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

Ha Tran¹,², Andrea Ficke¹, Theodore Asiimwe¹, Monica Höfte³ and Jos M. Raaijmakers¹

¹Laboratory of Phytopathology, Wageningen University, Wageningen, the Netherlands; ²Department of Plant Protection, Hue University of Agriculture and Forestry, Hue, Vietnam; ³Laboratory of Phytopathology, Ghent University, Ghent, Belgium

**Summary**

- *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, the role of the cyclic lipopeptide massetolide A of *Pseudomonas fluorescens* SS101 in biocontrol of tomato late blight was examined.
- *Pseudomonas fluorescens* SS101 was effective in preventing infection of tomato (*Lycopersicon esculentum*) leaves by *P. infestans* and significantly reduced the expansion of existing late blight lesions. Massetolide A was 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 was independent of salicylic acid signalling. Strain SS101 colonized the roots of tomato seedlings significantly better than its massA-mutant, indicating that massetolide A was 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*.

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


---

**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 preinfection process and a potential target to control *P. infestans* and other Oomycete pathogens (Donaldson & Deacon, 1993; Erwin & Robeiro, 1996; van West *et al.*, 2002). Late blight is traditionally controlled by a combination of cultural practices and chemical applications. To control late blight biologically, several antagonistic microorganisms have been tested for their activity against *P. infestans*, including nonpathogenic *Phytophthora cryptogea* (Stromberg & Brishammar, 1991) and endophytic microorganisms such as *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; Kloeper...
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 Phytophthora species (De Souza et al., 2003; De Bruijn et al., 2007). 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 Phytophthora 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.

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 supernatants are derivatives of massetolide A differing in amino acid composition of the peptide ring (I. de Bruijn et al., unpublished). 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 Tn5 insertion in massA, the first nonribosomal peptide synthetase (NRPS) gene required for the biosynthesis of massetolide A (I. de Bruijn et al., unpublished). Mutant 10.24 does not produce massetolide A, nor any of the other massetolide A derivatives produced by wild-type strain SS101. Mutant 10.24 is resistant to rifampicin (100 µg ml⁻¹) and kanamycin (100 µg ml⁻¹). For the bacterial inoculum used in the plant assays, strain SS101 and mutant 10.24 were grown on Pseudomonas agar (PSA) plates (Difco, Le Pont de Claix, France) at 25°C for 48 h. Bacterial cells were washed in sterile demineralized water before use. For treatment of tomato seeds, roots or leaves, washed cell suspensions of SS101 or 10.24 were diluted in sterile demineralized water to a final concentration of 10⁵ CFU ml⁻¹ (OD 600nm = 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 d in the dark at 18°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°C) for 1–2 h, after which the suspension was gently filtered (50 µm mesh) to remove sporangia. Zoospore density was determined microscopically at 100 × magnification and adjusted to a final concentration of 3–4 × 10⁵ swimming zoospores ml⁻¹.

Purification and detection of massetolide A

The cyclic lipopeptide massetolide A (molecular mass 1139 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 h at 25°C. Bacterial mats were suspended in sterile demineralized water and cells were pelleted by centrifugation at 5500 g for 20 min. 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 demineralized water. The precipitate was dissolved in sterile demineralized water by adjusting the pH to 8 with 0.5 M NaOH, lyophilized and stored at −20°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 & 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 six-point standard curve.

Plant cultivation and biocontrol assays

Tomato seeds (Lycopersicon esculentum Mill. cv. Moneymaker 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°C, 16:8 h day:night photoperiod) for 2 wk to stimulate germination. Seedlings were then transplanted to \(7 \times 7 \times 8\) cm (L x W x H) black plastic PVC pots containing the same soil mixture and kept in the climate chamber at 20°C for 3 wk. For the biocontrol assays with \(P.\ infestans\), plants were transferred to a 15°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, the Netherlands). Before use, tomato seeds were surface-sterilized, dried in a flow cabinet, and sown and maintained as already explained.

