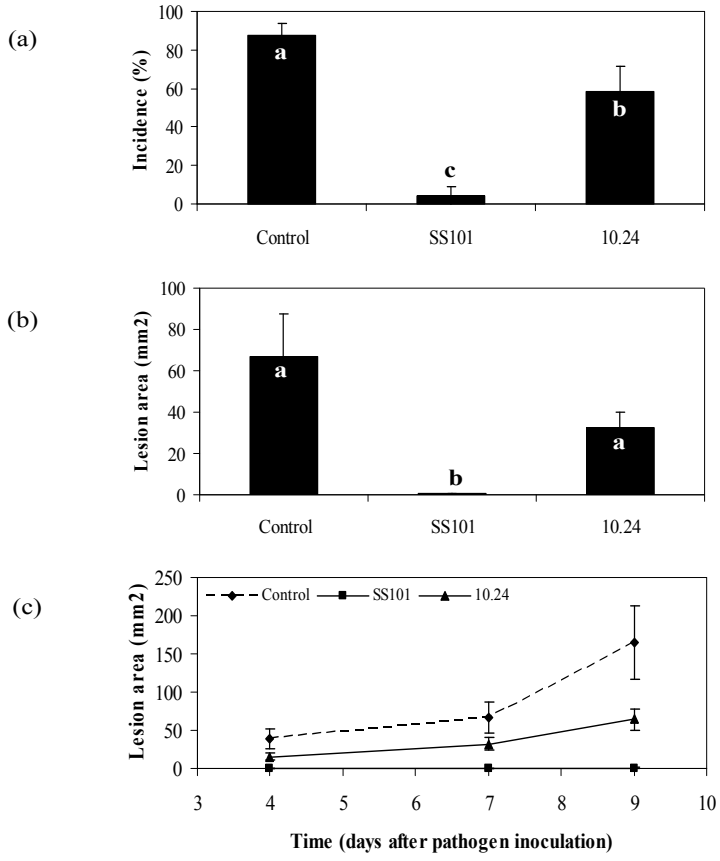

Figure 1: Direct effect of *P. fluorescens* SS101 and its massetolide A-deficient mutant 10.24 on tomato late blight. (a) disease incidence (%) refers to the percentage of zoospore droplets applied to tomato leaves that lead to infection; (b) lesion area (mm^2) at 7 days after inoculation with zoospores of *Phytophthora infestans*; (c) disease severity (lesion area) at different time points after pathogen inoculation. Means of 6 replicates are given and error bars represent the standard error of the mean. Means with a different letter are significantly different ($p<0.05$).

Effect of massetolide A on late blight infections of tomato leaves

To further investigate the role of massetolide A in preventing late blight disease of tomato, massetolide A was purified from cell-free culture supernatant of strain SS101. RP-HPLC analysis revealed that, based on peak area (206nm), massetolide A makes up on average 70% (ranging from 65 to 74%) of the purified extract from strain SS101 (Figure 2a1). The other 30% of the extract is composed, for most part (>95%), of four additional cyclic lipopeptides (retention times 14-20 min, Fig. 2a1), three of which were identified by LC-MS and NMR as derivatives of massetolide A (I. de Bruijn, M.J.D. de Kock and J.M. Raaijmakers, unpublished results). The derivatives of massetolide A are most likely the result of the flexibility of the adenylation domains in amino acid selection and activation (Stachelhaus *et al.*, 1999). Massetolide A and its derivatives were absent in cell-free culture supernatant of *massA*-mutant 10.24 (Fig. 2a2).

The results of subsequent bioassays showed that also partially purified massetolide A significantly reduces disease incidence when applied to tomato leaves at a concentration of 100 μg ml^{-1}; no significant effects on disease incidence were observed at concentrations of 50 μg ml^{-1} (Fig. 2b1). Application of cell suspensions of strain SS101 was significantly more effective than application of massetolide A alone (Fig. 2b1), suggesting that in addition to massetolide A other bacterial traits are involved in late blight control by strain SS101. In the control treatment, lesion area increased exponentially over a period of nine days after zoospore inoculation, whereas disease severity remained low for leaves treated with strain SS101 (Fig. 2b2); for leaves treated with different concentrations of massetolide A, disease progress and lesion areas at nine days after pathogen inoculation (dpi) were intermediate between the control treatment and the SS101 treatment (Fig. 2b2). To provide further evidence for the role of massetolide A in control of late blight of tomato, massetolide A was fractionated to purity and the structure was confirmed by LC-MS and NMR analyses (data not shown). The results of bioassays showed that also pure massetolide A significantly reduces disease incidence and lesion area when applied as a solution with a concentration of 100 μg ml^{-1} (equals 88 μM) (Fig. 2c). No phytotoxic effects on the tomato leaves were observed after application of strain SS101, mutant 10.24, or massetolide A at the concentrations used.

HPLC-analysis of leaf extracts treated with a relatively high and easily detectable concentration (350 μM) of massetolide A revealed that on average 37% (± 7.8, N=3) of the massetolide A is deposited on the tomato leaves directly after treatment. Based on the assumption that a similar fraction of massetolide A is deposited when leaves are treated with a solution of 44 μM (50 μg ml^{-1}) or 88 μM (100 μg ml^{-1}), the effective concentration of massetolide A on the leaves is 16 and 32 μM, respectively. Given that massetolide A has zoosporicidal activity at concentrations of 22 μM or higher may explain, at least in part, the difference in biocontrol efficacy between the two concentrations of massetolide A used in the experiments (Fig. 2c). RP-HPLC analysis of extracts of tomato leaves harvested at one and five days after treatment did not allow *in situ* detection and quantification of massetolide A due to interference of leaf-derived compounds that have similar retention times as massetolide A. Also from leaves treated

with cell suspensions of SS101, massetolide A could not be detected and quantified reliably due to background signals of compounds released from the tomato leaves during the extraction.

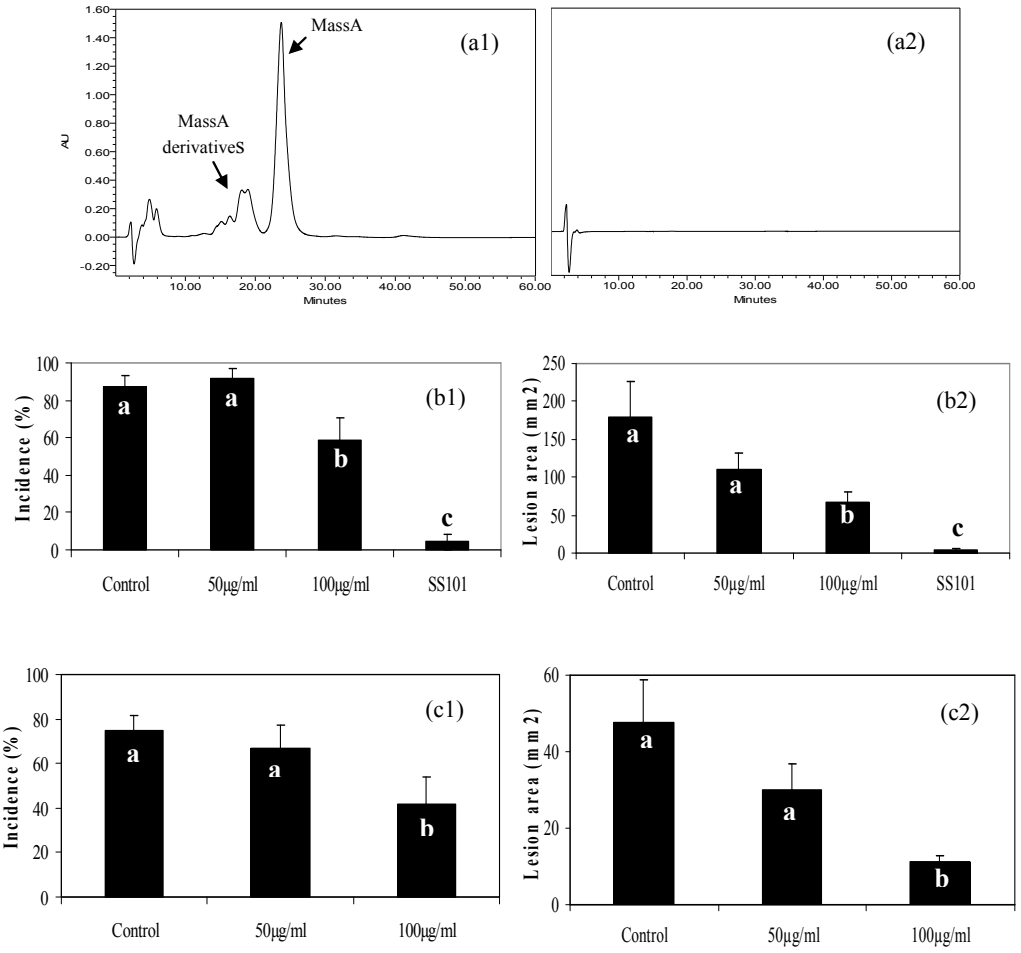

Figure 2: (a1) RP-HPLC chromatogram (206 nm) of the surfactant extract obtained from cell-free culture supernatants of *P. fluorescens* SS101. The predominant peak with a retention time of 24 min was identified by LC-MS and NMR as massetolide A (MassA). The peaks with retention times ranging from 14–20 min represent at least 4 other cyclic lipopeptide surfactants, three of which were identified by LC-MS and NMR as derivatives of MassA. (a2) RP-HPLC chromatogram (206 nm) of the extract obtained from cell cultures of massetolide A-deficient mutant 10.24. Panels b and c: direct effect of partially purified massetolide A and of HPLC-purified massetolide A on tomato late blight. Tomato leaves were treated with strain SS101 or with different concentrations of partially purified massetolide A (panels b1 and b2) or HPLC-purified massetolide A (panels c1 and c2); one day later, leaves were inoculated with *P. infestans* zoospores. Panels b1 and c1 show the disease incidence, and panels b2 and c2 the disease severity (lesion area) at 9 days after pathogen inoculation. Means of 6 replicates are given and error bars represent the standard error of the mean. Means with different letters are significantly different ($p<0.05$).

Effect of *P. fluorescens* SS101 and massetolide A on expansion of existing late blight lesions

To investigate if strain SS101 or massetolide A can reduce the development of existing late blight infections, tomato leaves with primary lesions of *P. infestans* were treated with cell suspensions of strain SS101, mutant 10.24, or with different concentrations of partially purified massetolide A. The results show that lesion area increased only three-fold on leaves treated with strain SS101, whereas lesion area increased more than seven-fold in the control treatment (Fig. 3). On leaves treated with mutant 10.24, lesion area increased almost 5-fold (Fig. 3b). Also application of massetolide A to tomato leaves significantly reduced the growth of existing lesions in a concentration-dependent manner (Fig. 3b).

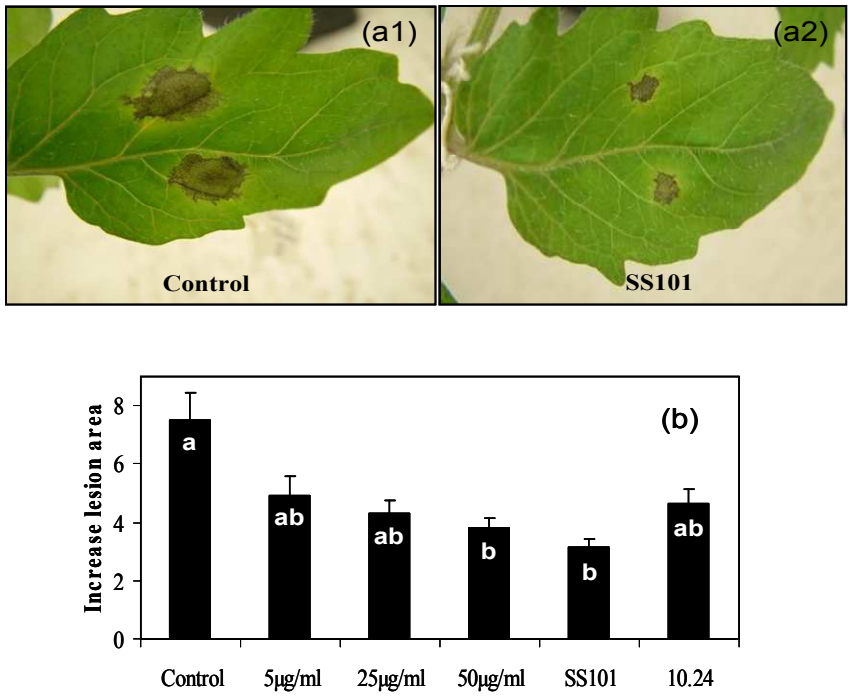

Figure 3: Effect of *P. fluorescens* SS101 on the growth of existing late blight lesions. Tomato leaves were first inoculated with *Phytophthora infestans* zoospores. After the initial late blight lesions were formed, lesion areas were measured and leaves were subsequently treated with cell suspensions of *P. fluorescens* SS101, its massetolide A-deficient mutant 10.24 or with different concentrations of partially purified massetolide A. Five days later, lesion sizes were determined again and the increase in lesion area was calculated. (a2) Typical effect of *P. fluorescens* SS101 on the growth of existing lesions compared to the non-treated control (a1); (b) effect of SS101, 10.24 and different concentrations of massetolide A on the increase in lesion size. The means of 6 replicates are shown and error bars represent the standard error of the mean. Means with a different letter are significantly different ($p<0.05$).

Induction of systemic resistance in tomato by *P. fluorescens* SS101 and massetolide A

To determine the role of induced resistance as a mechanism in late blight control by strain SS101 and massetolide A, two types of experiments were conducted to physically separate the inducing agents from the pathogen (Fig. S1). In one series of experiments, the inducing agents (bacterium or massetolide A) were applied to the lower leaf and the pathogen inoculated one day later on the upper leaves (Figs. 4, 5). In the second series of experiments, the inducing agents were applied to roots of tomato seedlings and two weeks later the tomato leaves were challenge-inoculated with *P. infestans* (Fig. S2). At the time disease severity was assessed, physical separation of strain SS101 and *P. infestans* was confirmed by dilution plating leaf suspensions onto agar media selective for the introduced bacterial strains. Both series of experiments showed that application of SS101 or massetolide A to leaves or roots significantly reduced lesion areas of tomato late blight, but did not reduce disease incidence (Fig. 4; Fig. S2). When applied to the lower leaf, mutant 10.24 was significantly less effective in reducing lesion area than wild-type SS101 or massetolide A (Fig. 4c). The results further showed that also in the *nahG* transgene, lesion area was significantly reduced upon treatment of lower leaves with SS101 or massetolide A to levels similar to that obtained in the wild-type progenitor tomato cultivar Moneymaker (Fig. 5). These results suggest that induction of resistance in tomato against *P. infestans* by *P. fluorescens* SS101 or by massetolide A is independent of salicylic acid signalling.

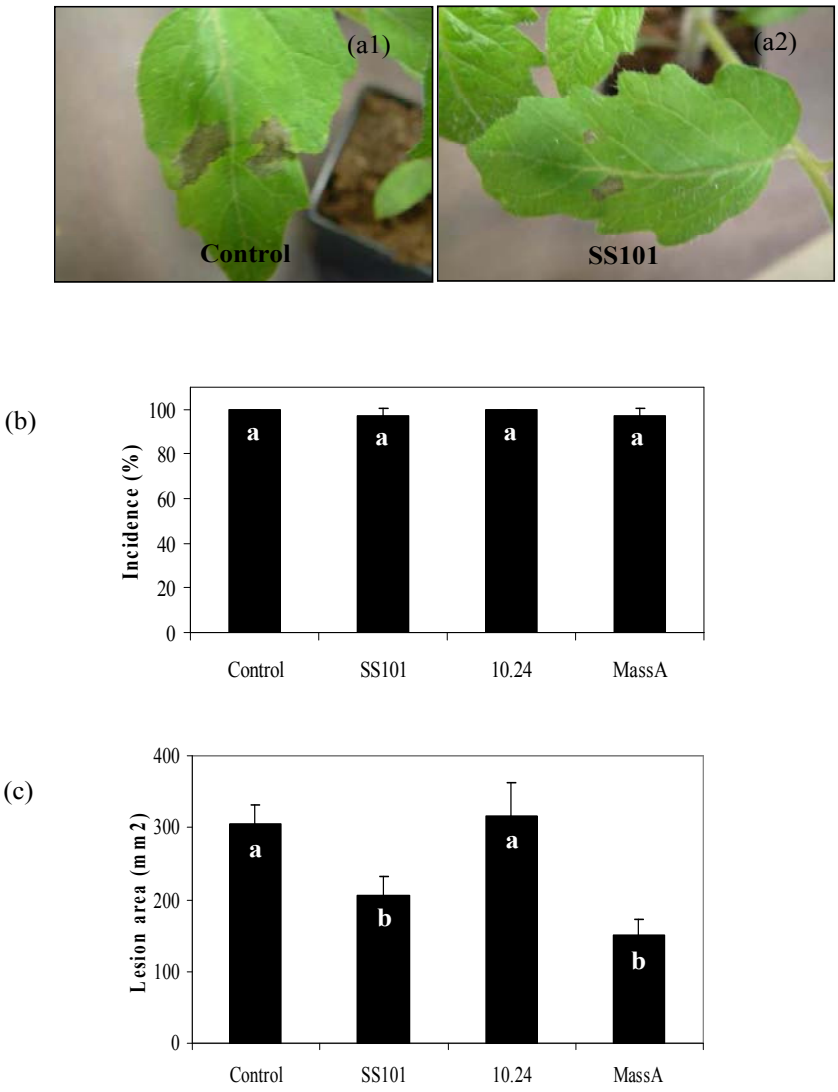

Figure 4: Induced resistance in tomato against late blight by application of *P. fluorescens* SS101 or massetolide A (MassA) to leaves. Twenty-four hours after treatment of the lower leaves of tomato plants with strain SS101, mutant 10.24, or MassA (50 µg ml^{-1} (44 µM)), upper leaves were challenged-inoculated with zoospores of *P. infestans*. (a) representative example of induced systemic resistance in tomato against late blight by strain SS101 applied to roots or leaves. At 7 days after pathogen inoculation, disease incidence (panel b) and lesion area (panel c) were determined. Means of 4 replicates are given. Error bars represent the standard error of the mean. Means with the same letter are not significantly different ($p<0.05$).

Role of massetolide A in plant colonization by *P. fluorescens* SS101

The role of massetolide A in colonization of tomato plants was investigated by comparing the distribution and population dynamics of wild-type strain SS101 on the surface and in the interior of different plant parts with that of its *massA*-mutant 10.24. Wild-type strain SS101 or mutant 10.24 were applied separately to tomato seeds to a final density of 6.5 Log CFU $seed^{-1}$ each. When treated tomato seeds were sown in non-sterile potting soil, both SS101 and 10.24 colonized the surfaces of roots, stems and cotyledons of 14-day-old seedlings (Fig. 6a1). On the surfaces of roots and cotyledons of tomato seedlings, strain SS101 established significantly higher densities than mutant 10.24 (Fig. 6a1). Neither

strain SS101 nor mutant 10.24 could be recovered from the interior tissue of roots and stem, but were present in the interior of cotelydons at a density of approximately 10^3-10^4 CFU g^{-1} (Fig. 6a2), which is approximately five to 20-fold lower than the density found on the surface of the cotyledons (Fig. 6a1). After 44 days of plant growth, strain SS101 and mutant 10.24 were still detectable on the surfaces of roots and cotyledons, although their densities on the root surface had declined by approximately 1000-fold compared to their densities on roots of 14-day-old tomato seedlings (Fig. 6a1, b1). SS101 maintained its density on the surface of cotelydons to a level that was significantly higher than that of mutant 10.24 (Fig. 6b1). Strain SS101 and mutant 10.24 were not detectable on the surfaces and in the interior of stem, and on and in true leaves infested with *P. infestans* (Fig. 6b1,2).

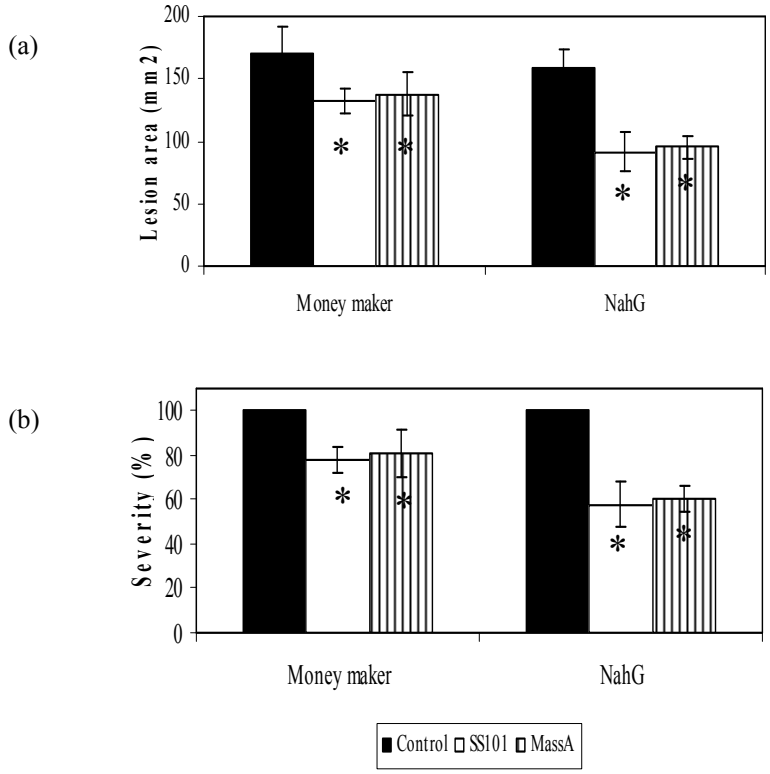

Figure 5: Induced resistance in tomato against late blight by application of *P. fluorescens* SS101 or massetolide A to leaves of cultivar Money Maker and its transgenic derivative *nahG*. Twenty-four hours after treatment of the lower leaves of the tomato plants with strain SS101 or massetolide A (MassA, 50 µg ml^{-1}), upper leaves were challenged-inoculated with zoospores of *P. infestans*. At 7 days after pathogen inoculation, lesion area (panel a) was determined. Panel b: the lesion areas in the SS101 and MassA treatments were expressed as a percentage of the lesion areas in the control treatments (set at 100%). Means of 6 replicates are given. An asterisk indicates a statistically significant reduction in disease severity ($p<0.05$) relative to the control. Error bars represent the standard error of the mean.

Effect of seed treatments on late blight incidence, lesion size, and sporangia formation

In the same experiment used to study plant colonization from treated seeds (Fig. 6), the effects of strain SS101 and mutant 10.24 on late blight incidence, lesion area and sporangia formation were determined (Fig. 7). True leaves of 35-day-old tomato plants raised from bacteria-treated seeds were inoculated with *P. infestans* zoospores and disease incidence and severity assessed nine days later. The results showed that treating tomato seeds with strain SS101 or mutant 10.24 did not reduce disease

incidence (Fig. 7a). Strain SS101 significantly reduced lesion area, whereas mutant 10.24 reduced lesion area to levels that were intermediate between the control and the SS101 treatments (Fig. 7b). Strain SS101 significantly reduced sporangia formation per unit of lesion area, whereas mutant 10.24 gave an intermediate reduction (Fig. 7c). Given that the applied bacterial strains SS101 and 10.24 could not be detected on the true leaves infested with zoospores of *P. infestans* (Fig. 6), these results indicate that the biocontrol effect of SS101 applied to tomato seeds is most likely mediated through systemic resistance elicited by the bacteria.

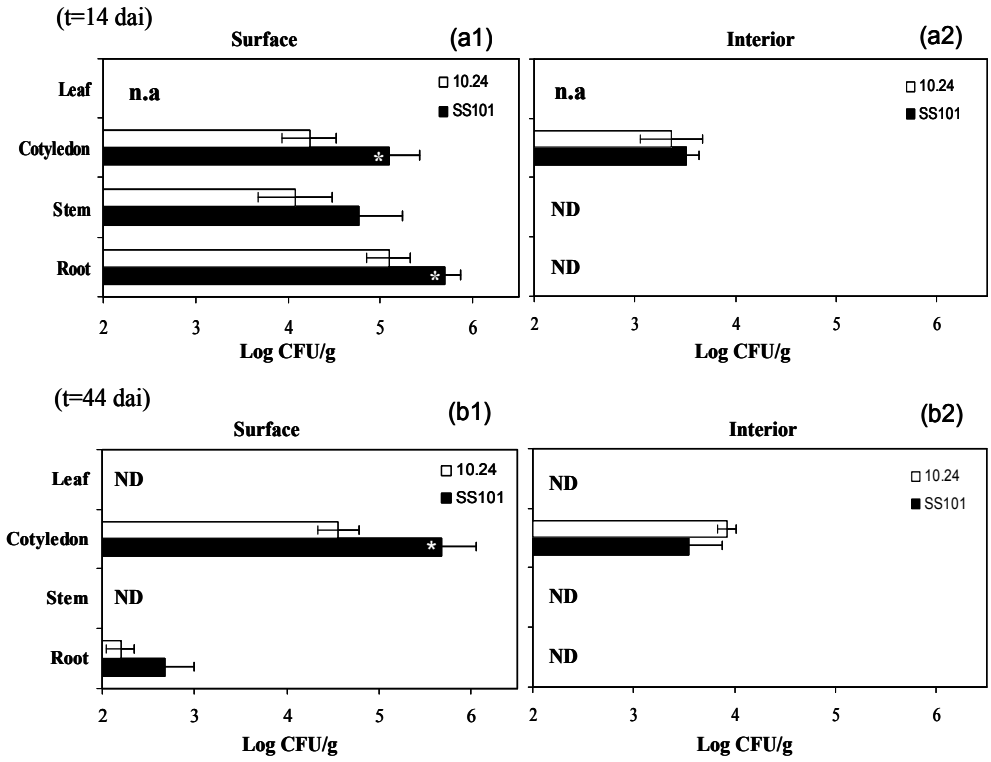

Figure 6: Colonization of tomato plants by *P. fluorescens* SS101 and its massetolide A-deficient mutant 10.24. Tomato seeds were treated with SS101 or 10.24 at a final density of 10^6 CFU seed^{-1} and sown in soil. After 14 (panels a1 and a2) and 44 days (panels b1 and b2) of plant growth, population densities of the applied bacterial strains were determined on the surface and in the interior of roots, stem, cotelydons and on true leaves infested with *P. infestans*. The means of 4 replicates are shown. For each pair of bars, an asterisk indicates a significant difference ($p<0.05$). n.a. not available; ND: not detectable (detection limit is Log 2.0 CFU/g).

Discussion

This study shows that *P. fluorescens* strain SS101 not only prevented infection of tomato leaves by *P. infestans*, but also significantly reduced expansion of existing late blight infections and sporangia formation. This is in contrast to results of previous studies on biocontrol of late blight, where the bacterial strains tested were effective mostly in preventing infections (Daayf *et al.*, 2003; Lourenço Júnior *et al.*, 2006). To date, biological control of plant diseases is mostly directed toward preventing infection of plants by pathogens and only few studies (Molina et al., 2003) have addressed the effects of biocontrol agents on plants already infected by pathogenic bacteria, fungi or Oomycetes. Given that

sporangia constitute an important primary and secondary inoculum source for *P. infestans*, the adverse effects of *P. fluorescens* strain SS101 on both lesion area and sporangia formation may lead to a reduction in disease development and epidemic progress of late blight of tomato.

The results also show that the cyclic lipopeptide surfactant massetolide A is an important component of the biocontrol activity of *P. fluorescens* SS101 against late blight of tomato. This conclusion is based on the observations that: (i) massetolide A-deficient mutant 10.24 was significantly less effective in biocontrol than wild-type strain SS101 (Figs 1, 3, 4, 7), and (ii) application of purified massetolide A to tomato leaves and roots provided significant control of *P. infestans* (Figs 2, 3, 4, 5, S2). Over the past decade, cyclic lipopeptides (CLPs) produced by *Pseudomonas* and *Bacillus* species have received considerable attention for their activity against a range of microorganisms, including, mycoplasmas, trypanosomes, bacteria, fungi, viruses, and Oomycetes (Nybroe & Sørensen, 2004; Raaijmakers *et al.*, 2006). In most of these studies, however, the antimicrobial effects of the CLPs were tested *in vitro* only and most biocontrol assays with plants did not include mutants deficient in CLP-biosynthesis. Work by Bais et al. (2004) was one of the first studies that included a mutant of *B. subtilis* strain 6051 defective in surfactin production and demonstrated that the wild-type strain was more effective in controlling root infection of Arabidopsis by *P. syringae* than the surfactin-deficient mutant. Another line of evidence that CLPs are important determinants of biocontrol activity was provided by Leclère et al. (2005), who showed that a derivative of *B. subtilis* strain BBG100 that overproduces the CLP mycosubtilin showed increased activity against *Pythium* on tomato seedlings. The present study further extends these findings and provides, for the first time, evidence that the CLP massetolide A is an important component of the biocontrol activity of *P. fluorescens* strain SS101.

In several of the experiments described in this study, extractions were performed on tomato leaves treated with cell suspensions of strain SS101 to determine the concentrations of massetolide A produced *in situ* by the applied bacterial strain. Nielsen and Sorensen (2003) showed that on sugar beet seeds, *P. fluorescens* strains produce massetolide A-like CLPs at concentrations ranging from 0.2 to 0.6 µg per seed. In the analyses performed in our study, however, relatively low concentrations of massetolide A could not be quantified accurately due to interference of plant-derived compounds. Therefore, it is not clear from our study if the concentrations of purified massetolide A applied to tomato leaves (Fig. 2) are representative of the massetolide A concentrations produced by strain SS101 *in situ*. To improve detection and quantification of massetolide A, antibody-based detection will be explored in future studies. Immunological detection has been succesfully adopted for *in situ* detection of syringopeptins: the competitive ELISA assay appeared to be approximately 100 times more sensitive than HPLC analysis and did not require extraction of plant material with organic solvents (Fogliano *et al.*, 1999). Antibodies will also be highly instrumental to study the localisation, fate and stability of the massetolide A applied to plant tissues or produced by *P. fluorescens* SS101 *in situ*.

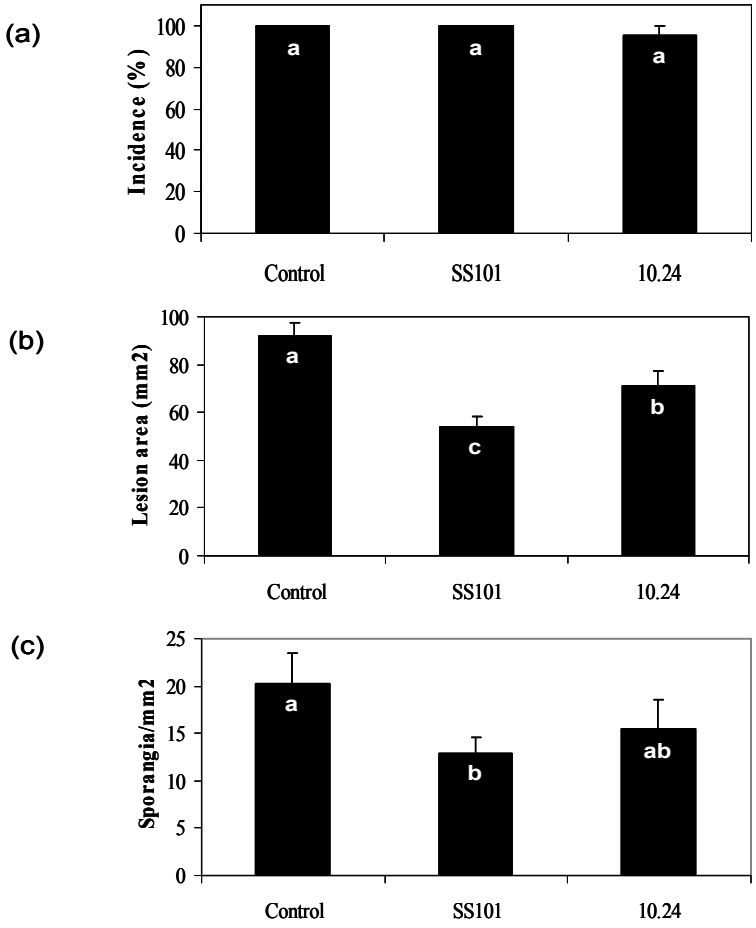

Figure 7: Effect of seed treatments on late blight infection of tomato and on sporangia production by *P. infestans*. Tomato seeds were treated with cell suspensions of *P. fluorescens* SS101 or its massetolide A -deficient mutant 10.24 and sown in potting soil. After five weeks of plant growth, tomato leaves were inoculated with zoospores of *P. infestans*. Disease incidence (panel a), lesion area (panel b) and the number of sporangia formed per unit of lesion area (panel c) were determined 9 days later. Means of 6 replicates are shown and error bars represent the standard error of the mean. Means with a different letter are significantly different ($p<0.05$).

Previous studies by De Souza et al. (2003) have shown that massetolide A disrupts zoospore membranes at concentrations of 25 μg ml^{-1} and higher, leading to lysis of entire zoospore populations within one minute of exposure. This zoosporicidal activity may explain, at least in part, the direct protection of tomato leaves against infection by zoospores of *P. infestans* (Figs. 1, 2), but does not explain the suppressive effects of SS101 or massetolide A on lesion growth and sporangia formation. Subsequent assays in which strain SS101 or massetolide A were physically separated from the pathogen (Figs. 4, 5, S2) demonstrated that induction of systemic resistance in tomato against late blight constitutes a main mode of the indirect activity against late blight. This was confirmed in assays with tomato plants raised from seeds treated with strain SS101 (Fig. 7). The observation that mutant 10.24 also reduced disease severity significantly but, in general, to a lesser extent than wild-type strain SS101, indicates that also bacterial determinants other than massetolide A play a role in induced systemic resistance in tomato by strain SS101.

Induced systemic resistance (ISR) is a common phenomenon among multiple strains of antagonistic bacteria representing various genera, including *Pseudomonas* and *Bacillus* (van Loon *et al.*, 1998; Kloepper *et al.*, 2004). Bacterial determinants shown to be involved in induction of resistance in plants by *Pseudomonas* and *Bacillus* strains include lipopolysaccharides (Leeman *et al.*, 1995), flagellin (Gomez-Gomez & Boller, 2002; Zipfel *et al.*, 2004; Meziane *et al.*, 2005), siderophores (Leeman *et al.*, 1996), salicylic acid (De Meyer & Höfte, 1997), pyocyanin (De Vleesschauwer *et al.*, 2006), an N-alkylated benzylamine derivative (Ongena *et al.*, 2005b), 2,4 diacetylphloroglucinol (Lavicoli *et al.*, 2003), the volatiles 2,3-butanediol and acetoin (Ryu *et al.*, 2004; Han *et al.*, 2006), and *N*-acylhomoserine lactones (Schuhegger *et al.*, 2006). Han *et al*. (2006) suggested that possibly multiple other bacterial determinants, different from those identified so far, are involved in the induction of systemic resistance. The results of our study show, for the first time, that the cyclic lipopeptide massetolide A is a bacterial determinant of induced resistance in tomato by a saprophytic *P. fluorescens* strain. Studies by Kováts et al. (1991), Yan et al. (2002) and Doke et al. (1987) suggested that the induced defense responses in tomato and potato against *P. infestans* operate in part by adverse effects on encystment or attachment of zoospores or sporangia to the plant surface. The results of our study, however, showed that the systemic protection induced by strain SS101 or massetolide A did not reduce initial infection of tomato leaves by zoospores of *P. infestans,* but limited growth of the pathogen in the leaves leading to smaller lesions and reduced sporangia formation (Fig. 7, S2). Whether structurally different CLPs produced by strain SS101 or other *Pseudomonas* species also induce resistance in plants against *P. infestans* or other pathogens is yet unknown and currently being investigated. Work by Ongena et al. (2005a) suggested that fengycins, CLPs produced by *Bacillus subtilis*, could be involved in eliciting induced resistance, whereas the structurally different CLP mycosubtilin most likely does not have resistance-inducing activities (Leclère *et al.*, 2005). It should be emphasized, however, that the capacity of specific bacterial determinants to induce resistance may be highly dependent on the host-pathogen system tested, as was demonstrated by Meziane et al. (2005) and De Vleesschauwer et al. (2006).

In many cases, signal transduction in rhizobacteria-mediated ISR has been shown to be independent of salicylic acid (SA), and dependent upon ethylene (ET) and jasmonic acid (JA) (Pieterse *et al.*, 1998; Ton *et al.*, 2001; Yan *et al.*, 2002). Our results with *nahG*, the transgenic derivative of cultivar MoneyMaker, suggest that also the systemic resistance induced in roots or leaves by *P. fluorescens* SS101 or massetolide A is independent of SA (Fig. 5). Bioassays with *Def-1*, a jasmonate-deficient mutant (Howe *et al.*, 1996), and with *Never Ripe*, a mutant deficient in ethylene signalling (Lanahan *et al.*, 1994), gave inconclusive results (data not shown). Future studies, involving Northern and microarray-based analyses, will be necessary for a more comprehensive identification of the signalling pathways, including ET and JA, involved in the resistance responses induced by cyclic lipopeptides.

Cyclic lipopeptide surfactants not only have zoosporicidal, antimicrobial and ISR-eliciting activities but have been postulated to play other important roles for the producing microorganisms, including

attachment and detachment to surfaces, biofilm formation, and colonization of plant tissue (Lindow & Brandl, 2003; Nybroe & Sørensen, 2004; Raaijmakers *et al.*, 2006). Several studies have shown that CLPs produced by *Pseudomonas* species are important in motility on soft agar media (Andersen *et al.*, 2003; Roongsawang *et al.*, 2003; de Bruijn *et al.*, 2007). The involvement of CLPs in bacterial motility may provide an advantage in colonization of plant tissue, in translocation from an inoculum source to new and more nutrient-rich niches on the plant surface, and in containment of plant pathogens (Andersen *et al.*, 2003). The results of our study showed that wild-type strain SS101, when applied to seeds, established significantly higher densities on roots and cotelydons of tomato seedlings than its CLP-deficient mutant 10.24 (Fig. 6). The presence of the introduced bacterial strains on and in cotelydons, but not on and in true leaves of tomato plants, is most likely due to passive colonization/contamination of the cotyledons during germination of the bacteria-treated seeds and subsequent seedling emergence (Raaijmakers *et al.*, 1995). These results indicate that massetolide A contributes to colonization of tomato plants by *P. fluorescens* SS101 and extend the findings of Nielsen et al. (2005) who showed that the CLP amphisin produced by *Pseudomonas* sp. strain DSS73 is an important trait in colonization of sugar beet seeds and roots.

In conclusion, the results of this study showed that the cyclic lipopeptide surfactant massetolide A is a metabolite with versatile functions in the ecology of producing strain *P. fluorescens* SS101 and with potential as a supplementary measure in the control of late blight.

Materials and methods

Microorganisms and growth conditions.

Pseudomonas fluorescens strain SS101 was originally isolated from the rhizosphere of wheat grown in a soil suppressive to take-all disease (de Souza *et al.*, 2003). Biochemical analysis revealed that SS101 produces at least five cyclic lipopeptide surfactants. Massetolide A is the main cyclic lipopeptide produced by SS101 (de Souza *et al.*, 2003) and the other cyclic lipopeptides detected in cell-free culture supernatans are derivatives of massetolide A differing in amino acid composition of the peptide ring (I. de Bruijn, M.J.D de Kock and J.M. Raaijmakers, unpublished results). 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 non-ribosomal peptide synthetase (NRPS) gene required for the biosynthesis of massetolide A (I. de Bruijn, M.J.D. de Kock and J.M. Raaijmakers, unpublished results). Mutant 10.24 does not produce massetolide A and none 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, France) at 25 ^{0}C for 48 h. Bacterial cells were washed in sterile demineralised water prior to use. For treatment of tomato seeds, roots or leaves, washed cell suspensions of SS101 or 10.24 were diluted in sterile demineralised water to a final concentration of 10^9 CFU ml^{-1} (OD 600_{nm}=1).

Phytophthora infestans strain 90128 (A2 mating type, race 1.3.4.6.7.8.10.11) was used in all bioassays. The strain was grown on rye sucrose agar (Latijnhouwers et al. 2004) for 7-9 days in the dark at 18^{o}C. To obtain zoospores, full-grown plates (9-cm-diameter) were flooded with 20 ml of sterile distilled water and hyphae were fully submerged with a glass spreader. Flooded plates were placed in the cold (4 ^{0}C) for 1-2 h after which the suspension was gently filtered (50 μm mesh) to remove sporangia. Zoospore density was determined microscopically at 100X magnification and adjusted to a final concentration of 3-4 x 10^3 swimming zoospores ml^{-1}.

Purification and detection of massetolide A

The cyclic lipopeptide massetolide A (molecular mass 1,139 Da) was extracted from cell cultures of *P. fluorescens* SS101 as described by De Souza et al. (2003). In summary, strain SS101 was grown on PSA agar plates for 48 hours at 25 ^{0}C. Bacterial mats were suspended in sterile demineralised water and cells were pelleted by centrifugation at 6000 rpm for 20 minutes. The cell-free culture supernatant was collected, acidified with HCl to pH 2 and incubated for 1 h on ice to precipitate massetolide A. The precipitate was obtained by centrifugation (5500 *g*, 30 min) and washed twice with acidified (pH 2) sterile demineralised water. The precipitate was dissolved in sterile demineralised water by adjusting the pH to 8 with 0.5 M NaOH, lyophilized and stored at -20 ^{0}C. The precipitate was analysed by Reverse-Phase High Performance Liquid Chromatography (RP-HPLC) linked to a photodiode-array detector as described by De Souza et al. (2003) and De Bruijn et al. (2007). For extraction of massetolide A from tomato leaves, the protocol of Asaka and Shoda (1996) was used. The extraction efficiency was tested by spiking 1 mg of massetolide A (70% purity) to tomato leaves submersed in the solvents used for extraction (HPLC-grade acetonitrile and trifluoroacetic acid (0.1% (v/v)). The concentration of massetolide A was determined based on peak area (at 206 nm) using a 6-point standard curve.

Plant cultivation and biocontrol assays

Tomato seeds (*Lycopersicon esculentum* cv. Money Maker Cf0) were sown in a tray containing a mixture of commercial potting soil and quartz sand (3:2, w/w). The initial water content of the soil mixture was adjusted to 40% (v/w). The tray was covered with a transparent lid and kept in the climate chamber (20 ^{0}C, 16h/8h day/night photoperiod) for 2 weeks to stimulate germination. Seedlings were then transplanted to 7-7-8-cm (LxWxH) black plastic PVC pots containing the same soil mixture and kept in the climate chamber at 20 ^{0}C for 3 weeks. For the biocontrol assays with *P. infestans*, plants were transferred to a 15 ^{0}C growth chamber to create conditions favourable for infection and disease development. For the induced resistance assays, the *nahG* transgene, which is unable to accumulate salicylic acid (Gaffney *et al.*, 1993), was included. The *nahG* derivative was kindly provided by Dr. Jan van Kan (Laboratory of Phytopathology, Wageningen University, Netherlands). Prior to use, tomato seeds were surface sterilized, dried in a flow cabinet, and sown and maintained as explained above.

Prevention of late blight infection of tomato leaves

To study the effects of *P. fluorescens* SS101 and massetolide A on late blight of tomato, two leaves located on the second branch from the stem base of 5-week-old tomato plants were immersed in bacterial suspension (10^9 CFU ml^{-1}) for 1 min or in a solution of massetolide A in sterile demineralised water (pH 8). Leaves immersed in sterile demineralised water (pH 8) for 1 min served as a control. Treated tomato plants were transferred to trays covered with transparent lids. After incubation for 1 day in a growth chamber at 15 ^{0}C, the lower side of each treated tomato leaf was inoculated with 3-µl-droplets of a *P. infestans* zoospore suspension (3-4 x10^3 swimming zoospores ml^{-1}) or 3-µl-droplets of sterile demineralised water (pathogen-free control). Two droplets were placed abaxially on each side of the leaf's mid vein. Tomato plants were incubated in the growth chamber and at several days after zoospore inoculation, disease incidence and lesion area were scored. Disease incidence was scored visually by counting the number of zoospore droplets that developed into a lesion. The area of the lesions was determined by an electronic marking gauge linked to the IBREXDLL software (IBR Prozessautomation) as described by Latijnhouwers et al. (2004). Each treatment had four or five replicates with one plant per replicate. For each plant, disease incidence and lesion area were assessed on two leaves, each treated with four zoospore droplets.

Effect of *P. fluorescens* SS101 and massetolide A on existing late blight lesions

Two tomato leaves in the second branch from the stem base of 5-week-old tomato plants were inoculated with 3-µl-droplets of a *P. infestans* zoospore suspension or sterile demineralised water (pathogen-free control). The droplets were placed abaxially on each side of the leaf's mid vein. Tomato plants were then incubated at 15 ^{0}C for 3-4 days to allow the late blight lesions to develop. The initial lesion area was determined as described above. Subsequently, the lesioned tomato leaves were dipped in bacterial suspension (10^9 CFU ml $^{-1}$) or in solutions with different concentrations of massetolide A for 1 min; sterile-

demineralised water served as a control. Tomato plants were then transferred to the climate chamber (15 ^{0}C) and the areas of the late blight lesions were measured again at 2 and 5 days after treatment with water (control), the bacterial suspension or with massetolide A. The increase in lesion area was calculated by dividing the lesion area after treatment by the initial lesion area assessed prior to treatment with water, the bacteria or with massetolide A.

Induced resistance assays

To determine the role of induced resistance as a mechanism in late blight control, two types of experiments were conducted to physically separate the inducing agents from the pathogen (Fig. S1). In one series of experiments, the inducing agents were applied to the lower leaf and the pathogen inoculated on the upper leaves. Therefore, two adjacent leaves on the first branch from the stem base of 5-week-old tomato plants were immersed in bacterial suspension or in a solution of massetolide A for 1 min. After incubation of the treated tomato plants for 24 h at 15 ^{0}C, two adjacent leaves on the second branch from the stem base were inoculated with 3-µl-droplets of a zoospore suspension of *P. infestans* as described above. In the second series of experiments, roots of 2-week-old tomato seedlings were washed gently with running tap water to remove adhering soil, blotted dry with sterile paper tissue, and dipped in a bacterial suspension or in a solution of massetolide A for 10 min. Sterile demineralised water (pH 8) served as a control. The seedlings were then transplanted and maintained as explained above. Approximately two weeks after treatment, the tomato leaves were challenge-inoculated with zoospores of *P. infestans* as described above. Disease incidence and lesion area were assessed at different time points after pathogen inoculation. For each treatment, six replicates were used.

Effect of seed treatment on late blight incidence, severity, and sporangia formation

The efficacy of seed treatments to control *P. infestans* was investigated by immersing tomato seeds in bacterial suspensions (10^9 CFU ml^{-1}) or in a solution of massetolide A for 1 h. Immersing seeds in sterile demineralised water for 1 h served as a control. After treatment, seeds were dried in a flow cabinet and sown in the soil mixture described above. The final density of strain SS101 and mutant 10.24 on the tomato seeds was 1 x 10^6 CFU seed^{-1} as was determined by dilution plating suspensions from seeds onto PSA medium supplemented with rifampicin. Tomato plants were maintained under the same growth conditions as indicated above. After 5 weeks of plant growth, tomato leaves were inoculated with zoospores of *P. infestans* as described above. Disease incidence and lesion area were assessed at different time points after pathogen inoculation. For each treatment, six replicates were used. Nine days after zoospore inoculation, tomato leaves were harvested and lesion areas determined as described above. The lesions were then excised from the leaves and transferred to 1.5 ml sterile tubes containing 1 ml of isotone II electrolytic buffer (Coulter Electronic Inc.). The sporangia were released from the sporangiophores by vigorously shaking on a vortex mixer for 1 min. The density of the sporangia was determined microscopically at 100X magnification in 5-µl-aliquots. Combined with the lesion area, sporangia formation per unit lesion area was calculated.

Plant colonization by introduced bacterial strains

Two-week-old tomato seedlings and six-week-old tomato plants raised from bacteria-treated seeds (described above) were used to study the role of massetolide A in plant colonization by *P. fluorescens* SS101. The parts of the seedlings and plants surveyed included roots, stems, cotyledons and true leaves. For each plant part, bacterial densities were determined for the surface and interior. To determine surface colonization, approximately 1 g (fresh weight) of roots was suspended in 5.0 ml of 0.01 M $MgSO_4$, vortexed for 1 min, sonicated for 1 min (Bransonic 12) and vortexed again for 15s prior to dilution plating. Surface colonization of stems, cotyledons and leaves was determined by suspending approximately 1 g of cotyledons, leaf or stem sections in 5.0 ml of 0.01 M $MgSO_4$ supplemented with 0.05% (v/v) Tween 80, and then vortexing vigorously for 1 min prior to dilution plating. Suspensions were plated onto PSA agar plates supplemented with rifampicin (for strain SS101) and on plates supplemented with rifampicin and kanamycin (for mutant 10.24). Delvocid (DSM, Delft, Netherlands) was added (100 µg ml^{-1}) to the agar plates to prevent fungal growth. Plates were incubated for 48-72 h at 25 ^{0}C, after which bacterial colonies were counted and population densities calculated.

For assessment of colonization of the root interior, root sections were surface sterilized with 10% H_2O_2 for 15s, rinsed twice with ample sterile demineralised water and blotted dry on sterile paper tissue. Surface-sterilized roots were homogenized with a mortar and pestle in 5.0 ml of 0.01 M $MgSO_4$ and serial dilutions were plated onto selective PSA medium as described above. The efficacy of surface sterilization was checked with additional samples by printing the surface-sterilized root sections onto selective PSA agar plates. To determine colonization of the interior of cotyledons, true leaves and stems, these tissues were surface sterilized with 10% H_2O_2 for 15s, blotted dry with sterile paper tissue and rinsed twice with ample sterile demineralised water. The efficacy of sterilization of leaf and stem surfaces was checked as described above. Surface-sterilized plant tissues were homogenized with a mortar and pestle in 5.0 ml of 0.01 M $MgSO_4$. Suspensions were dilution plated onto selective PSA media. Plates were incubated for 48-72 hours at 25 ^{0}C, after which bacterial colonies were counted and population densities calculated.

Statistical analysis

All experiments described in this study were performed at least two times. Representative results are shown. Population densities of the applied bacterial strains were $\log_{10}$ transformed prior to statistical analysis. Differences between treatments in disease incidence, lesion area, and population densities of the applied bacterial strains were analysed by ANOVA followed by student's t test ($p < 0.05$; SAS Institute, Inc, Cary, N.C.). Normal distribution of the data and homogeneity of variances were tested prior to ANOVA.

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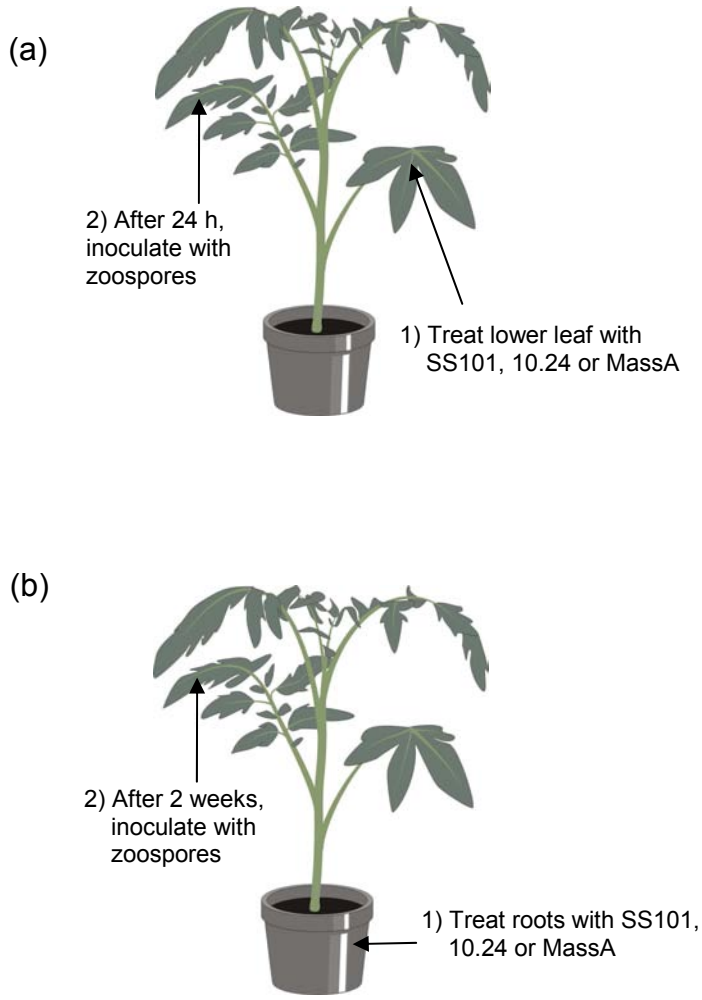
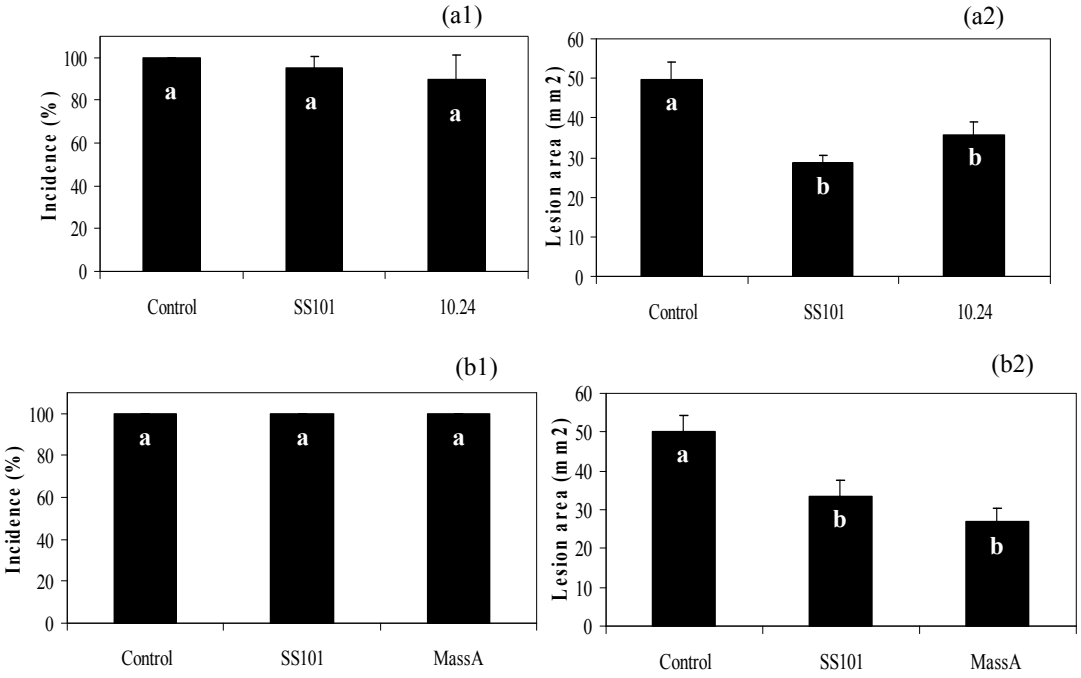


Supplementary figure 1: Schematic presentation of two experimental set-ups used to determine the role of *P. fluorescens* SS101, mutant 10.24 or massetolide A in induction of systemic resistance in tomato against *Phytophthora infestans*. (a) twenty-four hours after treatment of the lower leaves of the tomato plants with wildtype strain SS101, mutant 10.24, or massetolide (MassA, 50 µg ml^{-1}), upper leaves were challenged-inoculated with zoospores of *P. infestans*. Disease severity (lesion area) was determined 4 and 7 days after pathogen inoculation. (b) roots of tomato seedlings were treated with strain SS101, mutant 10.24 or massetolide A (50 µg ml^{-1}), transplanted and grown for two weeks under controlled conditions; then leaves were challenged-inoculated with zoospores of *P. infestans* and disease severity was determined 4 and 7 days after pathogen inoculation.