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-wk-old tomato plants were immersed in bacterial suspension \((10^9\ \text{CFU ml}^{-1})\) for 1 min or in a solution of massetolide A in sterile demineralized water (pH 8). Leaves immersed in sterile demineralized 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 d in a growth chamber at 15°C, the lower side of each treated tomato leaf was inoculated with 3 µl droplets of a \(P.\ infestans\) zoospore suspension \((3-4 \times 10^6\ \text{swimming zoospores ml}^{-1})\) or 3 µl droplets of sterile demineralized water (pathogen-free control). Two droplets were placed abaxially on each side of the leaf’s midvein. 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 lesion 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-wk-old tomato plants were inoculated with 3 µl droplets of a \(P.\ infestans\) zoospore suspension or sterile demineralized water (pathogen-free control). The droplets were placed abaxially on each side of the leaf’s midvein. Tomato plants were then incubated at 15°C for 3–4 d to allow the late blight lesions to develop. The initial lesion area was determined as already described. Subsequently, the lesioned tomato leaves were dipped in bacterial suspension \((10^9\ \text{CFU ml}^{-1})\) or in solutions with different concentrations of massetolide A for 1 min; sterile-demineralized water served as a control. Tomato plants were then transferred to the climate chamber (15°C) and the areas of the late blight lesions were measured again at 2 and 5 d after treatment with water (control), the bacterial suspension or massetolide A. The increase in lesion area was calculated by dividing the lesion area after treatment by the initial lesion area assessed before treatment with water, the bacteria or 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 (Supplementary material, 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-wk-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°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 earlier. In the second series of experiments, roots of 2-wk-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 demineralized water (pH 8) served as a control. The seedlings were then transplanted and maintained as explained above. Approximately 2 wk after treatment, the tomato leaves were challenge-inoculated with zoospores of \(P.\ infestans\), as already described. 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 \((10^9\ \text{CFU ml}^{-1})\) or in a solution of massetolide A for 1 h. Immersing seeds in sterile demineralized 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 \(1 \times 10^6\ \text{CFU per seed}\), as 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 wk of
plant growth, tomato leaves were inoculated with zoospores of 
P. infestans as described earlier. 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 earlier. The lesions 
were then excised from the leaves and transferred to 1.5 ml 
sterile tubes containing 1 ml of isotope II electrolytic buffer 
(Coulter Electronic Inc., Fullerton, CA, USA). 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 100× 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 6-wk-old tomato plants 
raised from bacteria-treated seeds (described earlier) 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, 
approx. 1 g (fresh weight) of roots was suspended in 5.0 ml of 
0.01 M MgSO₄, vortexed for 1 min, sonicated for 1 min 
(Bransonic 12) and vortexed again for 15 s before 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 MgSO₄ 
supplemented with 0.05% (v/v) Tween 80, and then vortexing 
vigorously for 1 min before 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, 
the Netherlands) was added (100 µg ml⁻¹) to the agar plates 
to prevent fungal growth. Plates were incubated for 48–72 h 
at 25°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% H₂O₂ for 15 s, 
rinsed twice with ample sterile demineralized 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 
MgSO₄. Suspensions were dilution-plated onto selective 
PSA media. Plates were incubated for 48–72 h at 25°C, after 
which bacterial colonies were counted and population densities 
calculated.

Statistical analysis

All experiments described in this study were performed at 
least twice. Representative results are shown. Population 
densities of the applied bacterial strains were log₁₀-transformed 
before 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, Cary, NC, USA). 
Normal distribution of the data and homogeneity of variances 
were tested before ANOVA.

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 1 d before 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 9 d 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 10 d after zoospore inoculation were 
8.3 and 8.4 log CFU g⁻¹ leaf, respectively.

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 (206 nm), massetolide A 
makes up, on average, 70% (ranging from 65 to 74%) of the 
purified extract from strain SS101 (Fig. 2a1). The other 30% of 
the extract is composed, for the most part (> 95%), of 
four additional cyclic lipopeptides (retention times 14– 
20 min, Fig. 2a1), three of which were identified by liquid 
chromatography/mass spectrometry (LC-MS) and nuclear
magnetic resonance (NMR) as derivatives of massetolide A (I. de Bruijn et al., unpublished). 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 also showed that 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 9 d 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 9 d 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 the 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 also showed that pure massetolide A significantly reduces disease incidence and lesion area when applied as a solution with a concentration of 100 µg ml⁻¹ (equal to 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.

High-performance liquid chromatography 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 concentrations of massetolide A on the leaves are 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 1 and 5 d after treatment did not allow in situ detection and quantification of massetolide A owing 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 because of background signals of compounds released from the tomato leaves during the extraction.

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 threefold on leaves treated with strain SS101, whereas lesion area increased more than sevenfold in the control treatment (Fig. 3). On leaves treated with mutant 10.24, lesion area increased almost fivefold (Fig. 3b). Moreover, application of massetolide A to tomato leaves significantly reduced the growth of existing lesions in a concentration-dependent manner (Fig. 3b).
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 1 d 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 2 wk 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 values similar to that obtained in the wild-type progenitor tomato cv. 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.
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 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 with the nontreated control (a1); (b) effect of SS101, 10.24 and different concentrations of massetolide A on the increase in lesion size. The means + SE of six replicates are shown. Means with a different letter are significantly different ($P < 0.05$).

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-d-old tomato plants raised from bacteria-treated seeds were inoculated with *P. infestans* zoospores. Twenty-four hours after treatment of the lower leaves of 35-d-old tomato (*Lycopersicon esculentum*) plants with strain SS101, mutant 10.24 or MassA (50 µg ml$^{-1}$ (44 µM)), upper leaves were challenge-inoculated with zoospores of *Phytophthora infestans*. At 7 d after pathogen inoculation, disease incidence (b) and lesion area (c) were determined. Means + SE of four replicates are given. Means with the same letter are not significantly different ($P < 0.05$).
Research

with *P. infestans* zoospores and disease incidence and severity assessed 9 d 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 areas to values 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.

**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 the 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 a 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.