Supplementary figure 2: Induced resistance in tomato against late blight by application of *P. fluorescens* SS101 or massetolide A to roots of tomato seedlings. Two weeks after root treatment, tomato leaves were challenged-inoculated with zoospores of *P. infestans*. Results of two experiments are presented. Panels a1 and a2 represent the results from experiment 1 (comparison wildtype SS101 and massetolide A-deficient mutant 10.24); panels b1 and b2 show the results from experiment 2 (comparison wildtype SS101 and partially purified massetolide A (50 µg ml^{-1}). Means of 6 replicates are given. Error bars represent the standard error of the mean. Means with the same letter are not significantly different ($p<0.05$).

Chapter 4

Distribution, frequency and control strategies of *Phytophthora* foot rot of black pepper in Vietnam

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Distribution, frequency and control strategies of *Phytophthora* foot rot of black pepper in Vietnam

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Abstract

Aims. Black pepper (*Piper nigrum* L.) is one of the main crops in Vietnam, but its production is limited by *Phytophthora capsici,* the causal agent of foot rot of black pepper. In this study, a survey was conducted in Quang Tri province to examine the distribution, frequency and current strategies to control Phytophthora foot rot of black pepper.

Methods. Three selected districts (Cam Lo, Gio Linh and Vinh Linh) were chosen for surveys conducted in February 2005 and 2006. Nintyfour households were interviewed and several pepper growing parameters were recorded each year. In addition, the number of black pepper plants and foot rot incidence was scored in each orchard to determine disease incidence.

Results. The overall disease incidence was on average 17-18% in 2005 and 2006. Disease incidence was highest in Vinh Linh district reaching approximately 25%, whereas disease incidence in Cam Lo and Gio Linh was almost 2-fold lower. The results clearly showed that treatment of black pepper stem cuttings used for propagation leads to a significant decrease in disease incidence. Also the cropping history had a significant impact on disease incidence. In contrast, fungicide applications to black pepper plants grown in the field had no significant effect on disease incidence

Conclusions. These results indicate that foot rot caused by *P. capsici* in Quang Tri Vietnam is one of the main constraints to production of black pepper. Fungicide treatment of stem cuttings used for propagation was shown to be an effective measure to reduce the incidence of Phytophthora foot rot of black pepper.

Key words: Black pepper, *Phytophthora capsici*, foot rot, nursery

Introduction

Black pepper (*Piper nigrum* L.) is a woody perennial climbing vine grown for its berries which are used as a spice and in medicine. Black pepper is grown throughout the tropics as it requires adequate rainfall and humidity. The crop grows well within a temperature range of 20-30 ^{o}C and tolerates a minimum of 10 ^{o}C and a maximum 40 ^{o}C. An annual rainfall of 1,000-3,000 mm spread throughout the year is considered ideal for black pepper production. Three types of aerial shoots can be distinguished, namely 1) the primary stem which clings to the support plant, 2) runner shoots which originate from the base of the vine, and 3) fruit bearing lateral branches. Pepper is propagated vegetatively and the runner shoots are mainly selected as planting materials. The shoots are cut into pieces of 2-5 nodes and leaves, if any, are cut off leaving a small portion of the petioles on the stem. Phytohormones, like 3-indole butyric acid (IBA), might be applied to the lower cut end of the shoots before planting to promote root development. Stem cuttings can be grown directly in the field or are grown in a nursery for 3-4 months in polyethylene bags before transplanting to the field. The cuttings need to be planted at least one node deep in the soil. Five or seven rooted cuttings are planted in the hole at a distance of about 30 cm away from the support plant. The growing portions of the cuttings are trailed and tied to the support plants to assist the start of the climbing process. Live plants, timber poles, or brick columns can be used as supports for the pepper vines.

Black pepper plants were first domesticated in India and from there spread to other Southeast Asian countries, including Indonesia, Malaysia, Thailand, SriLanka, Cambodia, Laos and Vietnam. In Vietnam, black pepper was first grown in the provinces Phu Quoc, Ha Tien, and Phuoc Tuy at the end of the 19th century (Phan *et al.*, 1988). In Vietnam, black pepper is grown in a wide range of soils with a pH of 4.5-6.5 (Phan *et al.*, 1988; Phan, 2000). In 2004, Vietnam had a growing area of black pepper of about 52,500 ha and a production of nearly 90,000 ton (Ton, 2005) and is now the second largest pepper exporting country. Black pepper is among the top ten export items of Vietnam by value and provides much needed income for subsistence farmers. However, black pepper production in Vietnam is hampered by several diseases, with foot rot caused by *Phytophthora capsici* considered the most destructive and economically most important (Nguyen, 2002; Drenth & Sendall, 2004). *Phytophthora capsici* Leonian is a soilborne heterothallic oomycete with two mating types, A1 and A2. Both mating types have been reported to coexist in several black pepper areas in Vietnam (Nguyen *et al.*, 2006). The pathogen can survive in soil as oospores for a long time under unfavourable conditions. Zoospores, produced from germinating oospores or from sporangia produced on sporulating leaves play an important role in dissemination. Zoospores are chemotactically attracted to the exudates released by the roots of the host plant. Once zoospores locate host plant tissue, they encyst, germinate and invade. *P. capsici* can attack all parts of black pepper plants at any stage of crop growth (Sarma, 2002). Crop losses caused by *P. capsici* can amount up to 40 or even 50% (Erwin & Ribeiro, 1996). In Vietnam, *P. capsici*

causes an estimated annual loss of 15-20% (Drenth & Sendall, 2004), but disease losses have not been accurately quantified for specific black pepper areas.

Current methods to control *P. capsici* include cultural practices, fungicide applications, root infusion of phosphorous acid and biological control (Wong, 2004; Anandaraj & Sarma, 2003; Manohara *et al.*, 2003; Drenth & Guest, 2004). There are no resistant commercial black pepper varieties available in Vietnam, although some varieties with different degrees of tolerance to Phytophthora foot rot have been reported (Erwin & Ribeiro, 1996; Manohara *et al.*, 2003). To date, detailed information concerning the occurrence and management practices of Phytophthora foot rot in Vietnam is limited. In order to improve disease management, a survey was conducted in Quang Tri province to determine the distribution, frequency and the effectiveness of current strategies employed by farmers to control Phytophthora foot rot in black pepper. Such information is important as it creates a baseline of disease incidence, severity and economic impact. This baseline can then be used to measure the effectiveness of future implemented disease management strategies.

Results

Description of observed disease symptoms

P. capsici attacks all parts of the plant: roots, basal stem or collar, stems (Fig. 1c3), vines (Fig. 1c2), leaves (Fig. 1c1), and flower and fruit spikes. Symptoms may vary depending on the plant part infected. With foliage infection, one or more circular lesions with a characteristic fimbriate edge appear on the leaves which later enlarge and coalesce leading to defoliation (Fig. 1c1). The infections on aerial stems are seen as black lesions. The fungus sporulates abundantly, forming a white covering on the blighted tender shoots before causing defoliation (Fig. 1c2). The infection of *P. capsici* on the main stems results in drooping of the leaves, followed by wilting, yellowing and drying. The branches become brittle and break up at the nodes resulting in entire vine collapses within 2-3 weeks (Fig. 1a1, b1 &b2). Infection on the roots and the basal stem leads to rapid yellowing and wilting resulting in sudden death of the pepper plant.

Table 1: Overview of the growing area of black pepper and the yield in three main districts in Quang Tri province in Vietnam from 2000-2005 *(Source: Quang Tri Statistics 2004)*.

Year	District		
	Cam Lo	Gio Linh	Vinh Linh
		Growing area (ha)	
2000	669	264	541
2002	804	411	638
2003	895	408	690
2004	923	398	720
2005	773	399	730
		Gross production (ton ha^{-1})	
2000	0.46	0.39	0.67
2002	0.49	0.57	0.63
2003	0.89	0.66	0.98
2004	0.91	0.81	1.05
2005	0.45	0.62	0.87

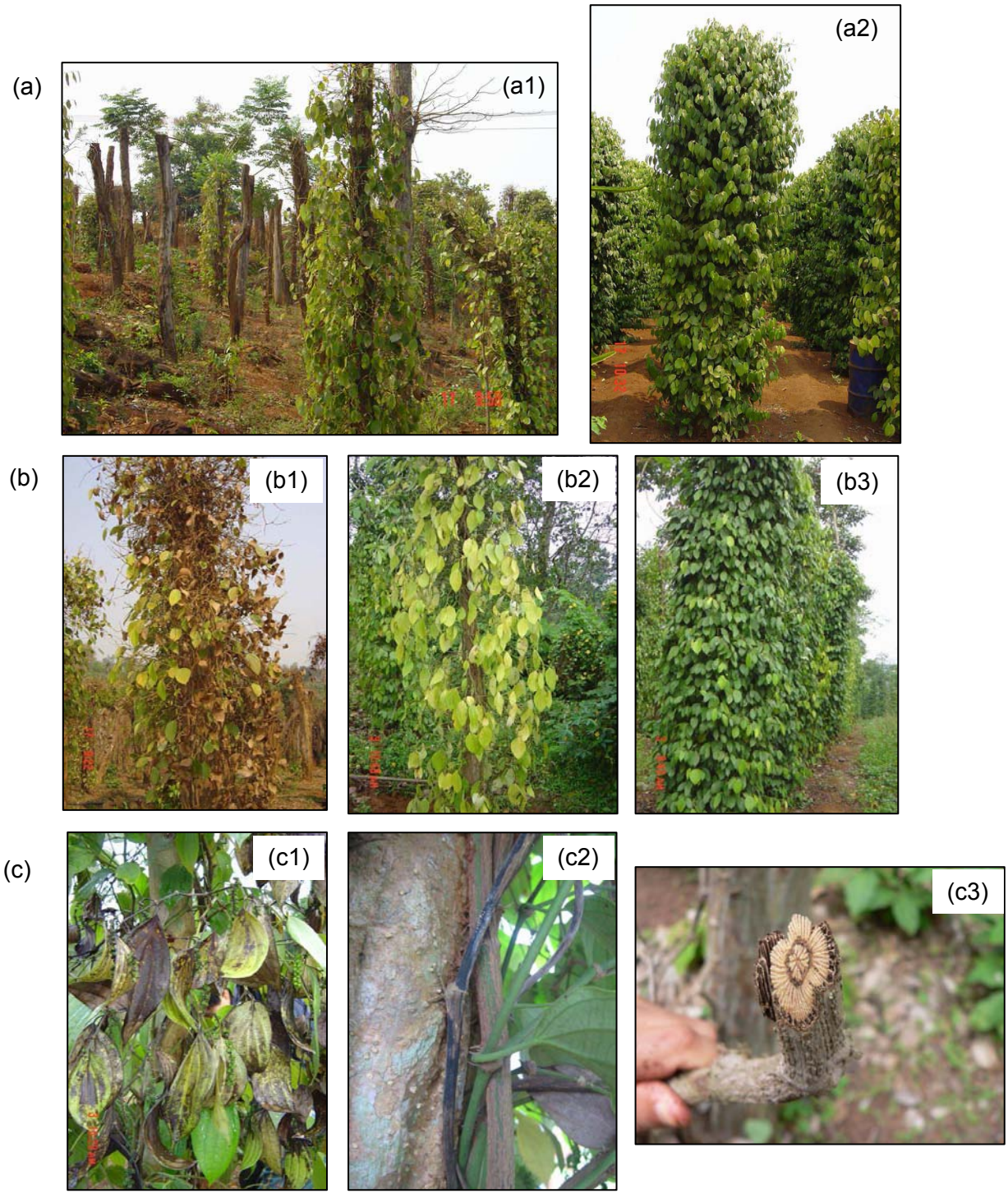

Figure 1: Pictures of black pepper and symptoms of Phytophthora foot rot

(a) Overview of a black pepper orchard heavily infested by *Phytophthora capsici* (a1); healthy black pepper orchard (a2)
(b) Black pepper plant infected by *P. capsici* showing typical symptoms of yellowing and defoliation (b1, 2); healthy black pepper plant (b3).
(c) *P. capsici* infects the leaves of black pepper (c1), the vine (c2) and the basal stem (c3)

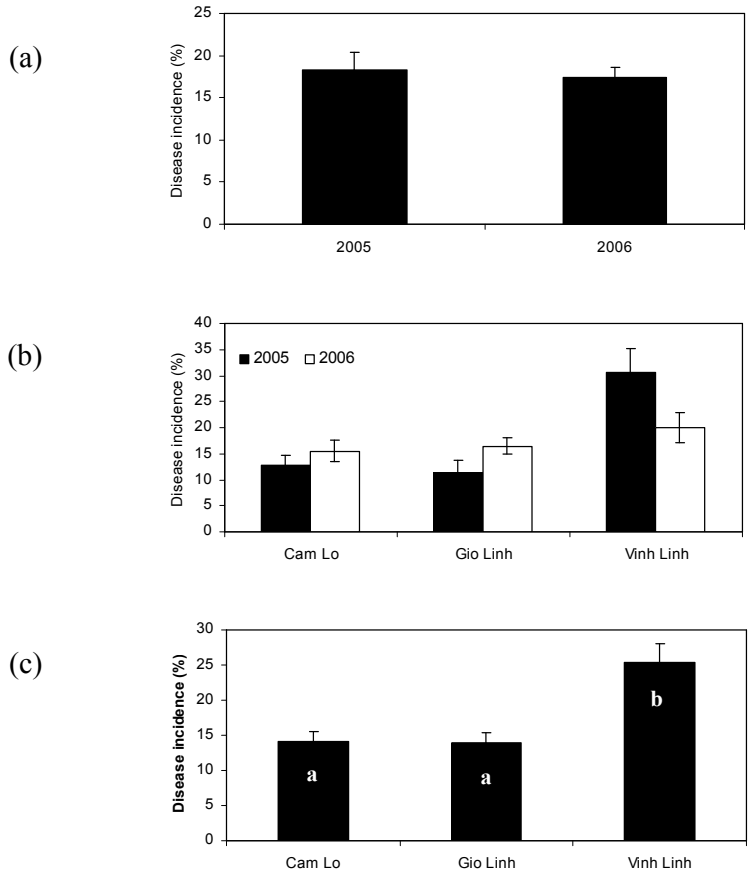

Figure 2: Incidence of Phytophthora foot rot in black pepper orchards in Quang Tri province, Vietnam. Error bars represent the standard error of the mean.

(a) Disease incidence in 2005-2006 combined for all three districts. Incidence was analysed by T-test (p=0.68).
(b) Disease incidence in 2005 and 2006 for each district. Incidence was analysed by T-test (Cam Lo, p= 0.33; Gio Linh, p= 0.82; Vinh Linh, p= 0.06).
(c) Disease incidence (%) combined for two years (2005 and 2006) and for each of the three districts (Cam Lo, Gio Linh, Vinh Linh). The incidence (%) was arcsin-transformed prior to statistical analysis. Means with the same letter are not statistically different ($p < 0.05$).

Distribution and frequency of Phytophthora foot rot of black pepper

In Quang Tri province, black pepper is mainly grown in the three districts Cam Lo, Gio Linh and Vinh Linh (Table 1). In all three districts and especially in Cam Lo, the yield per area (ton/ha) substantially decreased in 2005 when compared to 2004 (Table 1). For each of the three districts, the results of our survey showed that in the year 2005, the households had on average 2000 black pepper plants per ha. In the year 2006, the number of black pepper plants decreased in all three districts to approximately 1600 plants per ha (data not shown). This substantial reduction in the number of plants per ha is due in part to foot rot caused by *P. capsici*. Another important reason was the low market price for black pepper which significantly reduced the financial returns for farmers and resulted in replacement of black pepper plants by rubber trees or other crops. The results showed that the overall disease incidence was on average 17-18% in 2005 and 2006 (Fig. 2a). Comparison between the three districts showed that disease incidence was highest in Vinh Linh in 2005 reaching approximately 25 %,

whereas disease incidence in Cam Lo and Gio Linh was about half that level (Fig. 2b). In 2006, disease incidence in Vinh Linh decreased to approximately 20%, while in Cam Lo and Gio Linh disease incidence increased slightly to approximately 16% (Fig. 2b). When the data of 2005 and 2006 were pooled per district, disease incidence in Cam Lo was similar to that in Gio Linh, but significantly lower than in Vinh Linh (Fig.2c).

Effect of stem cutting treatment on disease incidence

Black pepper is propagated vegetatively and stem cuttings obtained from runner shoots serve as planting material. The stem cuttings are grown in the nursery for 3-4 months in polybags, but several local farmers plant the stem cuttings directly out in the field. In our survey, each of the households was asked if the black pepper stem cuttings were treated with fungicides and what type of fungicide (Table 2a,b) was used prior to transplantation to the nurseries. The results show that in the three districts on average 32% of the households do treat the stem cuttings with fungicides prior to planting in polybags or field soil (Table 2a). In Vinh Linh district, very few farmers (6.3%) treated the stem cuttings, compared to 34.5 and 54.5 % in Gio Linh and Cam Lo, respectively (Table 2a). The type of fungicide used varied and due to the small sample sizes the different fungicide treatments were pooled to evaluate the effect on disease incidence. The overall results for 2005 and 2006 show that there was a significant correlation between chemical treatment of stem cuttings and a decrease in disease incidence; disease incidence in black pepper orchards with nursery sanitation in both years was on average 10.9 % (n=40), while disease incidence in orchards without sanitation was on average 20% (n=148) (Fig. 3a1). When the data were analysed for each of the three districts separately, similar results were found for Cam Lo and to a lesser extent for Gio Linh (Fig. 3a2); for Vinh Linh district, the effect of nursery sanitation was highest, but could not be supported statistically due to a very small number of households that did apply nursery sanitation (Table 2a).

Table 2a: Frequency of households that treat black pepper stem cuttings with fungicides prior to transplantation in polybags or field soil.

District	Number of households sampled	Stem cutting treatment (%)
Cam Lo	33	54.5
Gio Linh	29	34.5
Vinh Linh	32	6.3
Total	**94**	**31.9**

Table 2b: Types of fungicides used on black pepper stem cuttings grown in nurseries in three districts in Quang Tri province in Vietnam.

Fungicide	Active ingredient
CaCO3	Carbonate canxi
Bordeaux	Copper sulfate
Vicarben	Carbendazim
Viben C	Copper oxychlorite + Benomyl
Others	unknown

Effect of fungicide applications on disease incidence in the field

The number of households in the three districts that did apply fungicides to the black pepper plants in the orchard was 56.9 % (Table 3). A relatively large number of households (22.4 %) applied 'unknown' fungicides and the majority of the households (24.0 %) applied furadan, a nematicide; only very few households applied metalaxyl-based fungicides (Table 3). Furadan is used frequently since several of the symptoms (e.g. yellowing of the leaves) associated with Phytophthora foot rot correspond to the symptoms caused by the nematodes *Meloidogyne incognita* and *Radopholus similis*. Overall and for each of the three districts separately, fungicide applications to black pepper plants grown in the field did not have a significant effect on disease incidence (Fig. 3b1,b2).

Table 3: Frequency and types of pesticides applied to black pepper plants grown in the field. Data were collected in 2005 and 2006 from three districts in Quang Tri province in Vietnam.

Name of pesticide	Active ingredient	Percentage (%)
No application	-	43.1
Furadan	Carbofuran	24.0
Unknown	-	22.4
Bordeaux	Copper sulfate	4.0
Ridomil	Metalaxyl	2.5
CaCO3	Carbonate canxi	2.5
Aliette	Fosetyl aluminium	0.5
Benlat C	Benomyl	0.5
Fortazeb	Metalaxyl + Mancozeb	0.5

Effect of cropping history on disease incidence

The results of the survey showed that for black pepper, a perennial crop, there was a significant correlation between the incidence of Phytophthora foot rot and the types of crops grown in the orchard prior to black pepper cultivation (Fig 3c1, c2). In Vinh Linh, most black pepper orchards (62.5 %) were established on soil with a long history of black pepper cultivation, whereas in Cam Lo and Gio Linh most black pepper orchards were established on soils grown to other crops (Table 4). When black pepper is grown in virgin soils or in soils grown prior with other crops, the overall disease incidence was significantly lower than in orchards with a history of black pepper cultivation (Fig. 3c1) The effect of the cropping history was clearly visible in the district Cam Lo and to some or no extent in Gio Linh and Vinh Linh (Fig. 3c2).

Effect of fertiliser application

Black pepper is a plant occurring naturally in forests and grown in soils with high levels of organic matter. The results of our survey showed that there was little difference between the three districts in the amounts of P_2O_5 and K_2O applied (Table 5). In Cam Lo, a significantly lower amount of nitrogen was applied and a significantly higher amount of cow manure (Table 5). Overall, no significant ($p< 0.05$) correlations were found between disease incidence and the amounts of cow manure, N, P_2O_5, or K_2O (data not shown).

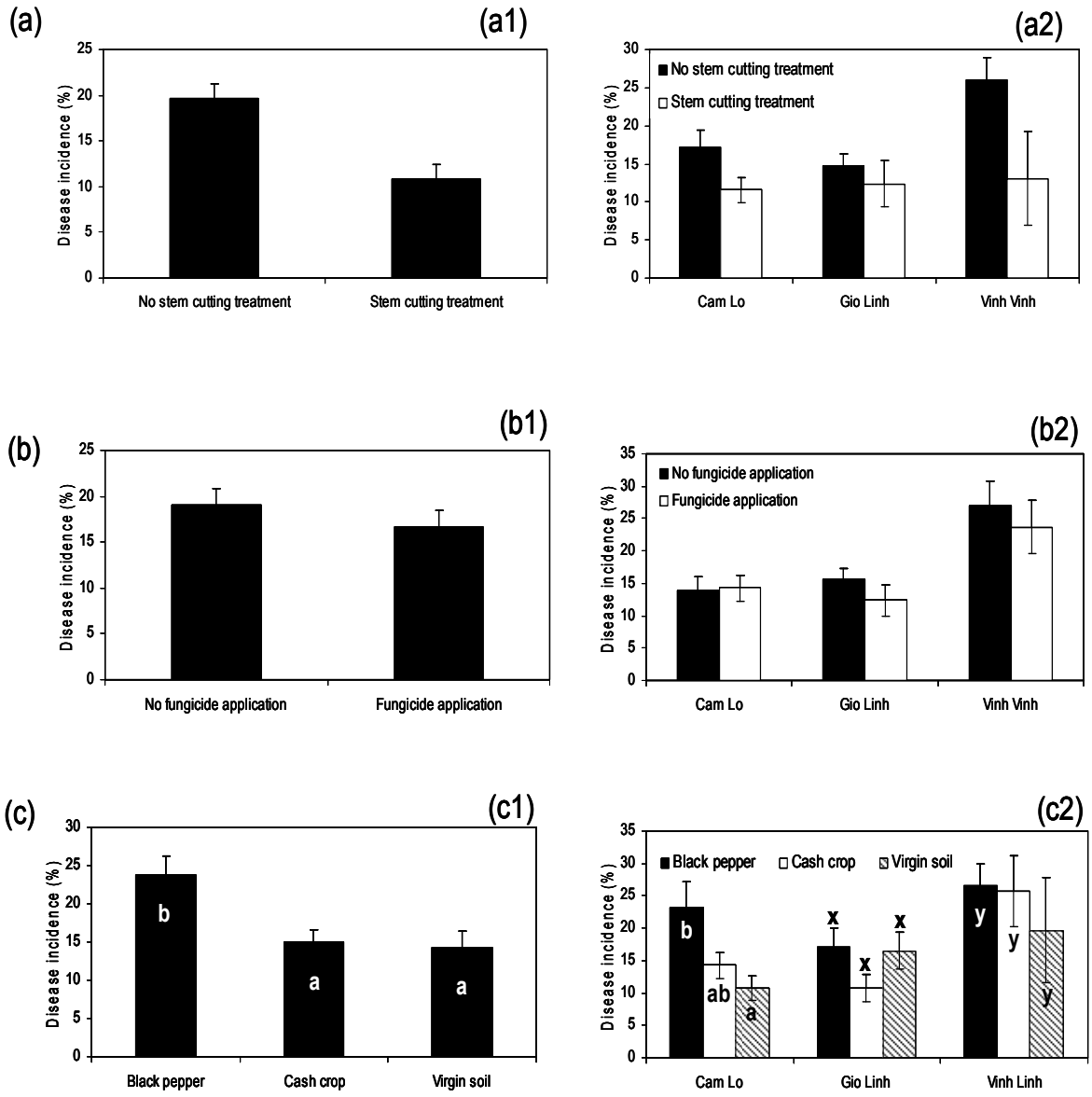

Figure 3: Effect of stem cutting treatment (a), fungicide applications (b) and cropping history (c) on incidence of Phytophthora foot rot of black pepper. The incidence (%) was arcsin-transformed prior to statistical analysis. Error bars represent the standard error of the mean.