Fig. 5  Induced resistance in tomato against late blight by application of *Pseudomonas fluorescens* SS101 or massetolide A to leaves of tomato (*Lycopersicon esculentum*) cv. Moneymaker and its transgenic derivative nahG. Twenty-four hours after treatment of the lower leaves of the tomato plants with strain SS101 or massetolide A (Messa, 50 µg ml⁻¹), upper leaves were challenge-inoculated with zoospores of *Phytophthora infestans*. At 7 d after pathogen inoculation, lesion area (a) was determined. (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 ± SE of six replicates are given. An asterisk indicates a statistically significant reduction in disease severity (*P* < 0.05) relative to the control.

Fig. 6  Colonization of tomato (*Lycopersicon esculentum*) plants by *Pseudomonas fluorescens* SS101 and its massetolide A-deficient mutant 10.24. Tomato seeds were treated with SS101 (closed bars) or 10.24 (open bars) at a final density of 10⁶ CFU per seed and sown in soil. After 14 d (a1, a2) and 44 d (b1, b2) of plant growth after inoculation (dai), population densities of the applied bacterial strains were determined on the surface and in the interior of roots, stem, cotyledons and on true leaves infested with *Phytophthora infestans*. The means ± SE of four 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⁻¹).
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 the 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 2−5, Fig. 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 (reviewed in 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 the 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 & Sørensen (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 because of 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 successfully adopted for in situ detection of syringopeptins: the competitive ELISA assay appeared to be approx. 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 localization, fate and stability of the massetolide A applied to plant tissues or produced by P. fluorescens SS101 in situ.

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 1 min 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, Fig. 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 bacterial determinants other than massetolide A also 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; Kloeper 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 (Ongen et al., 2005b), 2,4-diacytethoxylglucosinol (Lavici et al., 2003), the volatiles 2,3-butanediol and acetoin (Ryu et al., 2004), and N-acetylhomoserine lactones (Schuhberger 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 Kovats et al. (1991), Yan et al. (2002) and Doke et al. (1987) suggested that the induced defence 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, Fig. 52). Whether structurally different CLPs produced by strain SS101 or other *Pseudomonas* species also induce resistance in plants against *P. infestans* or other pathogens is as yet unknown and is currently being investigated. Work by Ongen 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; Tom et al., 2001; Yan et al., 2002). Our results with nubG, the transgenic derivative of cv. Moneymaker, suggest also that 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 JA-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 CLPs.

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 cotyledons of tomato seedlings than its CLP-deficient mutant 10.24 (Fig. 6). The presence of the introduced bacterial strains on and in cotyledons, but not on and in true leaves of tomato plants, is most likely the result of 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 CLP 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.

**Acknowledgements**

The first and second authors of this paper contributed equally to the initiation and setup of this study. This work is sponsored by the Vietnamese Ministry of Education and Training (MOET) through project 322, by the German Academic Exchange Service (DAAD) and by the Dutch Graduate School of Experimental Plant Sciences (EPS). We thank Evelyn van Heese for her input in the plant assays, Dr Teris van Beek and Pieter de Waard from Wageningen University (the Netherlands) for...
the LC-MS and NMR analyses, and Dr Renier van den Hoorn for the schematic drawings of the tomato plants. We are grateful to Prof. Pierre de Wit and Dr Jan van Kan for critically reading the manuscript and their valuable suggestions and discussions.

References


## Supplementary Material

The following supplementary material is available for this article online:

**Fig. S1** Schematic presentation of two experimental setups used to determine the role of *Pseudomonas fluorescens* SS101, mutant 10.24 or massetolide A in induction of systemic resistance in tomato against *Phytophthora infestans*. (a) 24 h after treatment of the lower leaves of the tomato plants with wild-type strain SS101, mutant 10.24, or massetolide (MassA, 50 µg ml⁻¹), upper leaves were challenge-inoculated with zoospores of *P. infestans*. Disease severity (lesion area) was determined 4 and 7 d 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 2 wk under controlled conditions; then leaves were challenge-inoculated with zoospores of *P. infestans* and disease severity was determined 4 and 7 d after pathogen inoculation.

**Fig. S2** Induced resistance in tomato against late blight by application of *Pseudomonas fluorescens* SS101 or massetolide A to roots of tomato seedlings. Two weeks after root treatment, tomato leaves were challenge-inoculated with zoospores of *Phytophthora infestans*. Results of two experiments are presented. (a1, a2) Results from Expt 1 (comparison of wild-type SS101 and massetolide A-deficient mutant 10.24); (b1, b2) results from Expt 2 (comparison of wild-type SS101 and partially purified massetolide A (50 µg ml⁻¹)). Means + SE of six replicates are given. Means with the same letter are not significantly different (*P* < 0.05).

This material is available as part of the online article from: http://www.blackwell-synergy.com/doi/abs/10.1111/j.1469-8137.2007.02138.x

(This link will take you to the article abstract.)

Please note: Blackwell Publishing are not responsible for the content or functionality of any supplementary materials supplied by the authors. Any queries (other than missing material) should be directed to the journal at *New Phytologist* Central Office.