(a1) Effect of stem cutting treatment on disease incidence in 2005-2006 for all three districts. Incidence was analysed by T-test (p= 0.001);
(a2) effect of stem cutting treatment on disease incidence in 2005-2006 for each district. Incidence was analysed by T-test (Cam Lo, p= 0.04; Gio Linh, p= 0.45; Vinh Linh, p= 0.26).
(b1) Effect of fungicide application on disease incidence in 2005-2006 for all three districts. Incidence was analysed by T-test (p= 0.32);
(b2) Effect of fungicide application on disease incidence in 2005-2006 for each district. Incidence was analysed by T-test (Cam Lo, p=0.93; Gio Linh, p=0.29; Vinh Linh, p=0.57).
(c1, c2) Effect of cropping history on disease incidence in 2005-2006 for all three districts together (c1) and separately (c2) Means with the same letter are not significantly different (p< 0.05).

Table 4: Overview of the cropping history in black pepper orchards in three districts in Quang Tri province, Vietnam. Cropping history was divided in three categories: orchards where black pepper was grown for at least 5 years or more were assigned to the first category designated 'black pepper'; category 2 (designated 'other crops') comprised orchards where other crops, including various vegetables, sweet potatoes, and groundnut, were grown at least 5 years or more prior to black pepper cultivation; orchards where no other crops were cultivated (virgin soil) prior to black pepper cultivation were assigned to the third category.

District	Number of households sampled	Cropping history		
		Black pepper (%)	Other crops (%)	Virgin soil (%)
Cam Lo	33	12.1	51.5	36.4
Gio Linh	29	24.1	48.3	27.6
Vinh Linh	32	62.5	21.9	15.6
Total	94	33.0	40.4	26.6

Table 5: Amount of inorganic and organic fertilizers applied to black pepper orchards in three districts in Quang Tri province in Vietnam. Data collected in 2005-2006 were pooled and analysed by ANOVA. Means with the same letter are not significantly different ($p < 0.05$).

District	N (kg/ha)	P_20_5 (kg/ha	K_20 (kg/ha)	Cow manure (kg/ha)
Cam Lo	91 a	160 a	162 a	34746 b
Gio Linh	205 b	203 a	162 a	22349 a
Vinh Linh	181 b	180 a	152 a	19722 a

Discussion

Black pepper is one of the main crops of Vietnam. The growing area of black pepper and its production increased rapidly from 9,800 ha with a production of 13,700 ton in 1997 to 52,500 ha with a production of nearly 90,000 ton in 2004 (Ton, 2005). This increase in the area of black pepper cultivation also led to an increase in the occurrence of a number of diseases and pests, causing considerable yield losses. One of the main and economically most significant diseases of black pepper is foot rot caused by *P. capsici*. Our survey conducted in 2005 and 2006 showed that in Quang Tri province, Phytophthora foot rot occurred in the field at a frequency of 17-18%. There was, however, a big variation in disease incidence between the three districts, ranging from 10-15% in Cam Lo to 20-30% in Vinh Linh (Fig. 2c). These results are consistent with the estimates of 15-20% given by Drenth and Sendall (2004) for Vietnam.

The results of this study show that fungicide treatment of stem cuttings used for propagation has a significant impact on disease incidence. Black pepper is propagated vegetatively and cuttings obtained from runner shoots serve as the main planting material. The runner shoots are grown in the nursery for 3-4 months and are then transplanted into the field. Alternatively, runner shoots are grown directly in the field. Stem cuttings are very susceptible to attack by *P. capsici* and other pathogens since the cutting wounds provide an ideal entry point for the pathogen. In the field, many farmers grow about 5-7 cuttings around one pole (support plant). They manually remove infected or dead stem cuttings but do not subsequently treat the soil, which may contain inoculum of the pathogen. Stem cutting treatment is also important when black pepper cuttings are grown in polybags. Recent studies have indicated that several

antagonistic bacterial strains isolated from roots of black pepper may serve as an alternative or supplementary measure in nursery sanitation to protect black pepper stem cuttings against diseases and, in addition, to promote root and shoot development (Tran *et al.*, 2007).

P. capsici infection of black pepper plants in the field causes serious yield losses (Erwin & Ribeiro, 1996; George et al. 2005). Farmers are often unsure about the cause of wilting of their pepper plants and lack access to information to select the appropriate fungicides. No significant effects of fungicide application on disease incidence of mature black pepper plants in the field were observed in this study (Fig. 3b1). There are a number of reasons why fungicides lack efficacy against foot rot in pepper. First, most of the fungicides used have little efficacy towards the oomycete *P. capsici* and metalaxyl-based fungicides are considered too expensive by the farmers. Second, most farmers apply fungicides when the symptoms start to appear; at that time, the pathogen has caused already irreversible damage to the stem base of the plant. According to Hausbeck and Lamour (2004), fungicides can not be relied upon alone to prevent diseases caused by *P. capsici*. In order to control foot rot more effectively, a more integrated approach to disease management is needed which involves management practices such as crop rotation, raised beds and proper drainage to reduce moisture levels. However, crop rotation is difficult to implement for a perennial crop such as black pepper, which is grown in orchards for a period up to 15 years. The results of this study did show that the cropping history of the orchard has a significant impact on disease incidence. In black pepper orchards with a history of black pepper cultivation, disease incidence was significantly higher than in orchards established on virgin soils or on soils grown to other crops. The absence of effective disease management strategies may give rise to a build-up and spread of *P. capsici* propagules in an orchard resulting in an increase in disease incidence and severity over time. Bowers et al (1990) mentioned that the long-term survival of oospores have been clearly demonstrated with a number of *Phytophthora* spp., including *P. capsici*. The study of Hausbeck and Lamour (2004) in Michigan further showed that even growers practicing lengthy rotations (>5 years) with non-susceptible host plants have experienced significant crop losses due to *P. capsici*. Our study also shows that black pepper plants grown in virgin soils suffered from Phytophthora root rot. This may be due to the planting of infected plantlets or due to prior presence of the pathogen on weeds, including common purslane (*Portulaca oleracea*), that may serve as an alternative host for *P. capsici* (Ploetz *et al.*, 2002).

Black pepper is a climbing vine that needs a support plant for climbing. Several types of support plants are used such as *Wrightia annamensis*, *Leucena leucocephala*, *Erythrina* spp., *Artocarpus heterophyllus*, *Spondias pinnata*, *Alstonia scholaris* and *Ceiba* spp., but also cement columns are used for support. This survey showed that in almost all orchards (97%) live plants were used as support for the black pepper vine. The type of support plants used depends on the region and climate. The density of support plants is about 2,000 - 2,500 per hectare and approximately 5-7 vines of black pepper climb in one support plant. In 2006, there was a significant reduction in the density of black pepper plants in the three districts from 2,071 plants ha^{-1} in 2005 to 1,618 plants ha^{-1} in 2006. This reduction in density is partly due to foot rot caused by *P. capsici* and also due to the low price of black pepper since 2000

(VnEconomy, 2007). In July 2005, the price of exported pepper was reduced to 1,140 USD ton^{-1} (Thông Tấn Xã Việt Nam, 2005) from a high point of 3,200 – 3,450 USD ton^{-1} prior (Nguyen & Bach, 2007). Therefore, many farmers started to replace black pepper plants with rubber and other high value crops. The Vietnamese Pepper Association (VPA) and Ministry of Agriculture and Rural Development currently recommend that farmers should not extend the area under cultivation for black pepper, but instead focus on selected existing black pepper areas and improve the quality and yield (Nông Thôn Ngày Nay, 2003). In order to increase and maintain the yield during the lifespan of the pepper orchard, an integrated approach to Phytophthora management is needed. This may include a combination of disease free planting material, the use of antagonistic and plant growing promoting micro-organisms, avoiding of highly susceptible pepper varieties, site selection, drainage, irrigation management, the use of cover crops, and orchard floor management in addition to application of fungicides. It is also important that disease symptoms are correctly identified at the early stage by the growers and that appropriate fungicides are applied in a timely and safe manner. The current use of a range of different chemical products with limited effect or efficacy is of real concern and needs urgent attention.

Materials and methods

According to the survey of the Institute of Planning and Agricultural Design (Ministry of Ariculture and Rural Development) conducted in 1999-2003, black pepper cultivation in Vietnam occurs mainly in the provinces Binh Phuoc (12,000 ha), Dak Lak (9,000 ha), Ba Ria-Vung Tau (5,400 ha), Dong Nai (4,100 ha), Gia Lai (3,800 ha) and Quang Tri (2,400 ha) (Nông Thôn Ngày Nay, 2003). In the central region of Vietnam, Quang Tri province is an area famous for black pepper production. In Quang Tri, black pepper is mainly grown in Cam Lo, Gio Linh and Vinh Linh (Table 1). These three districts include one highland (Cam Lo) and two lowland sites (Gio Linh and Vinh Linh), and were chosen for the surveys in February 2005 and 2006 on the occurrence and distribution of *Phytophthora* foot rot of black pepper. In each district, two communes were selected and 15 households per commune were randomly chosen with more than 100 black pepper plants per orchard. Each household was interviewed and several parameters, including the number of black pepper plants, the size of the orchard, the amount and frequency of fertilizer applications, nursery sanitation, and the types and frequency of fungicide applications to control foot rot disease were recorded for each year. In addition, the number of black pepper plants and foot rot incidence was scored in each orchard to confirm the data provided by the farmer and to obtain an accurate assessment of disease incidence. Foot rot caused by *P. capsici* was scored based on the typical symptoms described by Nguyen (2004) and shown in Figure 1. For each orchard, also the cropping history was surveyed and divided into three categories; orchards where black pepper was grown for at least 5 years or more were assigned to the first category; category 2 comprised orchards where other crops, including various vegetables, sweet potatoes, and groundnut, were grown at least 5 years prior to black pepper cultivation; orchards where no other crops were cultivated (virgin soils) prior to black pepper cultivation were assigned to category 3.

Disease incidence

The incidence of foot rot was assessed through visual inspection of all plants in each pepper orchard. Although 5-7 cuttings may have been used around one supporting pole or plant to produce a clump of vines, the presence of foot rot in one individual plant was rated as a positive for the whole clump for the purpose of this paper. The number of clumps per ha is about 2,000.

Statistical analysis

Each black pepper orchard was taken as an experimental unit. The wilt incidence (%) was arcsin-transformed prior to statistical analysis to meet the required conditions for analysis of variance (ANOVA). For 2005 and 2006, differences in wilt incidence between districts, year and orchards with different cropping histories and management strategies were analysed by one-way ANOVA followed by Tukey and T-test. The frequency of households that treat black pepper stem cuttings with fungicides and the cropping history in black pepper orchards were analysed by Chi-square (SPSS, version 12.01).

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Chapter 5

Diversity and activity of biosurfactant-producing *Pseudomonas* in the rhizosphere of black pepper in Vietnam

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Diversity and activity of biosurfactant-producing *Pseudomonas* in the rhizosphere of black pepper in Vietnam

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Abstract

Aims. *Phytophthora capsici* is a major pathogen of black pepper and zoospores play an important role in the infection process. Fluorescent Pseudomonads that produce biosurfactants with zoosporicidal activities were isolated from the black pepper rhizosphere in Vietnam, and their genotypic diversity and potential to control *P. capsici* root rot was determined.

Methods. Biosurfactant-producing Pseudomonads were genotypically and biochemically characterized by BOX-PCR, 16S-rDNA sequencing, RP-HPLC and LC-MS analyses.

Results. Biosurfactant-producing fluorescent Pseudomonads make up approximately 1.3% of the culturable *Pseudomonas* population in the rhizosphere of black pepper. Although BOX-PCR revealed substantial genotypic diversity, the isolates were shown to produce the same biosurfactants and were all identified as *Pseudomonas putida*. When applied to black pepper stem cuttings, several of the biosurfactant-producing strains provided significant disease control. In absence of the disease, several of the bacterial strains promoted shoot and root growth of black pepper stem cuttings.

Conclusions. Biosurfactant-producing Pseudomonads indigenous to the rhizosphere of black pepper plants are genotypically diverse and provide a novel resource for the control of *P. capsici* root rot and growth promotion of black pepper stem cuttings.

Significance and impact of study. The results of this study provide a strong basis for further development of supplementary strategies with antagonistic bacteria to control foot and root rot of black pepper and to promote plant growth.

Keywords: *Pseudomonas*, cyclic lipopeptide surfactants, *Phytophthora capsici*, black pepper

Introduction

Black pepper (*Piper nigrum* L.) is known as the 'King of Spices'. It is propagated vegetatively and stem cuttings used for propagation are raised mainly from runner shoots (George et al. 2005). *Phytophthora capsici* Leonian causes foot and root rot of black pepper referred to as 'sudden wilt'. It is a destructive and economically important pathogen of black pepper, occuring wherever the crop is grown (Drenth and Sendall 2004a; George et al. 2005). *P. capsici* can infect all plant parts during all growth stages. In black pepper cultivation, yield losses caused by *P. capsici* can amount up to 40-50% (Erwin and Robeiro 1996; Drenth and Sendall 2004a). In Vietnam, *P. capsici* causes an estimated annual loss of 15-20% (Erwin and Ribeiro 1996; Drenth and Sendall 2004a). This was confirmed in a survey conducted in 2005 and 2006 in the Quang Tri Province, one of the major black pepper areas in Vietnam, where *P. capsici* wilt incidence ranged from 12-30% (H. Tran and J.M. Raaijmakers unpublished data). Current methods to control *P. capsici* include cultural practices, the use of resistant varieties, fungicide application and root infusion with phosphonates (Wong 2004; Manohara et al. 2003; Drenth and Guest 2004; Nguyen 2004a). However, most of these control methods are not effective and many chemical control measures, including metalaxyl-based fungicides and phosphonates, are too expensive for local farmers. Moreover, in Vietnam no black pepper varieties with resistance to *P. capsici* are used (Dr. N.T. Ton, *pers. comm.*).

Biological control has been proposed as a potential (supplementary) strategy to control *P. capsici* on black pepper (Anandaraj and Sarma 2003). In this context, several antagonistic microorganisms, including *Trichoderma*, *Alcaligenes* and fluorescent *Pseudomonas* species, have shown promising results in the control of *P. capsici* on black pepper (Anandaraj and Sarma 1995; Jubina and Girija 1998; Anith and Manomahandas 2001; Anith et al. 2002, 2003; Diby et al. 2005a; Diby and Sarma 2006). An interesting group of microorganisms with potential for biocontrol of *P. capsici* are biosurfactant-producing bacteria. Biosurfactants, including rhamnolipids and cyclic lipopeptides (CLPs), disrupt the membrane integrity of *Phytophthora* zoospores leading to complete elimination of infectious zoospore populations within minutes (Stanghellini and Miller 1997; De Souza et al. 2003; De Jonghe et al. 2006; De Bruijn et al. 2007). Under moist conditions, motile zoospores of *P. capsici* play a crucial role in the infection of roots of black pepper and are a potential target to control foot and root rot. Moreover, purified rhamnolipids and CLPs also inhibit hyphal growth of *P. capsici* and other oomycete pathogens (Thrane et al. 1999, 2000; Kim et al. 2000; De Jonghe et al. 2006). In hydroponic systems, control of *P. capsici* root rot of pepper (*Capsicum annuum* L.) was achieved by addition of synthetic- or microbial biosurfactants to the recirculating nutrient solution (Stanghellini et al. 1996; Stanghellini and Miller 1997; Stanghellini et al. 2000; Nielsen et al. 2006). For the control of *P. capsici* foot and root rot of stem cuttings and black pepper plants grown in field soils, however, application of the pure biosurfactants will most likely be less effective due to the difficulty of delivering these compounds along the root system to the sites of pathogen infection and due to degradation by other soil microorganisms. In this context,

application of biosurfactant-producing bacteria that are well-adapted to the rhizosphere of black pepper plants is expected to be more effective in reaching the sites of pathogen infection and to effectively deliver the bioactive metabolites. Moreover, bacteria may colonize and persist on the roots, thereby providing long-term protection against pathogens.

In this study, fluorescent *Pseudomonas* bacteria that produce biosurfactants with zoosporicidal activities were isolated from the rhizosphere of healthy black pepper plants grown in the field in Vietnam. The genotypic diversity of the isolated bacteria was determined by BOX-PCR and 16S-rDNA sequencing. The biosurfactants produced by selected bacterial isolates, representative of the different genotypic groups, were classified by RP-HPLC and LC-MS analyses. The potential of these bacterial isolates to control foot and root rot caused by *P. capsici* and their ability to promote root and shoot development of black pepper plants was evaluated in greenhouse assays in Vietnam and compared to the activities of *P. fluorescens* strain SS101, a well-studied biosurfactant-producing strain with biocontrol activity against *Pythium* species (De Souza et al. 2003; De Boer et al. 2006) and *Phytophthora infestans* (Tran et al. 2007).

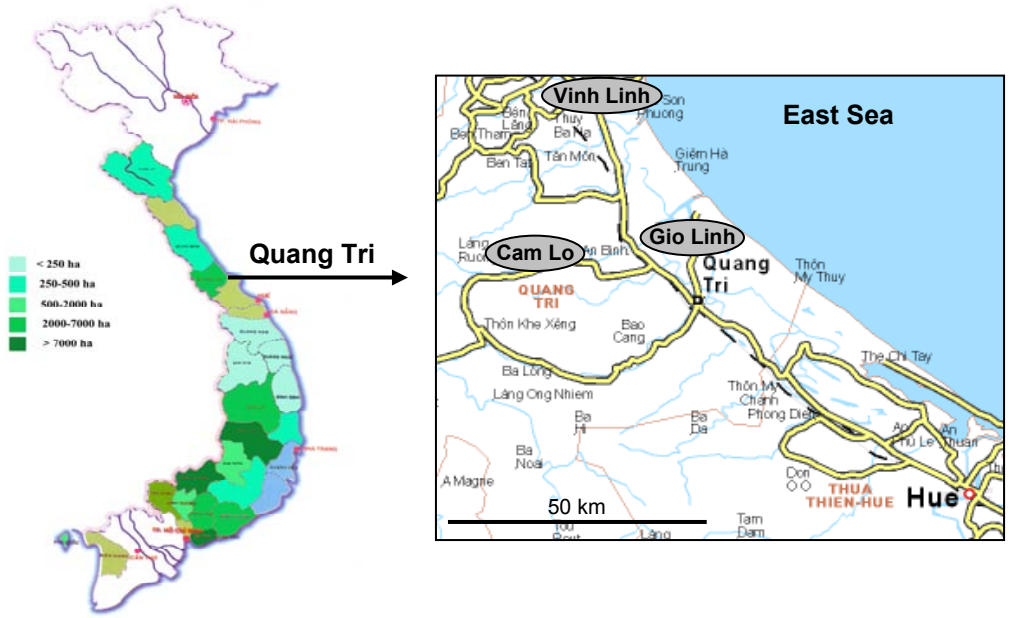

Figure 1: Map of the black pepper production areas in Vietnam in hectares per province. Rhizosphere samples were taken from roots of black pepper stem cuttings and plants grown in three districts in Quang Tri province indicated in the insert (obtained from www.multimap.com).

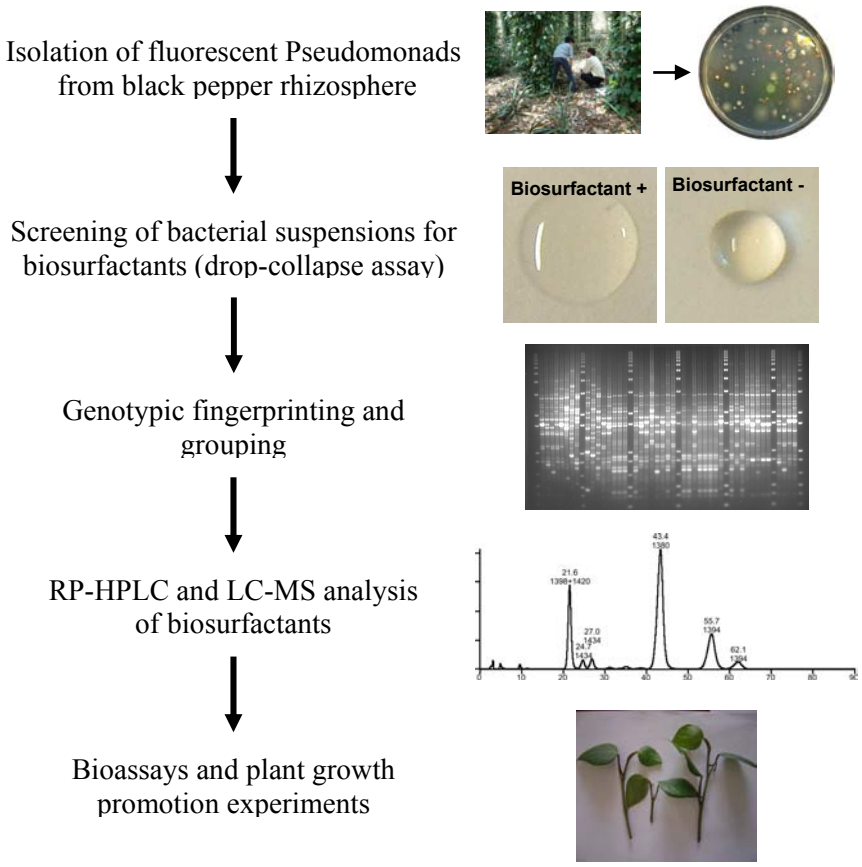

Figure 2: Schematic presentation of the approaches and techniques used to isolate, identify and characterize biosurfactant-producing Pseudomonads from the rhizosphere of black pepper plants grown in field soil in Vietnam.

Results

Frequency of biosurfactant-producing *Pseudomonas* in the black pepper rhizosphere

Sampling was done in three different districts (i.e. Vinh Linh, Gio Linh and Cam Lo) of the Quang Tri province located in North Central Vietnam (Figure 1). Rhizosphere samples were obtained from roots of black pepper plants grown in nurseries or from roots of 3- or 5-year-old plants. Nursery samples were collected in Cam Lo and Vinh Linh, but were not available in Gio Linh. For each of the samples, the strategy depicted in Figure 2 was followed to isolate and characterize biosurfactant-producing *Pseudomonas* isolates. In the rhizosphere of black pepper cuttings grown in nurseries, population densities of fluorescent *Pseudomonas* spp. ranged from $1.5x10^6$ to $4.2x10^6$ CFU g^{-1} root fresh weight (Table 1). The density of fluorescent Pseudomonads in the rhizosphere of 3- and 5-year-old plants was on average 10 times higher than for nursery plants. The frequency of biosurfactant-producing bacteria, determined on a total of nearly 14,500 randomly selected colonies, ranged from 0.5-2.4% (Table 1). All 219 biosurfactant-producing isolates, obtained from roots of nursery plants and from 3- and 5-year-old black pepper plants, were used for further analysis.

Table 1: Frequency of biosurfactant-producing fluorescent *Pseudomonas* spp. in the rhizosphere of black pepper stem cuttings grown in nurseries and of black pepper plants (3- and 5-year old) grown in the field. Samples were taken in three districts of Quang Tri province in Vietnam.

District	Nursery				3/5 year old			
	Pseudomonas[a]	biosurfactant[b]			*Pseudomonas*[a]	biosurfactant[b]		
		tested	bios$^+$	%		tested	bios$^+$	%
Cam Lo	$4.2x10^6 \pm 1.1x10^6$	1600	8	0.50	$1.0x\ 10^7 \pm 3.0x10^6$	3600	18	0.50
Gio Linh	na	na	na	na	$4.7x10^7 \pm 1.6x10^7$	3868	83	2.15
Vinh Linh	$1.5x10^6 \pm 0.1x10^6$	1394	13	0.93	$2.1x10^7 \pm 4.1x10^6$	3999	97	2.42
Total		**2974**	**21**	**0.71**		**11467**	**198**	**1.73**

[a]: Population density of fluorescent Pseudomonads expressed as CFU g^{-1} root fresh weight; ± refers to the standard error of the mean.
[b]: Number *Pseudomonas* isolates tested for biosurfactant production; bios$^+$ and % refer to the number and frequency of isolates, respectively, that were positive for biosurfactant production as was determined by the drop collapse assay.
na: not available.

Table 2: Genotypic and biochemical classification of biosurfactant-producing *Pseudomonas* isolates obtained from the rhizosphere of black pepper. A total of 177 isolates obtained from three different districts and from black pepper plants grown in nurseries or field soil were genotypically classified by BOX-PCR and were biochemically grouped on basis of biosurfactant HPLC-profiles.

HPLC-group	BOX-PCR group	Isolates	District[a]	Plant[b]
A	F	54	C G V	N 3 5
A	G	21	V	5
A	I	4	V	N 3 5
A	J	13	V	5
A	L	4	V	5
A	M	6	G	7
A	Q	6	C G	N 3
A	R	3	G	5
Total		**111**	**C G V**	**N 3 5**
B	K	13	G V	N 5
B	N	4	C G	N 3
B	O	3	C	3
B	P	5	C	N 3
Total		**25**	**C G V**	**N 3 5**
C	S	5	G	3
C	U	4	G	3
C	V	10	G	5
C	X	12	G	5
C	Z	6	G	5
Total		**37**	**G**	**3 5**
D	T	4	G	3
Total		**4**	**G**	**3**

[a]: District where the isolates were collected C: Cam Lo; G: Gio Linh; V: Vinh Linh.
[b]: Age of plants from which the isolates were collected. N: nursery; 3: 3-year old; 5: 5-year old; 7: 7-year old.

Genetic and biochemical characterization of biosurfactant-producing Pseudomonads

Based on the fingerprints obtained by BOX-PCR analysis of the 219 isolates, a total of 177 isolates was placed in 18 genotypic groups of three or more isolates with an identical BOX-PCR pattern (Table 2). The remaining 42 isolates consisted of double- or singletons (data not shown) and were not included in further analysis. Many of the 18 major BOX-groups were represented by isolates from all three districts, and from nursery and 3-5 year old plants (Table 2). To further identify the biosurfactants produced by the bacterial isolates, extractions were performed on cell-free culture supernatants of 19

isolates representing the 18 major BOX-groups (for the largest BOX-PCR group F, two isolates were used). Initial RP-HPLC analysis yielded only four different chromatographic patterns, designated HPLC groups A through D. Group A comprised of 111 isolates in total, whereas groups B, C, and D contained 25, 37 and 4 isolates, respectively (Table 2). HPLC group A contained 8, 70 and 33 isolates from Cam Lo, Vinh Linh and Gio Linh, respectively, whereas group B contained 12, 10 and 3 isolates, respectively. HPLC-groups C and D only harbored isolates from Gio Linh. Although four different HPLC patterns were identified, the differences were only observed in peak intensities and not in the peak number or peak retention times (data not shown). This suggests that all isolates tested produce the same biosurfactant(s), albeit in different quantities.

In subsequent analyses, five isolates were selected as representatives of HPLC groups A-D. As group A comprises over half of the biosurfactant-producing isolates, two representatives were chosen from group A. From the other groups, a single isolate was selected. The following isolates were selected: 150-A (BOX-group F, isolated from 5-year-old plant in Gio Linh), 269-A (BOX-group G, isolated from 5-year-old plant in Vinh Linh), 199-B (BOX-group P, isolated from 3-year-old plant in Cam Lo), 267-C (BOX-group X, isolated from 5-year-old plant in Gio Linh) and 214-D (BOX-group T, isolated from 3-year-old plant in Gio Linh). 16S-rDNA sequence analysis revealed that all five isolates are very closely related (99-100% identity) to reference strain *Pseudomonas putida* KT2440 and cluster distant from the *P. fluorescens* biocontrol strains SS101, SBW25 and Pf-5 (Figure 3). Consistent with the results obtained in HPLC analysis, the LC-MS profiles of the biosurfactant extracts were identical for the five isolates (Figure 4) and only differed in peak intensities (data not shown). The molecular masses of three of the peaks (retention times 43.4, 55.7 and 62.1 minutes; molecular masses of 1380 and 1394) in the LC-MS profile of bacterial isolate 150A correspond to those of putisolvin I and putisolvin II, two cyclic lipopeptide surfactants produced by *P. putida* strain PCL1445 (Kuiper et al. 2004).

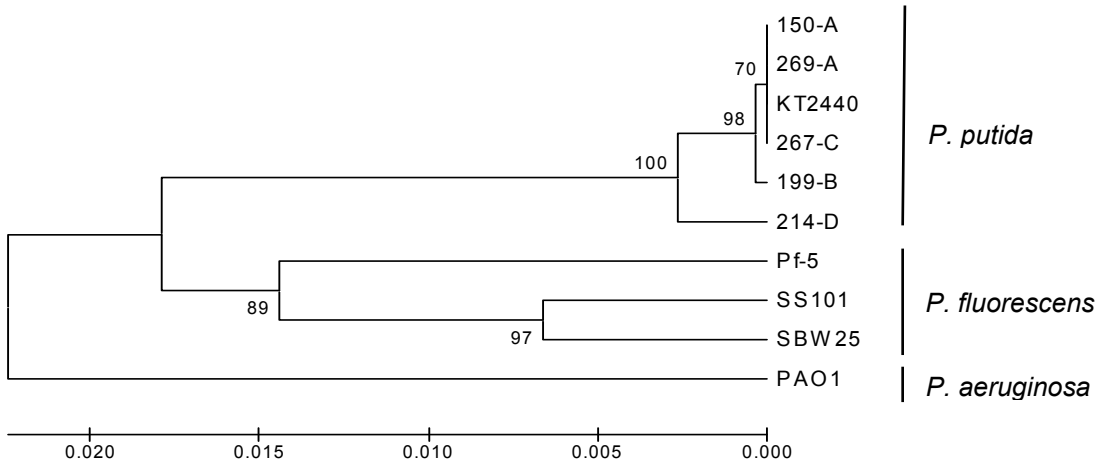

Figure 3: Phylogenetic relationships between 16S-rDNA sequences of five selected biosurfactant-producing isolates from black pepper rhizosphere (150-A, 269-A, 199-B, 267-C and 214-D) and reference strains *Pseudomonas putida* KT2440, *P. aeruginosa* PAO1 and *P. fluorescens* Pf-5, SBW25 and SS101. The branch length indicates the percentage of sequence dissimilarity and numbers at the nodes indicate bootstrap values. Bootstrap values above 70% are indicated.

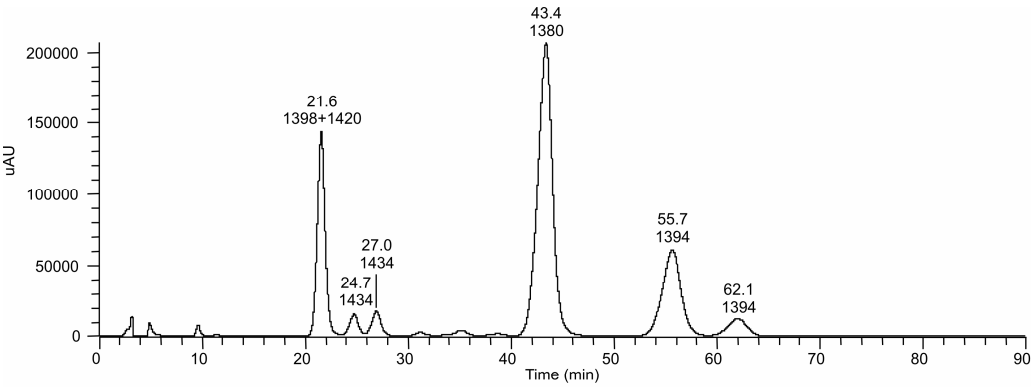

Figure 4: RP-HPLC chromatogram (206 nm) of the biosurfactant extract of *Pseudomonas putida* strain 150-A. Numbers above the peaks indicate the retention time (minutes) and the molecular masses as determined by LC-MS.

Zoosporicidal activity and phenotypic characterization

Cell suspensions (10^9 cells ml^{-1}) of each of the five representative isolates significantly reduced the surface tension of water (Table 3), consistent with the production of biosurfactants. Cell suspensions and cell-free culture supernatants of all five isolates caused cessation of zoospore motility of *P. capsici* and *P. infestans,* followed by rounding off of the zoospores. Lysis of *P. capsici* zoospores occurred within 90 seconds for each of the five isolates, whereas lysis caused by reference strain SS101 occurred within 60 seconds. In contrast, *P. infestans* zoospores were rendered immotile in 15-20 seconds and lysis occurred within 75 second when exposed to suspensions of the five isolates. When grown in dual plate cultures on $1/5^{th}$ strength potato dextrose agar and V8 agar, none of the five isolates nor reference strain SS101 showed inhibitory activity against hyphal growth of *P. capsici* (data not shown). None of the isolates were positive for the production of extracellular proteases or hydrogen cyanide (data not shown). However, all five isolates from the rhizosphere of black pepper were positive for the production of IAA (Table 3).

Biocontrol activity

The biocontrol activity of the five isolates 150-A, 269-A, 199-B, 267-C and 214-D was tested on stem cuttings transplanted into a soil from a black pepper plantation that was naturally and heavily infested with *P. capsici*. Strain *P. fluorescens* SS101 was included as a reference. Surface-sterilized stem cuttings were treated for 30 minutes with bacterial suspensions (10^9 cells ml^{-1}) or water (control) before transplanting. The effect of each of the applied bacterial strains on wilt incidence was determined 60 and 90 days after transplanting (Figure 5). Whether *P. capsici* was the sole causal agent of the wilting symptoms observed could not be confirmed in this study due to the inherent difficulty of isolation and subsequent identification of *P. capsici* from infested soil and plant tissue (Drenth and Sendall 2004b). At both time points, isolates 150-A and 214-D provided a significant level of disease control; also strain SS101 provided significant disease control. Isolate 267-C initially reduced wilt incidence significantly,

but this effect was not maintained at 90 days post inoculation (dpi). The population densities of the applied bacterial strains present on the cuttings (roots removed) at 30 and 60 dpi were (expressed as log CFU g^{-1} fresh weight) 4.30 and 3.04 (strain 150-A); 4.96 and 3.48 (strain 269-A); 3.82 and 3.35 (strain 199-B); 4.20 and 2.93 (strain 267-C); 4.33 and 3.28 (strain 214-D); and 4.11 and 3.17 (strain SS101), respectively.

Table 3: Phenotypic characteristics of selected biosurfactant-producing *Pseudomonas putida* isolates obtained from the rhizosphere of black pepper. *P. fluorescens* strain SS101 was used as a reference and water was used as a control.

Isolate	Drop collapse[a]	Surface tension[b]	Zoospore motility[c]	Zoospore lysis[d]	IAA production[e]
Water	-	71.2	-	-	-
150A	+	29.5	+	+	+
269A	+	32.5	+	+	+
199B	+	31.0	+	+	+
267C	+	30.0	+	+	+
214D	+	29.7	+	+	+
SS101	+	29.7	+	+	-

[a]: Droplets (5 µl) of bacterial cell suspensions (OD_{600nm} =1) were tested in the drop-collapse assay; + indicates a drop collapse.
[b]: Surface tension (mN/m) of the bacterial cell suspensions (OD_{600}=1).
[c]: Effect of bacterial isolates on zoospore motility was determined microscopically by mixing bacterial cell suspensions (OD_{600}=1) with zoospores (10^4 zoospores ml^{-1}) in a 1:1 ratio (v/v). Zoospores of *Phytophthora capsici* UQ6184, *P. capsici* UQ3691, and *P. infestans* 90128 were tested; "+" indicates cessation of zoospore motility for all oomycetes tested.
[d]: Effect of bacterial isolates on zoospore lysis was determined microscopically by mixing bacterial cell suspensions (OD_{600}=1) with zoospores (10^4 zoospores ml^{-1}) in a 1:1 ratio (v/v). Zoospore lysis was tested for *P. capsici* UQ3691 and *P. infestans* 90128; "+" indicates zoospore lysis.
[e]: + indicates *in vitro* production of indole acetic acid (IAA).

Plant growth promotion

To determine the effects of the five bacterial isolates and reference strain SS101 on shoot and root growth of black pepper stem cuttings, bioassays were conducted with a soil collected from a groundnut field in Vinh Linh where black pepper was never grown. Surface-sterilized stem cuttings were treated for 30 minutes with bacterial suspensions or water (control) before transplanting. During the course of the experiment, none of the plants showed disease symptoms nor did they show wilting symptoms typical for foot and root rot caused by *P. capsici*. All five isolates 150-A, 269-A, 199-B, 267-C and 214-D exhibited a positive effect on shoot height and weight (Figure 6) and on root growth (Figure 7). For several of the five *P. putida* strains, shoot height and weight increased significantly compared to the control treatment (Figure 6). Moreover, most of the bacterial treatments also led to a significant increase in the number of roots per stem cutting and root length (Figure 7). Reference strain SS101 also slightly increased shoot weight but not to the same level as most of the other bacterial isolates (Figure 6). Application of strain SS101 did not lead to significant increases in the number of roots per cutting and root length (Figure 7). Interestingly, most of the five *P. putida* strains induced root development at the base of the stem cuttings and the first node, whereas in the control treatment most of the new roots were formed at the first node (Figure 7A). After 60 days of plant growth, the population densities of the five *P. putida* strains on the roots were similar and ranged from 4.4-5.8 Log CFU g^{-1} of root fresh weight;

reference strain SS101 established on the roots of black pepper stem cuttings at a density of 5.8 Log CFU g^{-1}.

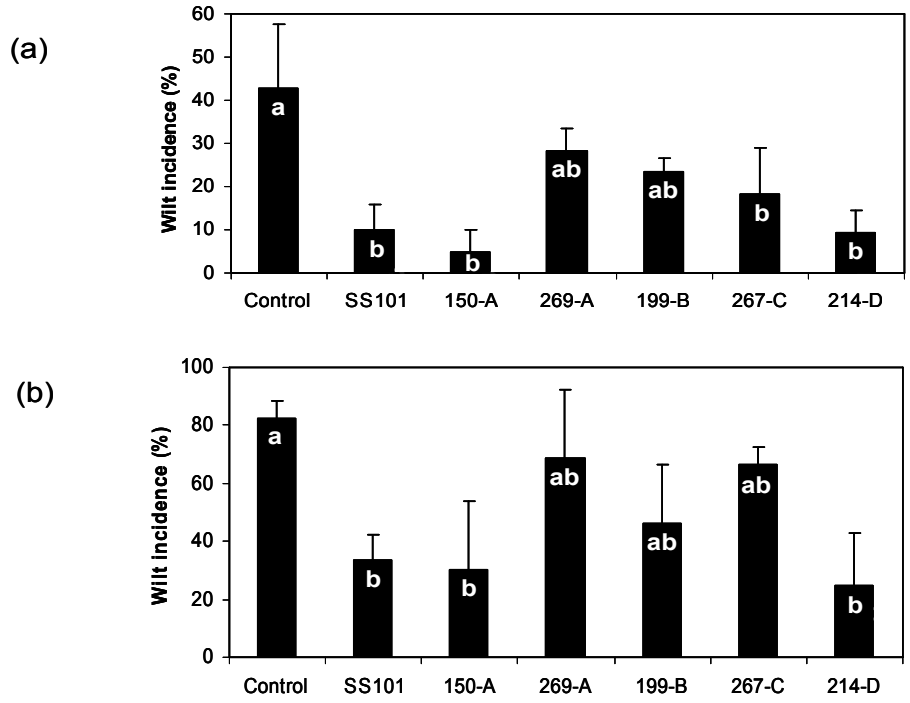

Figure 5: Effect of biosurfactant-producing bacteria on wilt incidence of black pepper stem cuttings grown in field soil naturally infested with *Phytophthora capsici*. Different bacterial strains (indicated below the bars) were applied to the black pepper stem cuttings and transplanted into infested soil. In the control treatment, no bacteria were applied. Wilt incidence of the stem cuttings was determined after 60 days (panel a) and 90 days (panel b) of plant growth. Means of 4 replicates are shown and error bars represent the standard errors. Different letters inside the bars indicate a statistically significant difference ($p<0.05$).

Discussion

Biosurfactants are versatile microbial metabolites with potential for a wide array of applications, including the control of plant and human pathogens (Nybroe and Sørensen 2004; Baltz et al. 2005; Raaijmakers et al. 2006). The results of this study show that among a total of nearly 14,500 fluorescent Pseudomonads, randomly selected from the rhizosphere of black pepper plants grown in nurseries or in the field in Vietnam, approximately 1.3 % produced biosurfactants. This is comparable to the frequency of 1-5% reported previously by De Souza et al. (2003) for biosurfactant-producing fluorescent Pseudomonads isolated from the wheat rhizosphere, but relatively low compared to the sugar beet rhizosphere, where frequencies of CLP-producing Pseudomonads ranged from 6 to 60% depending on the soil type (Nielsen et al. 2002). Indeed, both the soil type and the plant species are known to have a significant impact on the community structure and densities of *Pseudomonas* populations (Lemanceau et al. 1995; Latour et al. 1996; Bergsma-Vlami et al. 2005). It should be emphasized, however, that the frequencies reported in this and other studies are most likely underestimates of the actual number of

biosurfactant-producing Pseudomonads as culture conditions, used to grow the bacterial isolates prior to the drop-collapse assay, may influence the in vitro production of detectable levels of biosurfactants. biosurfactants. This is comparable to the frequency of 1-5% reported previously by De Souza et al. (2003) for biosurfactant-producing fluorescent Pseudomonads isolated from the wheat rhizosphere, but relatively low compared to the sugar beet rhizosphere, where frequencies of CLP-producing Pseudomonads ranged from 6 to 60% depending on the soil type (Nielsen et al. 2002). Indeed, both the soil type and the plant species are known to have a significant impact on the community structure and densities of *Pseudomonas* populations (Lemanceau et al. 1995; Latour et al. 1996; Bergsma-Vlami et al. 2005). It should be emphasized, however, that the frequencies reported in this and other studies are most likely underestimates of the actual number of biosurfactant-producing Pseudomonads as culture conditions, used to grow the bacterial isolates prior to the drop-collapse assay, may influence the in vitro production of detectable levels of biosurfactants.

The results also demonstrated that in spite of substantial genotypic diversity of the 219 selected biosurfactant-producing isolates, the isolates tested (representing 81% of all isolates) produced the same biosurfactants based on RP-HPLC and LC-MS analyses. In contrast to this uniformity in biosurfactant production among isolates from the rhizosphere of black pepper, CLP-producing Pseudomonads from the rhizosphere of sugar beet plants were grouped into 8 major groups, producing in total at least nine structurally different CLPs (Nielsen et al. 2002). Isolates from the Danish soils were classified as *Pseudomonas fluorescens* biotypes I, V and VI (Nielsen et al. 2002), whereas the representative isolates in this study were all classified as *Pseudomonas putida*. Based on the molecular masses of the individual HPLC peaks, the biosurfactants produced by the *P. putida* isolates from black pepper are most likely CLPs resembling the putisolvins I and II described for *P. putida* strain PCL1445 (Kuiper et al. 2004). MS-MS and NMR analyses will be required to further resolve the structures of the individual peaks and to verify if the produced biosurfactants are in fact CLPs. Putisolvins I and II contribute to swarming of the producing strain and inhibit the formation of biofilms of other *Pseudomonas* strains (Kuiper et al. 2004). A role for the putisolvins in biological control of plant diseases, however, has not yet been reported.

P. putida isolates 150-A, 269-A, 199-B, 267-C and 214-D and their cell-free culture supernatants resulted in lysis of zoospores of *P. capsici* and *P. infestans*. Several other surfactants, including massetolide A from *P. fluorescens* SS101 (De Souza et al. 2003), the viscosin-like CLP from *P. fluorescens* SBW25 (De Bruijn et al. 2007), the CLP orfamide from *P. fluorescens* Pf-5 (Gross et al. 2007) and rhamnolipids from *P. aeruginosa* (Stanghellini and Miller 1997; Kim et al. 2000) also have zoosporicidal activity, whereas other biosurfactants like viscosinamide induce encystment of zoospores (Thrane et al. 1999). Although lysis of zoospores by synthetic- or microbial surfactants may contribute, at least in part, to the control of several oomycete diseases (Stanghellini et al. 1996; Stanghellini and Miller 1997; Kim et al. 2000; Stanghellini et al. 2000; De Souza et al. 2003; Nielsen et al. 2006; Tran et al. 2007), biosurfactants may also reduce the growth and intracellular activity of oomycete and fungal

pathogens (Thrane et al. 1999, 2000; Kim et al. 2000) and induce resistance in plants leading to a reduction in disease severity (Ongena et al. 2007; Tran et al. 2007).

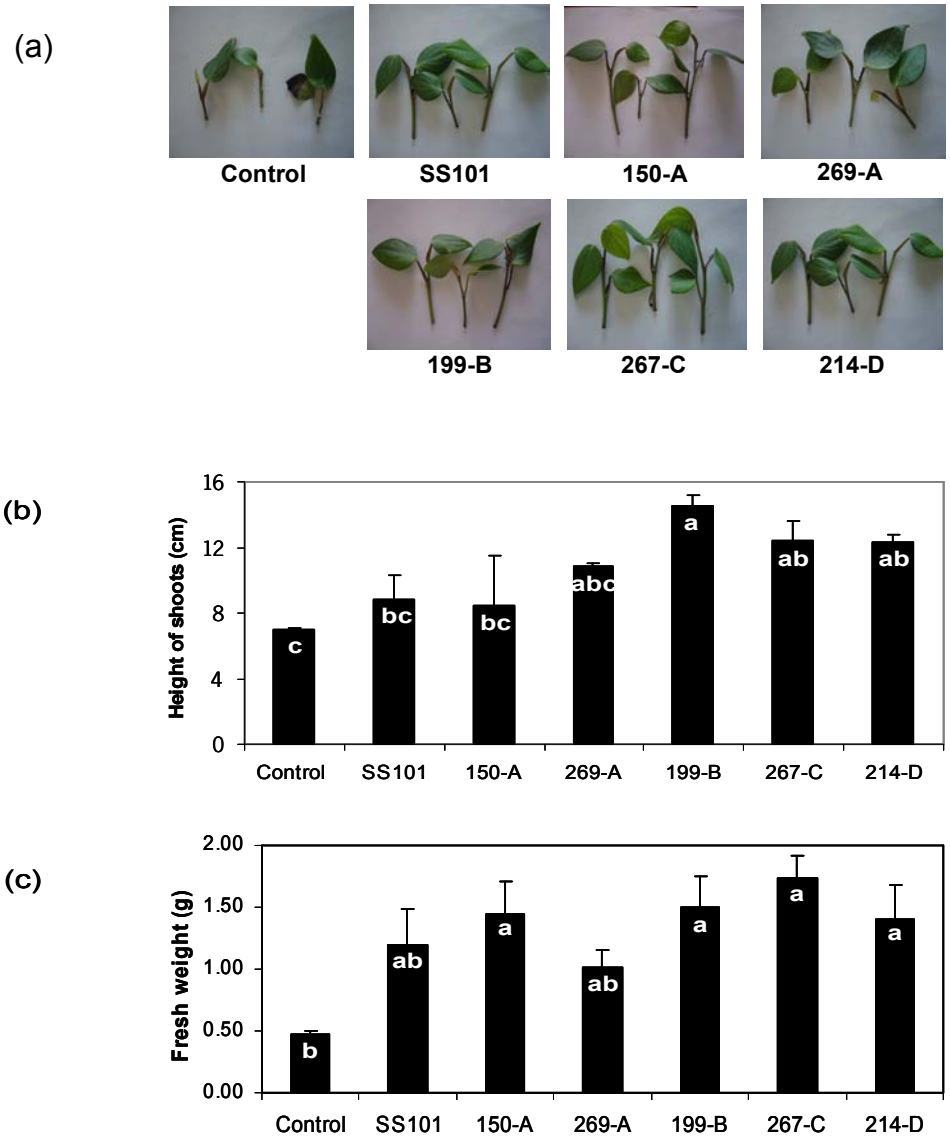

Figure 6: Effect of biosurfactant-producing bacteria on shoot growth of black pepper stem cuttings. Different bacterial strains (indicated below the bars) were applied to the black pepper stem cuttings and transplanted into field soil. In the control treatment, no bacteria were applied. Panel a shows representative pictures of the shoots of the stem cuttings 90 days after treatment with the different bacterial strains. Shoot height (panel b) and fresh weight (panel c) were determined afer 90 days of plant growth. Means of 4 replicates are shown and error bars represent the standard errors. Different letters inside the bars indicate a statistically significant difference ($p<0.05$).

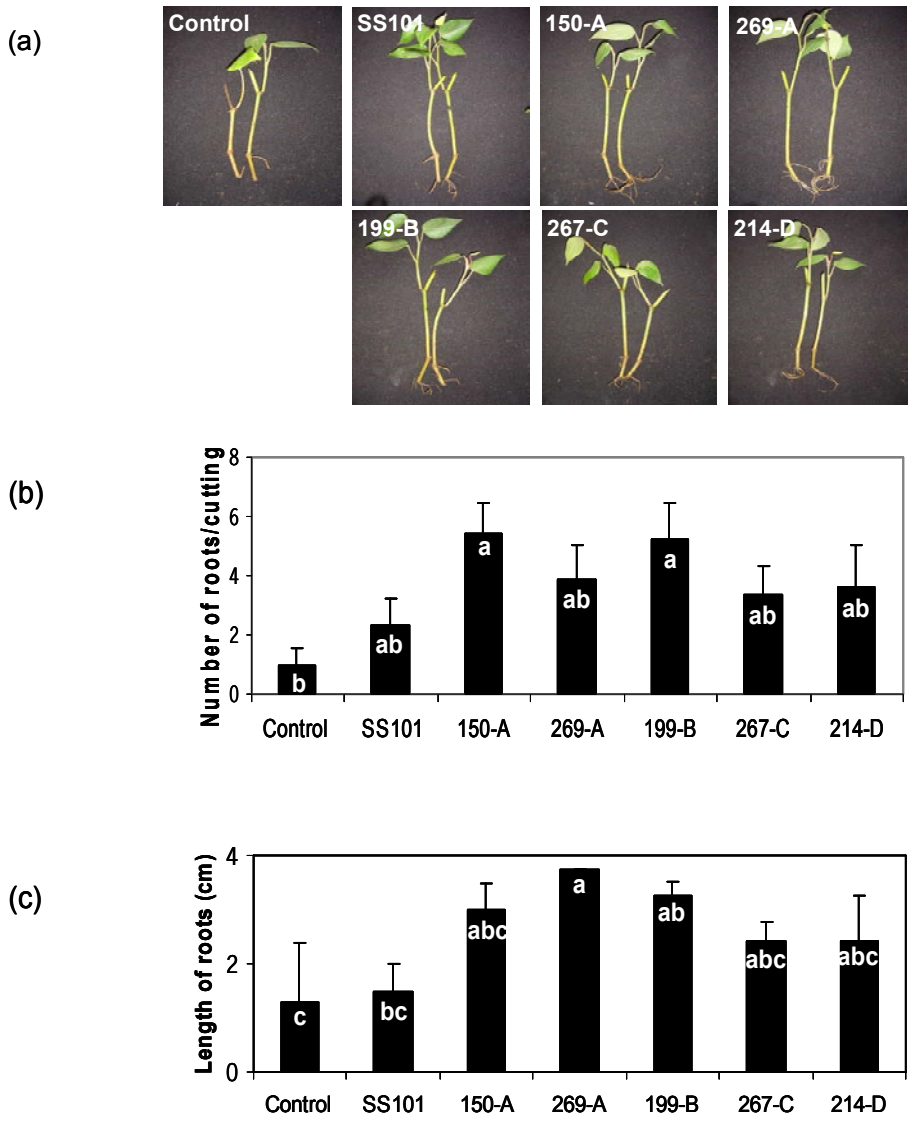

Figure 7: Effect of biosurfactant-producing bacteria on root development of black pepper stem cuttings. Different bacterial strains (indicated below the bars) were applied to the black pepper stem cuttings and transplanted field soil. In the control treatment, no bacteria were applied. Stem cuttings were planted in soil with the first node (from the base) located below the soil surface. Panel a shows representative pictures of the stem cuttings 90 days after treatment with the different bacterial strains. The number of roots per stem cutting (panel b) and root length (panel c) were determined afer 90 days of plant growth. Means of 4 replicates are shown and error bars represent the standard errors. Different letters inside the bars indicate a statistically significant difference ($p<0.05$).

In the present study, *P. putida* isolates 150-A and 214-D provided significant control of wilt incidence of black pepper stem cuttings grown in a field soil infested by *P. capsici*. Although isolates 269-A, 199-B, and 267-C colonized the roots of the black pepper stem cuttings to a similar level as isolates 150-A and 214-D (approximately 10^5 CFU g^{-1} after 60 days), they showed an intermediate level of disease control. Whether biosurfactant-mediated zoospore lysis is a main mode of action by which isolates 150-A and 214-D suppressed wilt incidence can not be concluded from the present study. Bioassays with well-characterized mutants deficient in biosurfactant production will be required to

further assess the role of the biosurfactants in control of wilt incidence of black pepper. Compared to other studies on biocontrol of *P. capsici* root rot of black pepper, the *P. putida* isolates obtained in this study provided equal or more promising effects. For example, in the study by Jubina and Girija (1998), *Trichoderma harzianum* and *Pseudomonas fluorescens* lost their biocontrol effect at 30 and 60 days, respectively. In contrast, *Bacillus* spp. isolates B5, B7 and B13, which were isolated from black pepper in Kerala, provided prolonged protection against root rot of black pepper in nurseries and reduced the wilt incidence from 100% in the control to 57-71% in the bacterial treatments (Jubina and Girija, 1998). In the study of Anith et al. (2003), *Pseudomonas* spp. isolate PN-026R reduced *P. capsici*-induced wilt incidence in nurseries at 90 days by approximately 55%.

Diby, Sarma and colleagues identified fluorescent *Pseudomonas* bacteria with both antagonistic as well as plant-growth promoting effects (Diby et al. 2005; Diby and Sarma 2006). In our study, the *P. putida* isolates also had a significant positive effect on shoot height and weight, and on root number and length. The observed effects on shoot and root growth suggest that in addition to their biocontrol activities, these isolates also may have a growth-promoting effect although suppression of other 'minor' pathogens can not be excluded in the experimental setup used. Interestingly, all five *P. putida* isolates were shown to induce the formation of roots at the base of the stem cuttings. In a recent survey in Vietnam, it was found that similar effects on root development are induced when stem cuttings are treated with naphthalene acetic acid (NAA) and indole butyric acid (IBA) (H. Tran and J.M. Raaijmakers, *unpublished results*). Combined with the observation that all five *P. putida* isolates are able to produce IAA in vitro, a potent plant hormone that stimulates root growth (Lambrecht et al. 2000), may suggest that this trait contributes to enhanced root growth of the black pepper stem cuttings treated with the bacterial isolates. Future studies, involving IAA-deficient mutants, will be needed to resolve the role of IAA in enhanced root development of black pepper stem cuttings.

In conclusion, this study provided for the first time an extensive survey of the frequency, diversity and activity of biosurfactant-producing Pseudomonads in the rhizosphere of black pepper, the most important spice crop in the world. In spite of the substantial genotypic diversity of the *Pseudomonas* isolates, the biosurfactants produced by the isolates appear to be the same, suggesting that the plant species, cultivar and/or the soil type exerted a specific selection pressure on the type of biosurfactant produced by the Pseudomonads. More rhizosphere samples, obtained from other black pepper regions in Vietnam will be collected and analysed to shed more light on the chemical and genotypic diversity within this group of microorganisms. From a more applied perspective, this study provides a strong basis for further development of supplementary strategies with indigenous antagonistic bacteria to suppress foot and root rot of black pepper and to promote shoot and root growth of the 'King of Spices'.

Materials and methods

Microorganisms and growth conditions

Pseudomonas isolates were routinely grown at 25 °C on Pseudomonas Agar (PSA, Difco, France). Spontaneous rifampicin-resistant derivatives of selected *Pseudomonas* strains were generated by plating an overnight liquid culture on PSA with rifampicin (100 µg·ml^{-1}). Strain integrity was confirmed by BOX-PCR. *Phytophthora capsici* strains UQ3691 and UQ6184 were kindly provided by Dr. André Drenth (University of Queensland, Brisbane, Australia). They were routinely grown at 25 °C on V8 juice agar (V8, N.V. Campell Foods, Puurs, Belgium), amended with 3 g l^{-1} $CaCO_3$ and 15 g l^{-1} agar. *P infestans* strain 90128 was obtained from Dr. Francine Govers (Wageningen University, The Netherlands). It was routinely grown at 18°C on rye sucrose agar (Latijnhouwers et al. 2004) for 7-9 days in the dark.

Isolation of biosurfactant-producing *Pseudomonas* species

Three areas (Cam Nghia, Gio An and Ho xa) in three different districts (Cam Lo, Gio Linh and Vinh Linh, respectively) in the Quang Tri Province in Vietnam were selected for sampling (Figure 1). The soil type in all these districts is red basalt. Quang Tri is located in central Vietnam and approximately 2,400 ha are used for black pepper production (Figure 1). Rhizosphere samples were collected from healthy black pepper plants in different growth stages (nursery, 3 and 5-year-old plants). For the nurseries, a total of 16 samples were collected from 6 orchards (3 orchards in Cam Lo and 3 in Vinh Linh). From 3 year-old black pepper plants, a total of 24 rhizosphere samples were obtained (8 samples per district) from 22 orchards (8 orchards in Cam Lo, 9 in Gio Linh, and 5 in Vinh Linh). From 5 year-old plants, 28 samples were obtained from 28 orchards (8 in Cam Lo, 8 in Gio Linh, and 12 in Vinh Linh). In total, 68 rhizosphere samples were collected and dilution plated. Roots with tightly adhering soil (rhizosphere samples) were obtained from a depth of at least 20 cm from the soil surface. Rhizosphere suspensions were prepared by grinding approximately 1 gram (fresh weight) of root sample in 5 ml of sterile water, followed by vortexing the suspensions vigorously for 1 min, sonicating for 1 min (ELMA Ultrasonics LC-130H, Singen, Germany), and vortexing again for 1 min. The suspensions were serially diluted and plated onto PSA supplemented with chloroamphenicol (13 µg·ml^{-1}) and ampicillin (40 µg·ml^{-1}), a medium semi-selective for *Pseudomonas* (Simon and Ridge 1974). Delvocid (DSM, Delft, The Netherlands) was added to the medium at 100 µg·ml^{-1} to prevent fungal growth. From each sample, 120 randomly selected fluorescent colonies (selected under near-UV light) were transferred to and purified on PSA and subsequently tested, in duplicate, for biosurfactant production by the drop-collapse assay described previously (De Souza et al. 2003).

Genotypic characterization and grouping of isolates

BOX-PCR was carried out as described by Rademaker et al. (1997). Bacterial suspensions were prepared from freshly grown bacterial colonies by transfer of a small bacterial colony to 50 µl sterile demineralised water. One µl of the bacterial suspension was used as a template in 25 µl BOX-PCR reactions. The BOX-PCR fragments were separated on a 25-cm-long, 1.5% agarose gel at 40 Volts for 16 h in 1x TAE buffer; the 1-Kb plus DNA ladder (Invitrogen, Frederick, Maryland, U.S.A.) was used as marker. The gel was stained afterwards for 30 min in 1x TAE supplemented with 20 ng ml^{-1} ethidium bromide. Fragments from 200 to 2250 bp were scored visually for presence or absence. The binary data were imported in FreeTree (Pavlicek et al. 1999) and phylogenetic analysis was performed using UPGMA and 1000 bootstraps. The resulting tree was visualized using TreeView 1.6.6 (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). 16S-rDNA sequencing of five selected isolates was carried out by Macrogen Inc. (Seoul, South Korea), yielding sequences of 1371 bp each. The 16S-rDNA sequences were deposited at GenBank with accession numbers EF615006-EF615010. 16S-rDNA sequences were submitted to the Ribosomal Database Project II (http://rdp.cme.msu.edu/ accessed May 15 2007; Cole et al. 2007) which allows classification of bacterial species. The 16S-rDNA sequences were aligned using MEGA 3.1 (Kumar et al. 2004) http://www.megasoftware.net/). A phylogenetic tree of the aligned 16S-rDNA sequences was generated with MEGA 3.1, using UPGMA with 1000 bootstraps.

Phenotypic characterization of bacterial isolates

Extracellular protease activity was assayed on Skim Milk Agar (per liter: 15 g skim milk powder; 4 g blood agar base; 0.5 g yeast extract; 15 g agar). Hydrogen cyanide (HCN) production was assayed by plating a bacterial suspension on PSA containing 4.4 $g \cdot l^{-1}$ glycine. Specific detection paper was used to monitor HCN production (Castric and Castric 1983). After 48 h, a piece of HCN detection paper was fitted on the inside of the lid of the plates, and the plates were sealed with Parafilm. HCN detection was scored after 2 h and *P. fluorescens* Pf-5 (Howell and Stipanovic 1979) was included as a positive control. Indole acetic acid (IAA) production was detected using a modification of the method developed by Bric et al. (1991). Strains were plated on 1/10-strength Tryptic Soy Agar (TSA) amended with 5 mM of L-tryptophan, overlaid with a Hybond-N+ membrane (Amersham Biosciences, Buckinghamshire, United Kingdom) and incubated at 25 ^{o}C for 24 h. The membrane was removed from the plate and treated with Salkowski reagent (2% (w/v) 0.5 M $FeCl_3$ in 35 % perchloric acid) for 15 min at room temperature. IAA-producing bacteria were identified on the membrane by the formation of a red halo surrounding the bacterial colony.

Zoospore lysis by bacterial isolates

Zoospores were obtained from V8 juice agar plates (145-mm-diameter) fully overgrown by *P. capsici* during 3-4 days of incubation in the dark at 25 °C. Plates were further incubated at room temperature (15-20 °C) for 5-6 days to stimulate the production of sporangia. The plates were flooded with 20 ml of sterile demineralised water and incubated at 4 °C for 30 min and subsequently incubated at room temperature for 30 min to release the zoospores. Similarly, zoospores of *P. infestans* were obtained by flooding 9-day-old cultures with 20 ml sterile demineralised water followed by incubation at 4 °C for 1-2 h. Zoospore dilutions were made in sterile water to obtain final concentrations of approximately 10^4 zoospores ml^{-1}. Lysis of zoospores by the bacterial isolates was tested by mixing 3 µl of zoospore solutions with 3 µl of bacterial suspensions (10^9 CFU ml^{-1}). Zoospore lysis was observed microscopically (100 X magnification) for 2-3 min.

Purification of biosurfactants

Biosurfactant extracts were prepared from cell cultures as described by De Souza et al. (2003) and De Bruijn et al. (2007). Briefly, bacterial strains were spread on PSA plates and grown for 48-72 h at 25 °C. The bacterial lawns were resuspended in sterile demineralised water and cells were pelleted by centrifugation at 6000 rpm (5500 g) for 20 min at 4 °C. The cell-free culture supernatant was collected, acidified with HCl to pH 2.0 and incubated for one hour on ice to precipitate the biosurfactants. The precipitate was recovered by centrifugation (5500 *g*, 30 min) and washed twice with acidified sterile demineralised water (pH 2.0). The precipitate was dissolved in sterile demineralised water by adjusting the pH to 8.0 with 0.5 M NaOH, lyophilized and stored at -20 °C. The extract was dissolved in sterile demineralised water and surface tension measurements were performed at 25 °C using a K6 tensiometer (Krüss GmbH, Hamburg, Germany).

RP-HPLC and LC-MS analyses

Lyophilized biosurfactant preparations were dissolved in the running solution (see below) at a concentration of 1 $mg \cdot ml^{-1}$. Analytical reverse-phase high-performance liquid chromatography (RP-HPLC) was carried out on a Alltech end-capped 5 µm C_{18}-columns of 250 or 4.6 mm (UV and evaporative light scattering detection (ELSD), respectively) or 2.1 mm (liquid chromatography mass spectrometry (LC-MS)). For the separations on the 4.6 mm column a Midas autosampler (Spark), two Spectroflow 400 pumps (Kratos), a Spectroflow UV detector 785 (Applied Biosystems), a Sedex 55 ELS detector (Sedere) and Dionex Chromeleon software was used. RP-HPLC was performed using a running solution of 15% HPLC-purified water, 45% acetonitrile, 40% methanol, and 0.1% trifluoroacetic acid (TFA). Online LC-MS studies were performed after RP-HPLC using a Finnigan LCQ ion trap mass spectrometer equipped with a Finnigan electrospray ionisation (ESI) interface. Data were processed with the Finnigan Xcalibur software system (ThermoQuest, Breda, The Netherlands).

Black pepper bioassays

For the bioassays, stem cuttings (approximately 15 cm in height) of the black pepper cultivar 'VinhLinh' were obtained from runner shoots of plants from a healthy black pepper orchard in Vinh Linh. For the biocontrol assays, field soil naturally

infested with *P. capsici* was collected from a black pepper plantation in Vinh Linh (Quang Tri, Vietnam); the soil was collected at a depth of 20-cm surrounding black pepper plants exhibiting the typical symptoms of foot rot caused by *P. capsici* as described by Nguyen (2004b). The infested soil was mixed with river sand in a 1:4 (v/v) ratio and transferred to polyethylene bags (10x15 cm (WxH)). Plants were watered 2 to 3 times per week (50 ml per plant) for the duration of the experiment to maintain soil moisture. To study the plant growth-promoting activities of the selected bacterial isolates, a natural soil was collected from a groundnut field in Vinh Linh (Quang Tri, Vietnam); black pepper was not grown in this soil before. This soil was also mixed with sand in a 1:4 ratio (v/v) and transferred to polyethylene bags.

For the bioassays, the disease-free cuttings (15-cm height) were surface sterilized with ethanol (70%) for 5 min followed by several washings with ample amounts of sterile water. Excess water adhering to the cuttings was removed using sterile facial tissues. Fresh cuts were made at the ends of the cuttings to yield sterile end tissues. The lower half of the stem cuttings, including the first node, was dipped in water (control) or bacterial suspensions (10^9 cells ml^{-1}) for 30 min prior to transplanting to the polyethylene bags. The bioassays were conducted in the greenhouse of Hue Fruit Research & Development Centre (Vietnam), without temperature and humidity control. The population dynamics of the applied rifampicin-resistant bacterial isolates on the roots of black pepper seedlings was assessed after 30 and 60 days of plant growth in the greenhouse. Population densities of the introduced bacterial strains were assessed by dilution plating of rhizosphere suspensions (prepared as described above) on PSA medium containing rifampicin (100 $\mu g \cdot ml^{-1}$) and delvocid (100 $\mu g \cdot ml^{-1}$). The height of the new black pepper shoots was scored after 90 days of plant growth. Additionally, the number of roots per cutting and the length of the roots were determined at 90 days.

Statistics

All experiments described (except the initial bacterial isolations from roots of field-grown black pepper plants) were performed at least two times and representative results are shown. Population densities of the applied bacterial strains were $\log_{10}$ transformed prior to statistical analysis. The wilt incidence was transformed to arcsin prior to statistical analysis. Statistical differences ($p < 0.05$) between treatments were analyzed by ANOVA followed by student's t test (SAS Institute, Inc, Cary, N.C.). Normal distribution of the data and homogeneity of variances was tested prior to ANOVA.

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Chapter 6

Characterization and activity of biosurfactants produced by *Pseudomonas putida* 267

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Characterization and activity of biosurfactants produced by *Pseudomonas putida* 267

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Abstract

Aims. *Pseudomonas putida* strain 267, originally isolated from the rhizosphere of black pepper, produces biosurfactants that cause lysis of zoospores of *Phytophthora capsici,* the causal agent of foot rot of black pepper and damping-off disease of cucumber. In this study, the biosurfactants produced by *P. putida* strain 267 were further characterized and the genes involved in their biosynthesis partially sequenced. Bioassays were performed to determine the role of the biosurfactants in control of Phytophthora damping-off of cucumber.

Methods. Biosurfactants were identified by LC-MS and ESI-MS-MS analysis. Genes involved in biosurfactant production were identified by random mutagenesis followed by cloning and sequencing. A biosurfactant-deficient mutant of strain 267 was tested in cucumber bioassays and its biocontrol efficacy was compared to that of wild type strain *P. putida* 267

Results and conclusions. The biosurfactants from *P. putida* 267 reduce the surface tension of water and lyse zoospores of *P. capsici* at concentrations of 50 µg ml^{-1} and higher. LC-MS analysis revealed at least 5 distinct peaks with molecular masses ranging from 1380–1434. Subsequent MS-MS analysis indicated that the peak with a molecular mass of 1380 is most likely the cyclic lipopeptide surfactant putisolvin I; two other peaks with a molecular mass of 1394 are most likely putisolvin II. Characterization of four biosurfactant-deficient mutants of strain 267 revealed that the transposon had integrated into non-ribosomal peptide synthetase genes. Mutant EP1 lacked the ability to reduce the surface tension of water, to swarm on soft agar medium and to cause lysis of *P. capsici* zoospores. Both wildtype strain 267 and its biosurfactant-deficient mutant EP1 significantly reduced pre- and post-emergence damping-off of cucumber. The biosurfactants of strain 267 did not provide any disease control. These results indicate that, in spite of their zoosporicidal activity, the biosurfactants are not involved in the biocontrol activity of *P. putida* strain 267 against Phytophthora damping-off of cucumber.

Keywords: *Pseudomonas putida*, biosurfactants, *Phytophthora capsici*

Introduction

The genus *Pseudomonas* harbors plant and human pathogenic species, as well as species that degrade xenobiotic compounds, promote plant growth, antagonize plant pathogenic fungi and oomycetes, or induce systemic resistance in plants (Ramos, 2004). The interest in *Pseudomonas* species is partly due to their ability to produce a wide variety of antimicrobial metabolites, including enzymes, volatiles, antibiotics and cyclic lipopeptides (CLPs) (Raaijmakers *et al.*, 2002; Haas & Defago, 2005). CLPs are composed of a fatty acid tail connected to a cyclic peptide and are produced non-ribosomally. There is considerable structural variation in both the fatty acid tail and the peptide ring (Nybroe & Sørensen, 2004; Fishbach & Walsh, 2006; Raaijmakers *et al.*, 2006). Numerous plant-associated Pseudomonads are known to produce CLPs with versatile functions (Nybroe & Sørensen, 2004; Raaijmakers *et al.*, 2006). For plant pathogenic Pseudomonads, CLPs act as virulence factors, whereas for antagonistic isolates they contribute to biocontrol of plant diseases (Raaijmakers *et al.*, 2006). Control of oomycete pathogens by CLP-producing Pseudomonads is in part attributed to the ability of CLPs to lyse the infectious zoospores (de Souza *et al.*, 2003; Raaijmakers *et al.*, 2006; de Bruijn *et al.*, 2007; Tran *et al.*, 2007a). The studies with *Pseudomonas* sp. strain DR45 further showed that the CLP viscosinamide adversely affected mycelium growth, reduced intracellular activity, caused hyphal swellings, increased branching and rosette formation in *Rhizoctonia solani* and the oomycete *Pythium ultimum* (Thrane *et al.*, 1999; Hansen *et al.*, 2000; Thrane *et al.*, 2000). In most studies with antagonistic CLP-producing *Pseudomonas* strains, however, a comparison between the biocontrol activity of the wildtype strain and CLP-deficient mutants was not included.

In a recent study on the diversity and activity of biosurfactant-producing Pseudomonads in the rhizosphere of black pepper plants grown in the field in Vietnam, more than 200 isolates were collected and characterized (Tran *et al.*, 2007b). Representative isolates were identified by 16S-rDNA sequencing as *P. putida*, and were shown to produce the same biosurfactants. Based on the molecular masses, some of the biosurfactants were predicted to be closely related to the CLPs putisolvin I and II produced by *P. putida* strain PCL1445 (Kuiper *et al.*, 2004; Tran *et al.*, 2007b). The *P. putida* strains isolated in Vietnam were shown to provide significant control of *Phytophthora capsici* root rot and to promote shoot and root development of black pepper stem cuttings. Moreover, the partially purified biosurfactants were shown to lyse zoospores of *P. capsici* and *P. infestans* (Tran *et al.*, 2007b). However, the role of the biosurfactants produced by these isolates in control of diseases caused by *P. capsici* was not determined.

In this study, the biosurfactants produced by *P. putida* strain 267 were further characterized by ESI-MS-MS analysis. To asses the contribution of biosurfactant production in control of *P. capsici*, mutants of strain 267 deficient in biosurfactant production were generated by random plasposon mutagenesis. The biosurfactant-deficient mutants were characterized genetically and one of the mutants was tested for

activity against zoospores of *P. capsici* in vitro and for biocontrol activity in growth chamber assays with cucumber as the host plant.

Results

Zoospore lysis and surface activity of biosurfactants from *P. putida* 267

Previously, a cell suspension of *P. putida* 267 was shown to cause lysis of zoospores of *P. capsici* and *P. infestans* within 90 sec (Tran *et al.*, 2007b). Dose-response experiment performed in this study with partially purified biosurfactants of *P. putida* 267 showed that lysis of *P. capsici* zoospores occurred within 90 sec at biosurfactant concentrations of 50 µg ml^{-1} or higher (Figure 1). Surface tension measurements showed a response curve typical for biosurfactants. The results indicate that the critical micelle concentration (CMC) of the biosurfactants from strain 267 is approximately 50 µg ml^{-1} (Figure 1).

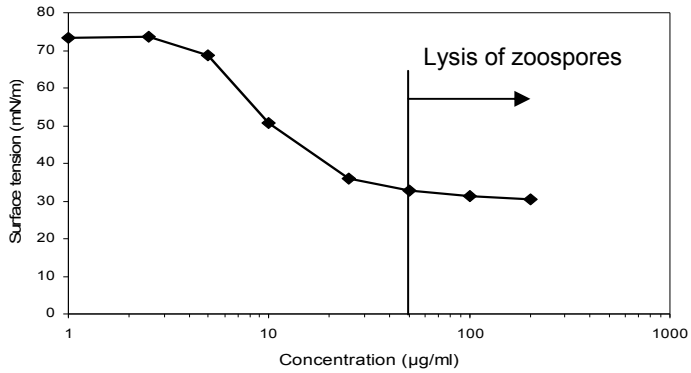

Figure 1: Dose-response relationship between the concentration of the biosurfactants of *Pseudomonas putida* 267 and the surface tension of distilled water. The biosurfactants were dissolved in sterile distilled water at different concentrations. Mean values of 3 replicates are shown. Error bars represent the standard error of the mean. The vertical line indicates the minimum concentration at which lysis of zoospores of *Phytophthora capsici* LT3239 occurs.

RP-HPLC, LC-MS and MS-MS analysis of the biosurfactants of *P. putida* 267

Previously, six biosurfactant peaks were identified in *P. putida* strain 150 isolated from black pepper (Tran *et al.*, 2007b). RP-HPLC analysis of the biosurfactant extract of strain 267 also showed six peaks with retention times similar to that of strain 150 (Figure 2). LC-MS revealed that peaks 1, 2, and 3 (Figure 2) have molecular masses of 1380 (peak 1) and 1394 (peaks 2 and 3). Subsequent ESI-MS-MS analysis showed a fragmentation pattern for peak 1 identical to that of putisolvin I (Table 1). Putisolvin II differs from putisolvin I by substitution of a valine by a leucine/isoleucine residue, resulting in an increase of the molecular mass by 14 (Kuiper *et al.*, 2004). The ESI-MS-MS profile of both peaks 2 and 3 showed several fragments with masses identical to those of putisolvin I, whereas the molecular masses of other fragments were 14 higher than that of putisolvin I (Table 1). Therefore, peaks 2 and 3 are most

likely putisolvin II. ESI-MS-MS analysis was not performed for the peaks with molecular masses of 1398, 1420 or 1434.

Generation of biosurfactant-deficient mutants of *P. putida* 267 and identification of genes

A total of 1296 mutants of strain 267, obtained by random mutagenesis, were screened for loss of biosurfactant production in a drop-collapse assay. Four mutants were identified that had completely lost the ability to collapse a droplet of water. Strain integrity of the mutants was confirmed by BOX-PCR. Plasposon rescue was performed on these four mutants and flanking sequences on both sides of the plasposon insertion were obtained. From the biosurfactant-deficient mutants CP2, DP2, EP1 and HB1, sequences of 231, 648, 939 and 933 bp, respectively, were obtained after editing. All translated sequences yielded highest blastp hits (McGinnis & Madden, 2004) with non-ribosomal peptide synthetases (NRPS) genes from Pseudomonads. The coding sequence of HB1 showed highest identity (73%) to the first 160 amino acids of PsoA, the synthetase responsible for putisolvin biosynthesis in *P. putida* strain PCL1445 (Table 2; (Dubern *et al.*, 2006), whereas the sequence upstream of the open reading frame did not reveal any homology to Genbank sequences.

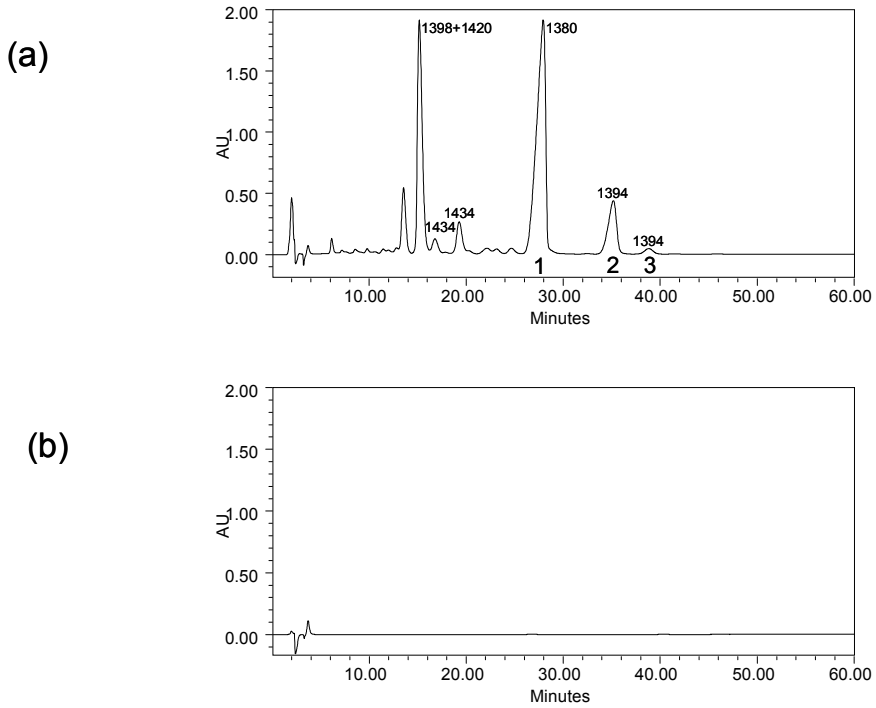

Figure 2: RP-HPLC chromatogram (210 nm) of the biosurfactant extract of *Pseudomonas putida* isolate 267 (a) and its biosurfactant-deficient mutant EP1 (b). The numbers above the peaks indicate the molecular masses as determined by LC-MS analysis.

Table 1: Comparison of the masses of the MS-MS fragments of putisolvins I and II, and three putative biosurfactants produced by *Pseudomonas putida* 267.

Putisolvin I and II[a]	Peak 1[b]	Peaks 2 & 3[b]
1380* (parent ion)	+	+
341	+	+
454	+	+
567	+	+
686*	+	+
695	+	+
782	+	+
814*	+	+
863	+	+
881	+	+
927*	+	+
994	+	+
1040*	+	+
1081	+	nd
1169*	+	+

[a]: The molecular masses of the MS-MS fragments and the parent ion of putisolvin I are according to Kuiper et al. (2004). Fragments of putisolvin II (molecular mass of the parent ion: 1394) with an expected mass of +14 relative to putisolvin I are indicated with an asterisk (*).
[b]: For RP-HPLC peaks 1, 2 and 3 of *P. putida* 267 (Figure 2), ESI-MS-MS analysis was performed. A "+" indicates detection of a fragment with a mass identical to MS-MS fragments of putisolvin I (peak 1) or putisolvin II (peaks 2 & 3); nd: not detected.

Table 2: Results of blastp analyses of the genes disrupted in four biosurfactant-deficient mutants of *Pseudomonas putida* 267.

Mutant	Size amino acid sequence	Blastp hit [a]	E-value
CP2	77	NRPS (*Pseudomonas entomophila* L48)	1e-11
		NRPS (*Pseudomonas fluorescens* Pf-5)	3e-08
		Amphisin synthetase (*Pseudomonas* sp. DSS73)	1e-07
		Massetolide A synthetase MassB	1e-07
DP2	206	NRPS (*Pseudomonas syringae* pv. *syringae* B728a)	1e-78
		NRPS (*Pseudomonas syringae* pv. *tomato* DC3000)	1e-78
		NRPS (*Pseudomonas entomophila* L48)	4e-77
EP1	312	NRPS (*Pseudomonas entomophila* L48)	6e-135
		Syringopeptin synthetase B (*Pseudomonas syringae* pv. *syringae*)	3e-112
		NRPS (*Pseudomonas syringae* pv. *syringae* B728a)	7e-111
HB1	160	Putisolvin synthetase (*Pseudomonas putida* PCL1445)	3e-54
		NRPS (*Pseudomonas entomophila* L48)	3e-42
		NRPS (*Pseudomonas syringae* pv. *tomato* DC3000)	5e-37

[a]: NRPS: non-ribosomal peptide synthetase.

Characteristics of biosurfactant-deficient mutant EP1

Mutant EP1 does not reduce the surface tension of water (Table 3) and does not produce any of the biosurfactants produced by wildtype strain 267 (Figure 2). Zoospore assays revealed that, in contrast to wildtype strain 267, mutant EP1 did not cause cessation of zoospore motility and did not cause zoospore lysis (Table 3). Swarming and biofilm formation are two traits associated with biosurfactant production in different *Pseudomonas* strains (Raaijmakers *et al.*, 2006). In vitro assays showed that wildtype strain 267 was able to swarm and was able to overgrow a soft agar plate in 24 h, whereas mutant EP1 had lost this ability completely (data not shown). Biofilm assays showed that mutant EP1 was capable of forming a significant biofilm, whereas wildtype strain 267 showed only marginal biofilm formation (data not shown).

Table 3: Phenotypic characteristics of *Pseudomonas putida* 267 and its biosurfactant-deficient mutant EP1.

Isolate	Drop collapse[a]	Surface tension[b]	Zoospore motility[c]	Zoospore lysis[d]
Water	-	71.2	-	-
P. putida 267	+	31.5	+	+
EP1	-	74.0	-	-

[a]: 5 µl droplets of bacterial cell suspensions (OD_{600}=1) were tested in a drop-collapse assay on Parafilm; "+" indicates a drop collapse.
[b]: Surface tension (mN/m) of bacterial cell suspensions (OD_{600}=1).
[c]: Zoospore motility was observed microscopically after addition of bacterial cell suspensions (OD_{600}=1) to zoospores (10^4 zoospores ml^{-1}) of *P. capsici* LT3239 in a 1:1 (vol/vol) ratio. A "+" indicates cessation of zoospore motility.
[d]: Zoospore lysis was observed microscopically after bacterial cell suspensions (OD_{600}=1) were mixed with zoospores (10^4 zoospores ml^{-1}) of *P. capsici* LT3239 in a 1:1 (vol/vol) ratio. A "+" indicates zoospore lysis.

Role of biosurfactants in the biocontrol activity of *P. putida* 267

Growth chamber assays were conducted to determine the biocontrol activity of strain 267, its biosurfactants and mutant EP1 against pre- and post-emergence damping-off of cucumber caused by *P. capsici.* Massetolide A-producing strain *P. fluorescens* SS101, partially purified massetolide A and the massetolide A-deficient mutant 10.24 were included as references. The results showed that wildtype strains 267 and SS101 significantly reduced pre- and post-emergence damping-off of cucumber (Figures 3, 4; Table 4). The biosurfactant-deficient mutants EP1 and 10.24 were as effective as their parental strains, whereas addition of the biosurfactants of strain 267 or massetolide A did not provide any control of pre- and post-emergence damping-off of cucumber (Figures 3, 4; Table 4). These results indicate that in spite of their zoosporicidal activities, the biosurfactants produced by strain 267 and massetolide A produced by strain SS101 do not contribute to the biocontrol activity of both strains against *P. capsici* on cucumber.

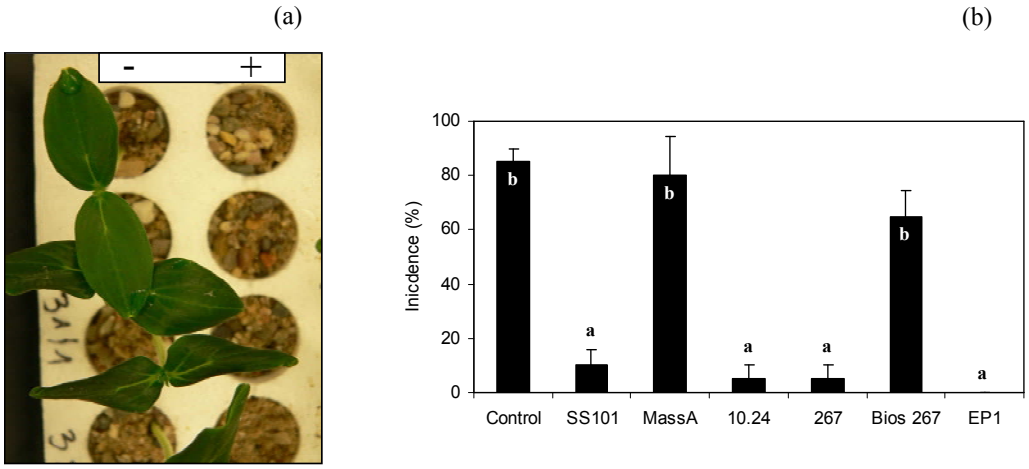

Figure 3: Pre-emergence damping-off of cucumber caused by *Phytophthora capsici*. "-": no *P. capsici* zoospores were applied; "+", cucumber seeds inoculated with zoospores of *P. capsici* LT3931 (a). Effect of application of *Pseudomonas putida* 267, its biosurfactants and the biosurfactant-deficient mutant EP1 on pre-emergence damping-off of cucumber (b). In the control treatment, only zoospores of *P. capsici* were applied. *Pseudomonas fluorescens* SS101, massetolide A and the massetolide A-deficient mutant 10.24 were included as references. Pre-emergence damping-off was scored 5 days after sowing. Means of 4 replicates are shown and error bars represent the standard errors. Different letters indicate a significant difference ($p<0.05$).

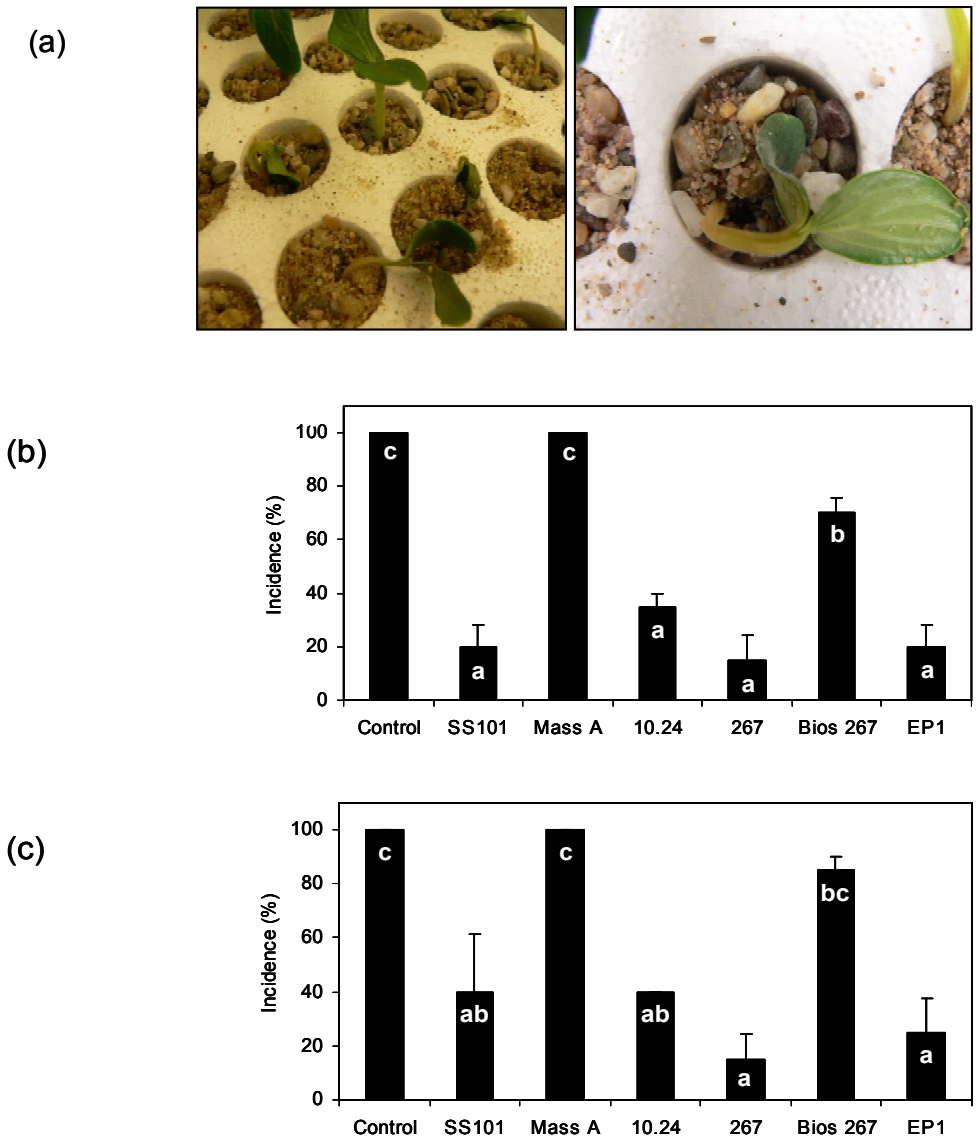

Figure 4: Post-emergence damping-off of cucumber caused by *Phytophthora capsici* (a). Effect of application of *Pseudomonas putida* 267, its biosurfactants and the biosurfactant-deficient mutant EP1 on post-emergence damping-off of cucumber caused by *P capsici* (b and c). In the control treatment, only zoospores of *P. capsici* LT3931 were applied. *Pseudomonas fluorescens* SS101, massetolide A and the massetolide A-deficient mutant 10.24 were included as references. Post-emergence damping-off was scored 5 days (b) and 13 days after sowing (c). Means of 4 replicates are shown and error bars represent the standard errors. Disease incidence at 7 and 10 days after sowing was identical to the data provided in panel c. Different letters indicate a significant difference ($p<0.05$).

Discussion

The oomycete plant pathogen *Phytophthora capsici* Leonian infects solanaceous and cucurbitaceous hosts, including cucumber, tomato, eggplant, pumpkin, squash, and melon (Erwin & Ribeiro, 1996; Hausbeck & Lamour, 2004). Zoospores are important propagules in the infection process and a potential target to control *P. capsici*. Previously, *P. putida s*train 267 was shown to lyse zoospores of *P. capsici,* provide control of foot rot of black pepper stem cuttings grown in soil infested with *P.*

capsici and to promote root and shoot development (Tran *et al.*, 2007b). The results of this study show that, in spite of their zoosporicidal activity, the biosurfactants produced by *P. putida* strain 267 do not contribute to biocontrol of *P. capsici* damping-off of cucumber. Similarly, the biosurfactant massetolide A of *P. fluorescens* strain SS101 did not play a significant role in control of damping-off of cucumber. This is in contrast to results obtained in previous studies where massetolide A did contribute to the biological control of late blight of tomato by *P. fluorescens* SS101 (Tran *et al.*, 2007a) and where application of pure rhamnolipid B, produced by *P. aeruginosa* B5, suppressed diseases caused by *P. capsici* and *Colletotrichum orbiculare* on pepper and cucumber plants (Kim *et al.*, 2000). One possible explanation may be that the amount of biosurfactants of strain 267 applied to the rockwool plugs was not sufficient to lyse all of the zoospores of *P. capsici* or that they encysted rapidly after introduction rendering them insensitive to lysis by the biosurfactants. The substantial level of control achieved by wild type strain 267 and the biosurfactant-deficient mutant EP1 as well as reference strain SS101 and its massetolide A-deficient mutant 10.24, however, indicates that mechanisms other than biosurfactant production are involved in the biocontrol activity.

Table 4: Area under the disease progress curve (AUDPC) of post-emergence damping-off of cucumber caused by *Phytophthora capsici.*

Treatment[a]	AUDPC[b]	Tukey test[c]
Control	700	c
P. fluorescens SS101	280	ab
Massetolide A	700	c
10.24	280	ab
P. putida 267	105	a
Biosurfactant 267	595	bc
EP1	175	a

[a]: Cucumber seeds were sown in rockwool plugs amended with water (control), cell suspensions of *Pseudomonas fluorescens* SS101, massetolide A, massetolide A-deficient mutant 10.24, *P. putida* 267, biosurfactants produced by strain 267, or biosurfactant-deficient mutant EP1. Rockwool plugs were inoculated with zoospores of *P. capsici* LT3239 and pre-emergence damping-off was assessed after 5 days of incubation.
[b]: Post-emergence damping-off of cucumber seedlings was monitored after 5, 7, 10 and 13 days of plant growth and, based on these data, the Area Under the Disease Progress Curve (AUDPC) was calculated for each of the treatments. Means of 4 replicates are shown.
[c]: Differences between treatments were analyzed by the Tukey test and means with different letters are significantly different ($p<0.05$).

Sequence analysis of the four biosurfactant-deficient mutants of 267 indicated that the biosurfactants are produced via non-ribosomal synthesis and most likely are CLPs. LC-MS and ESI-MS-MS indicated that several of the biosurfactants produced by strain 267 are most likely the CLPs putisolvin I and II, although NMR analysis will be required to confirm the exact structures of the individual peaks. Activity assays with each of the purified putisolvins are required to study their characteristics and activity spectrum in more detail.

Ron and Rosenberg (2001) proposed several natural roles for CLPs and other biosurfactants, including a function in antimicrobial activity, regulation of attachment and detachment to and from surfaces, and swarming. For *P. putida* strain 267, the biosurfactants contribute to surface motility and

inhibit biofilm formation. These results are consistent with the data obtained for putisolvin-producing strain PCL1445 and its putisolvin-deficient mutant (Kuiper *et al.*, 2004); the putisolvin mutant had lost its motility and had an increased biofilm. Addition of purified putisolvin I to the growth medium prior to incubation reduced biofilm formation by the wildtype strain in a concentration-dependent manner (Kuiper *et al.*, 2004). Furthermore, putisolvins I and II also adversely affected biofilm formation of at least two other *Pseudomonas* strains and were showed to break down existing biofilms (Kuiper *et al.*, 2004). Similarly, an arthrofactin-deficient mutant of *Pseudomonas* sp. MIS38 formed unstable, but more, biofilms than the wildtype (Roongsawang *et al.*, 2003). In contrast, viscosin is essential in biofilm formation by *P. fluorescens* SBW25 (de Bruijn *et al.*, 2007); viscosin-deficient mutants were impaired in biofilm formation and when viscosin was added to the growth medium, biofilm formation was restored (de Bruijn *et al.*, 2007). Surfactin plays an important role in adherence of *Bacillus subtilis* strain 6051 to a synthetic surface and in biofilm formation on roots of Arabidopsis (Bais *et al.*, 2004). Given that the wild type strain was more effective in controlling root infection of Arabidopsis by *P. syringae* than a surfactin-deficient mutant, Bais et al. (2004) postulated that CLP production may enable bacteria to efficiently colonize plant roots, thereby providing protection to their host (Bais *et al.*, 2004).

In this study, the biosurfactants of *P. putida* strain 267 did not contribute to biocontrol of *P. capsici* damping-off of cucumber. To control *P. capsici*, crop rotation in conjunction with cultural and chemical control strategies are recommended in practice. However, these strategies have not yet provided significant economic control for diverse drops (Lamour & Hausbeck, 2003; Drenth & Guest, 2004; Hausbeck & Lamour, 2004). In a previous study (Tran *et al.*, 2007b) and in the present study, *P. capsici* disease control was achieved on both black pepper and cucumber by application of antagonistic *Pseudomonas* strains 267 or SS101. Although the CLPs do not contribute to the control of *P. capsici* damping-off of cucucmber, the high levels of disease control achieved by strains 267 and SS101 may provide an attractive supplementary strategy to control *P. capsici*.

Materials and Methods

Microorganisms and growth conditions

P. fluorescens SS101 (de Souza *et al.*, 2003) and *P. putida* 267 (Tran *et al.*, 2007b) were routinely grown at 25°C on Pseudomonas Agar (PSA; Difco, France). For both strains, spontaneous rifampicin resistant derivatives were used. Mutant 10.24 is a transposon mutant of strain SS101 deficient in the production of the CLP massetolide A (de Souza *et al.*, 2003). *Phytophthora capsici* strain LT3931, originally isolated from pumpkin, was kindly provided by Dr. Kurt H. Lamour (University of Tennessee, Knoxville, USA). It was routinely grown at 25 °C on V8 juice medium (V8, N.V. Campell Foods Puurs, Belgium) amended with 3 g l^{-1} $CaCO_3$ and 15 g l^{-1} agar. *P. capsici* zoospores were obtained from V8 juice agar plates (145-mm-diameter) fully overgrown by *P. capsici* during 3-4 days of incubation in the dark at 25 °C. Plates were further incubated at room temperature (~ 20 °C) for 4-5 days to stimulate the production of sporangia. The plates were flooded with 20 ml of sterile distilled water and incubated at 4 °C for 30 min and subsequently incubated at room temperature for 30 min to release the zoospores. Zoospore dilutions were made in sterile distilled water. The density of the zoospores was determined microscopically (100x magnification) and adjusted to a concentration of 10^4 zoospores ml^{-1}.

Zoosporicidal activity of biosurfactant-producing *Pseudomonas* strains

Bacterial cell suspensions (10^9 CFU ml^{-1}) were prepared from colonies grown on PSA plates for 48 h at 25 °C. A 10-µl-aliquot of the bacterial cell suspension was mixed on a glass slide with an equal volume of *P. capsici* zoospores (10^4 ml^{-1}). Zoospore lysis was observed microscopically at 100x magnification for up to 2 min.

Purification of biosurfactants

Biosurfactant extracts were prepared from cell cultures as described by De Souza et al. (2003) and De Bruijn et al. (2007). Briefly, bacterial isolates were spread on PSA plates and grown for 48-72 h at 25 °C. The bacterial lawns were resuspended in sterile demineralised water and cells were pelleted by centrifugation at 6000 rpm (5500 *g*) for 20 min at 4 °C. The cell-free culture supernatant was collected, acidified with HCl to pH 2.0, and incubated for one hour on ice to precipitate the biosurfactants. The precipitate was recovered by centrifugation (5500 *g*, 30 min) and washed twice with acidified sterile demineralised water (pH 2.0). The precipitate was dissolved in sterile demineralised water by adjusting the pH to 8.0 with 0.2 M NaOH, lyophilized and stored at -20 °C. To determine the critical micelle concentration (CMC), the extracts were dissolved in sterile demineralised water at different concentrations and surface tension measurements were performed at 25 °C with a K6 tensiometer (Krüss GmbH, Hamburg, Germany). Extracts for reverse phase high performance liquid chromatography (RP-HPLC) were prepared in the same way, but were dissolved in a mixture of 15% HPLC-purified water, 45% acetonitrile, 40% methanol and 0.1% trifluoroacetic acid (the HPLC running solution).

RP-HPLC, LC-MS and ESI-MS-MS analyses

RP-HPLC analysis was performed as described previously (de Souza *et al.*, 2003; de Bruijn *et al.*, 2007). For determining the molecular masses of the individual HPLC peaks, liquid chromatography-mass spectrometry (LC-MS) was performed as described previously (de Bruijn *et al.*, 2007; Tran *et al.*, 2007b). For evaporative ionization tandem mass-spectrometry (ESI-MS-MS) analysis, 200 µg of the biosurfactant extract was dissolved in 6 ml dimethylsulfoxide. After membrane filtration, five injections of each 1150 µl were carried out on a Shimadzu autopreparative system with an Alltech end-capped 5 µm C_{18} column of 250 x 22 mm at 23 ml min^{-1} using an isocratic flow of 15% HPLC-purified water with 0.1% trifluoroacetic acid, 45% acetonitrile with 0.1% trifluoroacetic acid and 40% methanol. Fractions containing peaks 1, 2 and 3 were collected on the basis of their UV signal and the eluent was removed using a rotary evaporator. All three fractions were investigated by means of analytical RP-HPLC (Tran *et al.*, 2007b)) and (+)-ESI-MS-MS. ESI-MS-MS was performed using helium as a collision gas. Only a single parent ion was kept in resonance (isolation *m/z* 3) and all other ions were ejected from the trap without mass analysis. The ion was then agitated and allowed to fragment by collision-induced dissociation (CID).

Plasposon mutagenesis and gene sequencing

Random plasposon mutagenesis of *P. putida* strain 267 was performed by triparental mating with pTn*Mod*-OTc (Dennis & Zylstra, 1998) as a donor and pRK2013 (Figurski & Helsinki, 1979) as a helper. Mutants were selected on King's B agar medium supplemented with 50 µg ml^{-1} tetracycline and 100 µg ml^{-1} rifampicin. Mutants were transferred to microtiter plates containing 100 µl King's B agar medium per well. After 2 days of growth at 25 °C, colonies were dissolved in 100 µl of sterile distilled water and 10 µl droplets were tested in a drop-collapse assay. Mutant cultures that did not show the typical drop-collapse were selected for further identification and sequencing of the regions flanking the plasposon insertion. Strain integrity of these mutants was confirmed by BOX-PCR (Rademaker *et al.*, 1997) as described by Tran et al. (2007b).

Genomic DNA was isolated from 1.5 ml overnight cultures (Sambrook & Russell, 2001) and digested with *Bam*HI or *Pst*I (Promega, Madison, Winsconsin, USA). Total digested genomic DNA was self-ligated and transformed by electroporation to *Escherichia coli* DH5α. Colonies were selected on LB plates containing 50 µg ml^{-1} tetracycline. Plasmid mini-preps were performed on 1.5 ml overnight cultures (Sambrook & Russell, 2001), and the rescued plasposons were digested with *Bam*HI or *Pst*I to assess the insert sizes. The rescued plasposons were sequenced (BaseClear, Leiden, The Netherlands) using primers OTc-SEQ4 (5'-acggttcctggccttttgc-3') and OTc-SEQ6 (5'-tgataaactaccgcattaaagc-3'). The obtained sequences were trimmed to remove plasposon and poor quality sequences, and subsequently joined to obtain a single sequence.

Swarming and biofilm assays

Swarming experiments were performed on soft agar plates (King's B medium with 0.6 % (w/v) agar). Bacterial cells grown for 24 h on PSA agar plates were dissolved in sterile distilled water to a final density of 10^9 CFU ml^{-1} (OD_{600}= 1), pelleted by centrifugation and washed once with sterile distilled water. Five µl of the cell suspension was placed in the centre of a soft agar plate and the diameter of the spreading bacterial colony was determined after 24 and 48 h of incubation at 25 °C.

The biofilm assays were performed in flat-bottom 96-wells plates (Greiner-Bio One GmbH, Frickenhausen, Germany) according to the methods described by O' Toole et al. (1999) and De Bruijn et al. (2007). Wells where filled with 150 µl of KB broth and biofilm formation was measured after 24 h of incubation at 30 °C. Biofilms were stained with crystal violet and measured quantitatively at an optical density of 600 nm (de Bruijn *et al.*, 2007).

Biocontrol activity of *P. putida* 267 against *P. capsici* on cucumber

To allow testing of biosurfactant-deficient mutants in disease assays, cucumber was chosen as a host for *P. capsici*. This pathosystem allows rapid bioassays under controlled conditions, including application of mutant bacterial strains. Among several tested *P. capsici* strains, strain LT3239 was most virulent on cucumber. Cucumber seeds (cv. Chinese Slangen; Pieterpikzonen BV Holland, Heerenveen, the Netherlands) were surface sterilized and subsequently dried under continuous airflow prior to use. Trays with rockwool plugs (20-mm diameter and 25-mm height) were used for the bioassay. Bacterial inoculum was prepared by growing the *Pseudomonas* wildtype and mutants strains on PSA plates at 25 °C for 48 h. Bacterial cells were collected and washed in sterile distilled water and adjusted to a final density of 10^8 CFU ml^{-1} or 10^9 CFU ml^{-1}. Four ml of the bacterial suspension or four ml of biosurfactant solution (50 µg ml^{-1}) was added to each rockwool plug. Next, one ml of zoospores of *P. capsici* LT3239 (10^4 ml^{-1}) was added and one cucumber seed was sown in each rockwool plug and covered with a 1-cm layer of river sand. The rockwool trays were placed in boxes with a transparent cover and incubated in a climate chamber (25 °C, 16 h photoperiod). Germination of the cucumber seeds was scored 5 days after sowing, and post-emergence damping-off was scored at 5, 7, 10 and 13 days after sowing. The disease incidence (%) was calculated by dividing the number of plants suffering from damping-off disease by the total number of seedlings. Each treatment had four replicates with 10 plants per replicate.

Statistics

All experiments described were performed at least two times and representative results are shown. Disease incidence (%) was arcsin-transformed prior to statistical analysis. Statistical differences ($p < 0.05$) between treatments were analyzed by ANOVA followed by the Tukey test (SAS Institute, Inc, Cary, N.C.). Normal distribution of the data and homogeneity of variances was tested prior to ANOVA.

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Chapter 7

General discussion

General discussion

Phytophthora species cause substantial yield losses in a variety of crops worldwide. Various strategies, including disease resistance breeding, chemical control, cultural practices, and biological control continue to be explored for their potential to protect seedlings, plants and plant produce against *Phytophthora* species. The appearance of highly aggressive and fungicide-insensitive strains of *Phytophthora* species has led to an increased demand for new control measures. This thesis focused specifically on the interaction between CLP-producing *Pseudomonas* strains and *P. infestans* and *P. capsici*.

Responses of *Phytophthora* to cyclic lipopeptides

Phytophthora species can infect plant tissue by means of mycelium, sporangia, zoospores and oospores. Most studies on the sensitivity of plant pathogenic *Phytophthora* species to bioactive metabolites produced by antagonistic microorganisms focus only on one particular stage in the life cycle of the pathogen, usually mycelial growth. The results of this study showed that swimming zoospores of *P. infestans* were the most sensitive to the CLP massetolide A followed by mycelium and zoospore cysts (**chapter 2**). Massetolide A caused lysis of zoospores of *P. infestans* and *P. capsici* at concentrations of 25 µg ml^{-1} and higher, leading to complete elimination of zoospore populations within minutes after exposure. Massetolide A caused increased branching of hyphae of *P. infestans* and a significant reduction of sporangia formation. At concentrations below the critical micelle concentration (CMC), massetolide A induced encystment of zoospores. Similar observations were described for the CLP viscosinamide (Thrane *et al.*, 1999; Thrane *et al.*, 2000) and were postulated to result from an increased influx of Ca^{2+} and H^{+} due to formation of ion channels in the membrane. From the experiments described in this thesis, however, no conclusive evidence was obtained for ion channel formation in zoospore membranes by massetolide A. Assays with erythrocytes or artificial membranes are currently ongoing to provide evidence for ion channel formation by massetolide A. Alternatively, fluorescence microscopy with specific stains can be used to monitor the ability of massetolide A to cause structural disorders or to form pores in membranes of zoospores or hyphae of *Phytophthora*.

Massetolide A was also shown to adversely affect autoaggregation of swimming zoospores. Autoaggregation has been reported for *Phytophthora* and *Pythium* species (Ko & Chase, 1973; Porter & Shaw, 1978; Reid *et al.*, 1995) and was proposed to enhance the inoculum potential at the infection site and to serve as a survival mechanism when internal energy reserves become limiting, permitting the germination of some zoospores in the population by mobilization of resources of other zoospores in the aggregate. The results of this study (**chapter 2**) show that massetolide A interferes with autoaggregation of zoospores and may therefore have an adverse effect on survival or plant colonization by *P. infestans* and other oomycete pathogens. How massetolide A interferes with autoaggregation is still unclear. The observation that 2-fold higher concentrations of massetolide A are required to prevent autoaggregation

of zoospores from *P. infestans* R2, a mutant that constitutively produces an active form of the α subunit of the heterotrimeric G-protein, suggests that G-protein signalling plays a role in the response of zoospores to massetolide A (**chapter 2**). Given that G proteins are key regulators of ion channels in animal (Wickman & Clapham, 1995) and plant cells (Wang *et al.*, 2001), in particular K^+ and Ca^{2+} channels, we postulate that constitutive expression of the Gα protein interferes, to some extent, with ion channel formation by massetolide A thereby reducing the influx of Ca^{2+} and/or K^+. These two ions have been shown to induce encystment of zoospores (Deacon & Donaldson, 1993; Warburton & Deacon, 1998; Connolly *et al.*, 1999; Appiah *et al.*, 2005) and to affect autoaggregation (Reid *et al.*, 1995). Studies with Ca^{2+} channel blockers and chelators are currently ongoing to support the hypothesis that massetolide A leads to increased Ca^{2+} influx in zoospores thereby inducing encystment.

Role of cyclic lipopeptides in biological control of *Phytophthora infestans*

To date, biological control of plant diseases is mostly directed toward preventing pathogen infection and only few studies (Molina *et al.*, 2003) have addressed the effects of biocontrol agents on plants already infected by pathogenic bacteria, fungi or oomycetes. **Chapter 3** shows that *P. fluorescens* strain SS101 not only prevented infection of tomato leaves by *P. infestans*, but also significantly reduced expansion of existing late blight infections and sporangia formation. Given that sporangia constitute an important primary and secondary inoculum source for *P. infestans*, the adverse effects of *P. fluorescens* strain SS101 on both lesion area and sporangia formation may lead to a reduction in the epidemic progress of late blight disease of tomato.

In most of the studies described to date (Nybroe & Sørensen, 2004; Raaijmakers *et al.*, 2006), the antimicrobial effects of CLPs have been tested *in vitro* only and most biocontrol assays with plants did not include mutants deficient in CLP-biosynthesis. The results of this study provide, for the first time, evidence that the CLP massetolide A is an important component of the biocontrol activity of *P. fluorescens* strain SS101 against late blight of tomato (**chapter 3**). This conclusion is based on the observations that massetolide A-deficient mutant 10.24 was significantly less effective in biocontrol than wild-type strain SS101, and that application of purified massetolide A to tomato leaves and roots provided significant control of *P. infestans*. The zoosporicidal activity of massetolide A, produced by *P. fluorescens* strain SS101, may explain, at least in part, the direct protection of tomato leaves against infection by zoospores of *P. infestans.* Additional experiments further indicated that strain SS101 and massetolide A also induced systemic resistance in tomato against late blight. These results show, for the first time, that the CLP massetolide A is a bacterial determinant of induced systemic resistance (ISR) in tomato by an antagonistic *P. fluorescens* strain (**chapter 3**). In many cases, signal transduction in rhizobacteria-mediated ISR has been shown to be independent of salicylic acid (SA) and dependent on ethylene (ET) and jasmonic acid (JA) (Pieterse *et al.*, 1998; Ton *et al.*, 2001; Yan *et al.*, 2002). Our results with *nahG*, the transgenic derivative of cultivar Moneymaker, suggest that also the systemic resistance induced in tomato plants by *P. fluorescens* SS101 or massetolide A is independent of SA.

Northern and microarray-based analyses are planned to further identify the signalling pathways, including ET and JA, involved in the resistance responses in tomato induced by massetolide A.

Frequency and diversity of CLP-producing *Pseudomonas* spp.

In a comprehensive survey, Nielsen and associates (2002) showed that among the Pseudomonads isolated from the sugar beet rhizosphere, the frequencies of CLP-producing isolates ranged from 6 to 60% depending on the soil type. In the study by De Souza et al. (2003), the frequency of biosurfactant-producing Pseudomonads in the wheat rhizosphere ranged from 1-5%. Apart from these studies, information on the occurrence and densities of CLP-producing Pseudomonads in natural environments is fragmentary (Raaijmakers *et al.*, 2006). The results obtained in this thesis (**chapter 5**) showed that among a total of nearly 14,500 fluorescent Pseudomonads, randomly selected from the rhizosphere of black pepper plants grown in nurseries or in the field in Vietnam, on average 1.3 % produced biosurfactants. Recent analyses of the genomes of *Pseudomonas* species, however, indicated that CLP biosynthesis genes can be found in a relatively large number of strains (de Bruijn *et al.*, 2007), suggesting that this trait is more frequent among Pseudomonads than shown in the screenings performed in this and other studies. This presumed underestimation of the actual number of biosurfactant-producing Pseudomonads in natural environments may be explained by the fact that the culture conditions, used to grow the bacterial isolates prior to the drop-collapse assay, can have a significant influence on the in vitro production of detectable levels of biosurfactants.

The results of this thesis also demonstrated that in spite of substantial genotypic diversity of the biosurfactant producers from the rhizosphere of black pepper, the isolates tested produced the same biosurfactants (**chapter 5**). In contrast to this uniformity in biosurfactant production among isolates from the rhizosphere of black pepper, CLP-producing Pseudomonads from the rhizosphere of sugar beet plants were grouped into eight major groups, producing in total at least nine structurally different CLPs (Nielsen *et al.*, 2002). Whether the black pepper plant and/or the soil type exerted a specific selection pressure on the type of biosurfactant produced by the Pseudomonads remains to be investigated. Isolates from the Danish soils were classified as *Pseudomonas fluorescens* (Nielsen *et al.*, 2002), whereas the representative isolates in this study were all classified as *Pseudomonas putida*. LC-MS analysis revealed that the biosurfactants produced by the *P. putida* strains from black pepper are most likely CLPs resembling the putisolvins I and II described for *P. putida* strain PCL1445 (Kuiper *et al.*, 2004). MS-MS analysis of the biosurfactants produced by *P. putida* strain 267 further supported this conclusion (**chapter 6**).

Role of cyclic lipopeptides in biocontrol of *Phytophthora capsici*

Foot rot caused by *Phytophthora capsici* is one of the most destructive and economically most important diseases of black pepper in Vietnam and other countries (Nguyen, 2002; Drenth & Sendall, 2004). The survey conducted in 2005 and 2006 showed that in Quang Tri province, Phytophthora foot

rot occurred in the field at a frequency of 17-18% (**chapter 4**), which is consistent with the estimates of 15-20% given by Drenth and Sendall (2004) for Vietnam. One other important finding of the survey was that fungicide treatment of stem cuttings used for propagation significantly reduced the incidence of foot rot of black pepper plants grown in the field (**chapter 4**). In contrast, there were no significant effects on disease incidence when fungicides were applied on mature black pepper plants grown in the field. Since fungicides can not be relied upon alone to prevent foot rot caused by *P. capsici* (Hausbeck & Lamour, 2004), the CLP-producing strains isolated from black pepper plants were subsequently tested for their ability to protect stem cuttings from foot rot (**chapter 5**). The results showed that several of the *P. putida* strains reduced disease incidence of stem cuttings grown in soil heavily infested with *P. capsici.* Interestingly, several of the strains also promoted root and shoot development (**chapter 5**). Combined with the observation that all five *P. putida* strains produce indole acetic acid (IAA) *in vitro*, a potent plant hormone that stimulates root growth (Lambrecht *et al.*, 2000), may suggest that this trait contributes to enhanced root growth of the black pepper stem cuttings treated with these strains. Future studies, involving IAA-deficient mutants, will be needed to resolve the role of IAA in enhanced root development of black pepper stem cuttings.

The observation that the CLPs produced by the five *P. putida* strains cause lysis of zoospores of *P. capsici*, suggested that this zoosporicidal activity was one of the modes of action by which these strains controlled the disease. However, bioassays with cucumber as a host for *P. capsici* clearly showed that a well-characterized CLP-deficient mutant of *P. putida* strain 267 was as effective in biocontrol of Phytophthora damping-off as its parental strain (**chapter 6**). Although it cannot be excluded that CLPs play, at least in part, a role in biocontrol of *P. capsici* on black pepper stem cuttings, these results indicated that the CLPs are of no importance in the biocontrol activity of *P. putida* strain 267 against Phytophthora damping-off of cucumber. Similarly, the biosurfactant massetolide A of *P. fluorescens* strain SS101 did not play a significant role in the control of damping-off of cucumber (**chapter 6**). Mechanisms responsible for the biocontrol activity of *P. putida* strain 267 and *P. fluorescens* strain SS101 against *P. capsici* on cucumber are yet unknown and will be subject of future studies.

Role of cyclic lipopeptides in motility and plant colonization

Ron and Rosenberg (2001) proposed several natural roles for CLPs and other biosurfactants, including a function in antimicrobial activity, regulation of attachment and detachment to and from surfaces, and swarming. For *P. putida* strain 267, the biosurfactants contribute to surface motility since the mutant deficient in CLP-biosynthesis lost its ability to swarm on soft agar media (**chapter 6**). These results are consistent with the data obtained for putisolvin-producing strain PCL1445 (Kuiper *et al.*, 2004), for arthrofactin-producing *Pseudomonas* sp. MIS38 (Roongsawang *et al.*, 2003), for viscosin-producing *P. fluorescens* SBW25 (de Bruijn *et al.*, 2007), for surfactin-producing *Bacillus subtilis* 6051 (Bais *et al.*, 2004), and for massetolide A-producing *P. fluorescens* SS101 (De Bruijn et al. unpublished). The involvement of CLPs in bacterial motility may provide an advantage in colonization

of plant tissue, in translocation to new and more nutrient-rich niches on the plant surface, and in containment of plant pathogens. The results of this study showed that wild-type strain SS101, when applied to seeds, established significantly higher densities on roots and cotelydons of tomato seedlings than its CLP-deficient mutant (**chapter 3**). These results indicate that massetolide A contributes to colonization of tomato plants by *P. fluorescens* SS101 and extend the findings of Nielsen et al. (2005) who showed that the CLP amphisin is an important trait in colonization of sugar beet seeds and roots by *Pseudomonas* sp. strain DSS73.

Future perspectives

P. infestans and *P. capsici* continue to cause immense economical damage. Current control measures involve mostly fungicide applications which are too expensive for many local farmers in Vietnam. The results of this thesis showed that the CLP massetolide A is a metabolite with versatile activities and functions, and has potential as a supplementary measure in the control of late blight of tomato caused by *P. infestans*. This study also provided, for the first time, an extensive survey on the frequency, diversity and activity of biosurfactant-producing Pseudomonads in the rhizosphere of black pepper, the most important spice crop in the world. The promising results obtained with indigenous Pseudomonads in greenhouse experiments conducted in Vietnam provide a strong basis for further development of these bacterial strains as a supplementary strategy to suppress foot and root rot of black pepper and to promote shoot and root growth of the 'King of Spices'. Future studies will have to focus on how to produce and formulate these bacterial biocontrol agents and how to include them in an affordable and integrated management practice to control Phytophthora foot rot of black pepper. The broad-spectrum activity of these antagonistic Pseudomonads and their CLPs may also provide new opportunities to apply these agents for the protection and growth promotion of other crops.

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Summary

Fluorescent *Pseudomonas* species produce a wide variety of antimicrobial metabolites, including cyclic lipopeptide surfactants (CLPs). Several CLP-producing *Pseudomonas* strains have shown promising results in the biological control of plant pathogens. Among the plant pathogenic oomycetes, *Phytophthora* species continue to cause immense economical damage to a range of crops. Current control measures mostly involve frequent fungicide applications. The results of this thesis showed that CLPs and CLP-producing *Pseudomonas* strains have potential as a supplementary measure in the control of Phytophthora diseases, in particular of late blight of tomato caused by *P. infestans* and of foot rot of black pepper caused by *P. capsici*.

Phytophthora species can infect plant tissue by means of mycelium, sporangia, zoospores and oospores. Swimming zoospores of *P. infestans* were the most sensitive to the CLP massetolide A produced by *Pseudomonas fluorescens* SS101, followed by mycelium and zoospore cysts. Massetolide A caused lysis of zoospores of *P. infestans* and *P. capsici* at concentrations of 25 μg ml^{-1} and higher within minutes after exposure. Massetolide A increased branching of hyphae of *P. infestans* and significantly reduced sporangia formation. At concentrations below the critical micelle concentration, massetolide A also induced encystment of zoospores and interfered with autoaggregation of swimming zoospores. Autoaggregation of zoospores of a transformant of *P. infestans,* that constitutively produces an active form of the α subunit of the heterotrimeric G-protein, was less affected by massetolide A. These results suggest that G-proteins play a role in the interaction between CLPs and *P. infestans*.

To date, biological control of plant diseases is mostly directed toward preventing pathogen infection. The results presented in this thesis showed that *P. fluorescens* strain SS101 not only prevented infection of tomato leaves by *P. infestans*, but also reduced expansion of existing late blight infections. The zoosporicidal activity of massetolide A, produced by *P. fluorescens* SS101, may explain, at least in part, the direct protection of tomato leaves against infection by zoospores of *P. infestans.* Other results presented in this thesis also showed, for the first time, that the CLP massetolide A induced resistance in tomato against *P. infestans*. Assays with *nahG* transgenic tomato plants indicated that the systemic resistance response induced by strain SS101 or massetolide A is independent of salicylic acid signaling. Strain SS101 colonized the roots of tomato seedlings significantly better than its *massA*-mutant, indicating that massetolide A is an important trait in plant colonization.

Biosurfactant-producing fluorescent Pseudomonads make up approximately 1.3% of the culturable *Pseudomonas* population isolated from the rhizosphere of black pepper plants grown in the field in Vietnam. Although BOX-PCR revealed substantial genotypic diversity, the isolates were shown to produce the same biosurfactants and were all identified as *Pseudomonas putida.* Biochemical analysis further revealed that the biosurfactants produced by these *P. putida* strains are most likely CLPs resembling the putisolvins I and II described for *P. putida* strain PCL1445. Several of the CLP-producing *P. putida* strains significantly reduced disease incidence of black pepper stem cuttings grown in field soil infested with *P. capsici,* and in absence of this disease, promoted root and shoot development. Subsequent bioassays with cucumber and *P. capsici* showed that CLP-deficient mutants of *P. putida* strain 267 and *P. fluorescens* SS101 were as effective in biocontrol of Phytophthora damping-off as their parental strains, indicating that CLPs do not contribute to the biological control of Phytophthora damping-off of cucumber.

The promising results obtained with indigenous Pseudomonads in greenhouse experiments conducted in Vietnam provide a strong basis for further development of these bacterial strains as a supplementary strategy to suppress foot rot of black pepper and to promote shoot and root development of the 'King of Spices'. Future studies will focus on how to implement these biocontrol agents in an integrated management practice to control Phytophthora diseases. The broad-spectrum activity of these antagonistic Pseudomonads and their CLPs may also provide new opportunities to apply these agents for the protection and growth promotion of other crops.

Samenvatting

Pseudomonas bacteriën produceren diverse antimicrobiële metabolieten waaronder cyclische lipopeptide surfactants (CLPs). CLP-producerende *Pseudomonas* stammen blijken zeer effectief te zijn in de bestrijding van plantenpathogene schimmels en oomyceten. De oomyceet *Phytophthora* omvat diverse soorten die grote economische schade kunnen toebrengen aan diverse gewassen. De huidige beheersmaatregelen bestaan voornamelijk uit het veelvuldig toedienen van fungiciden. De resultaten beschreven in dit proefschrift tonen aan dat CLPs en CLP-producerende *Pseudomonas* stammen toegepast kunnen worden als een additionele maatregel om ziekten veroorzaakt door *Phytophthora* soorten te onderdrukken, in het bijzonder *P. infestans* op tomaat en *P. capsici* op zwarte peper.

Phytophthora soorten kunnen planten infecteren via mycelium, sporangiën, zoösporen en oösporen. De zwemmende zoösporen van *P. infestans* waren het meest gevoelig voor de CLP massetolide A geproduceerd door *Pseudomonas fluorescens* SS101, gevolgd door mycelium en geëncysteerde zoösporen. Bij concentraties van 25 µg ml^{-1} en hoger veroorzaakte massetolide A binnen enkele minuten lysis van zoösporen van *P. infestans* en *P. capsici*. Massetolide A veroorzaakte een toename in de vertakkingen van hyphen van *P. infestans* en leidde tot een significante reductie in de vorming van sporangiën. Bij concentraties lager dan de kritische micel-concentratie induceerde massetolide A tevens de encystering van zoösporen en interfereerde met autoaggregatie van zwemmende zoösporen. Massetolide A had minder effect op autoaggregatie van zoösporen afkomstig van een transformant van *P. infestans* die constitutief het Gα-eiwit to expressie brengt. Deze resultaten suggereren dat 'G-proteins' een rol spelen in de interactie tussen CLPs en *P. infestans.*

Tot op heden is biologische bestrijding van plantenziekten voornamelijk gericht op het voorkomen van infectie. De resultaten beschreven in dit proefschrift tonen aan dat *P. fluorescens* SS101 niet alleen infectie van tomatenbladeren door *P. infestans* kan voorkomen, maar ook de uitgroei van bestaande infecties kan beperken. Lysis van zoösporen door massetolide A kan de preventieve werking tegen infectie van tomatenbladeren door *P. infestans* slechts tendele verklaren. De resultaten beschreven in dit proefschrift tonen voor de eerste keer aan dat de CLP massetolide A tevens resistentie induceert in tomatenplanten tegen *P. infestans*. Experimenten met *nahG* transgene tomatenplanten laten zien dat de systemische resistentie geïnduceerd door stam SS101 onafhankelijk is van de salicylzuur-afhankelijke signaaltransductie. Stam SS101 koloniseerde de wortels van tomatenplanten significant beter dan de *massA*-mutant, hetgeen een indicatie is dat massetolide A een rol speelt in kolonisatie van plantenoppervlakten.

Van de cultiveerbare *Pseudomonas* populatie, geïsoleerd uit de rhizosfeer van zwarte peperplanten in Vietnam, produceerde ongeveer 1,3 % surfactants. Op basis van BOX-PCR analyse bleek dat deze populatie van surfactant-producerende *Pseudomonas* soorten genotypisch divers was. Echter, ze bleken dezelfde surfactants te produceren en werden alle geïdentificeerd als *Pseudomonas putida*. Biochemische analyse toonde vervolgens aan dat de surfactants geproduceerd door deze *P. putida* stammen zeer waarschijnlijk CLPs zijn, nauw verwant aan putisolvins I en II beschreven voor *P. putida* PCL1445. Een aantal van deze CLP-producerende *P. putida* stammen waren effectief in de bescherming van stengelstekken van zwarte peper tegen infectie door *P. capsici.* In afwezigheid van deze ziekteverwekker bevorderden de *P. putida* stammen de groei en ontwikkeling van de stengelstekken. Biotoetsen met komkommerzaailingen toonden aan dat CLP-deficiënte mutanten van *P. putida* 267 en van *P. fluorescens* SS101 net zo effectief waren in biologische bestrijding van *P. capsici* als hun wildtype stammen, hetgeen aantoont dat CLPs geen rol van betekenis speelden.

De veelbelovende resultaten verkregen met de CLP-producerende *Pseudomonas* stammen in kasexperimenten in Vietnam vormen een goede basis voor verdere ontwikkeling van deze bacteriestammen om *P. capsici* te onderdrukken en de groei van stengelstekken van zwarte peperplanten te bevorderen. Toekomstige studies zullen zich richten op het implementeren van deze stammen in een geïntegreerde aanpak om plantenziekten veroorzaakt door *Phytophthora* soorten te beheersen. De breed-spectrum activiteit van deze antagonistische Pseudomonaden en de CLPs kunnen tevens nieuwe mogelijkheden bieden voor de beheersing van andere plantenziekten en voor de groeibevordering van andere gewassen.

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Tran Thi Thu Ha
Wageningen, The Netherlands
October 2007

Curriculum Vitae

Tran Thi Thu Ha was born on November 20, 1972 in Ha Noi, Vietnam. She obtained her bachelor degree of Agronomy in 1996 at Hue University of Agriculture and Forestry, Vietnam. She has worked at Hue University of Agriculture and Forestry as assistant lecturer since September 1996. From September 1997 – November 1999 she conducted her MSc study on wilt diseases of groundnut at the same University where she worked. She then continued working as a lecturer on plant pathology at the Department of Plant Protection of Hue University of Agriculture and Forestry.

In October 2001, she got a PhD scholarship of the Vietnam Ministry of Education and Training through project 322 for study abroad. She joined the Section Molecular Ecology at the Laboratory of Phytopathology, Wageningen University, the Netherlands. From July 2003 – October 2007, she conducted her PhD study on biological control of *Phytophthora* species by biosurfactant-producing *Pseudomonas*.

After finishing her PhD study in the Netherlands, she will be back in her home country and continue her work at the Department of Plant Protection of Hue University of Agriculture and Forestry.

Education Statement of the Graduate School
Experimental Plant Sciences

Issued to: Tran Thi Thu Ha
Date: 9 October 2007
Group: Phytopathology, Wageningen University

1) Start-up phase	date
► **First presentation of your project**	
Interaction between biosurfactant-producing *Pseudomonas* and *Phytophthora* species	Sep 6, 2004
► **Writing or rewriting a project proposal**	
Interaction between biosurfactant-producing *Pseudomonas* and *Phytophthora* species	Sep 2004
► **Writing a review or book chapter**	
► **MSc courses**	
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*7.5 credits**

2) Scientific Exposure	date
► **EPS PhD student days**	
EPS PhD student day - Free University of Amsterdam	Jun 3, 2004
EPS PhD student day - Wageningen University	Sep 19, 2006
► **EPS theme symposia**	
Theme 2: Interactions between plants and biotic agents, University of Amsterdam	Feb 2, 2007
Theme 3: Metabolism and Adaptation, University of Amsterdam	Nov 10, 2006
► **NWO Lunteren days and other National Platforms**	
ALW plaform Molecular Genetics - Lunteren	Oct 13, 2005
► **Seminars (series), workshops and symposia**	
A window to the past: Tracking historic migration of the Irish potato famine pathogen -Jean Beagle Ristaino	Nov 2, 2004
Symposium on Systems biology in honor of Prof. dr. Pierre de Wit - Aula, Wageningen	Nov 4, 2004
Management of Phytophthora blight (*P. capsisci*) of cucurbits and peppers-Mohammad Babadoost	July 18, 2005
Phytophthora capsici - Kurt Lamour	Nov, 2006
Management of Phytophthora disease in central part of Vietnam workshop, Hue-Vietnam	April 7-9, 2007
► **Seminar plus**	
► **International symposia and congresses**	
Rhizosphere meeting 2004, Munich (Germany)	Sep 12-17, 2004
IOBC-meeting 'Multitrophic Interactions in Soil and Integrated Control', Wageningen (The Netherlands)	Jun 6-8, 2005
5th micoarray usergroup meeting, Ghent (Belgium)	Nov 16-18, 2005
IOBC-meeting 'Fundamental and practical approaches to increase biocontrol efficacy', Spa (Belgium)	Sep 6-10, 2006
► **Presentations**	
IOBC-meeting - Multitrophic Interactions in Soil and Integrated Control - Oral	Jun 6-8, 2005
IOBC-meeting - Fundamental and practical approaches to increase biocontrol efficacy - Poster	Sep 6-10, 2006
EPS PhD student day 2006 - Poster	Sep 19, 2006
PhD excursion in London - Oral	March 9, 2007
Workshop - Management of Phytophthora disease in central part of Vietnam - Oral	April 9, 2007
► **IAB interview**	Sep 18, 2006
► **Excursions**	March 4-8, 2007
Subtotal Scientific Exposure	*13.5 credits**

3) In-Depth Studies	date
► **EPS courses or other PhD courses**	
PhD course 'Molecular phylogenies: Reconstructions and Interpretation', Wageningen	Oct 6 -10, 2006
PhD course 'Basics statistics', Wageningen	Dec 18-22, 2006
► **Journal club**	
Literature studygroup Phytopathology	2003-2007
► **Individual research training**	
Subtotal In-Depth Studies	*4.5 credits**

4) Personal development	date
► **Skill training courses**	
Techniques for writing and presenting a scientific paper	Jun 14-17, 2005
Project and time management (3 days)	Nov-Dec 2005
Teaching and Supervising thesis students	Jan 25-26, 2006
► **Organisation of PhD students day, course or conference**	
IOBC-meeting 'Multitrophic Interactions in Soil and Integrated Control', Wageningen, The Netherlands	Jun 6-8, 2005
► **Membership of Board, Committee or PhD council**	
Subtotal Personal Development	*4.7 credits**

TOTAL NUMBER OF CREDIT POINTS*	**30.2**

Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 credits

** A credit represents a normative study load of 28 hours of study*

The research described in this thesis was performed at the Laboratory of Phytopathology of Wageningen University, The Netherlands and Hue Biotechnology Laboratory, Hue Fruit Research and Development Centre and Laboratory of Plant Protection Department of Hue University of Agriculture and Forestry, Vietnam, under the Training and Supervision Plan of the Graduate School 'Experimental Plant Sciences'. This research was financially supported by the Vietnamese Ministry of Education and Training (MOET) through project 322.

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