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# Induction of systemic resistance against bacterial wilt in *Eucalyptus urophylla* by fluorescent *Pseudomonas* spp

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*Key words:* biological control, pseudobactin, *Ralstonia solanacearum*, salicylic acid, siderophore

**Abstract**

The ability of selected strains of fluorescent *Pseudomonas* spp. to cause induced systemic resistance (ISR) in *Eucalyptus urophylla* against bacterial wilt caused by *Ralstonia solanacearum* was investigated. Four of the five strains used can produce salicylic acid (SA) *in vitro* and, therefore, chemical SA, that is known to induce resistance in many plant species, was used as a reference treatment. Whereas a soil drench with SA did induce systemic resistance in *E. urophylla*, infiltration of SA into leaves did not. None of the fluorescent *Pseudomonas* spp. strains caused ISR against bacterial wilt when applied to the soil, but two strains, *P. putida* WCS358r and *P. fluorescens* WCS374r triggered ISR when infiltrated into two lower leaves 3–7 days before challenge inoculation. A mutant of strain WCS358r defective in the biosynthesis of the fluorescent siderophore pseudobactin, did not cause ISR, while the purified siderophore of WCS358r did, suggesting that pseudobactin358 is the ISR determinant of WCS358. A siderophore-minus mutant of WCS374r induced the same level of disease resistance as its parental strain, but the purified siderophore induced resistance as well, indicating that both the siderophore and another, unknown, inducing determinant(s) of WCS374r can trigger ISR in *Eucalyptus*. A possible role of WCS374r-produced SA remains uncertain. Transformation of a siderophore-minus mutant of WCS358 with the SA biosynthetic gene cluster from WCS374 did not enable this transformant to cause ISR in *E. urophylla*.

## Introduction

Specific strains of non-pathogenic, plant growth-promoting rhizobacteria (PGPR) are known to suppress diseases by induced systemic resistance (ISR) in plants (van Peer et al., 1991; Wei et al., 1991; Kloepper et al., 1992; van Loon et al., 1998). During the past two decades, bacterial determinants responsible for this induction have been identified and shown to be differentially effective in different plant species. This differential effectiveness is clearly illustrated by results from three PGPR strains, *Pseudomonas putida* WCS358, *Pseudomonas fluorescens* WCS374, and *P. fluorescens* WCS417, in different plant–pathogen systems. Strain WCS358 triggers ISR in *Arabidopsis thaliana* against *Pseudomonas syringae* pv. *tomato* (*Pst*) and against *F. oxysporum* f. sp. *raphani* (For) (van Wees et al., 1997), but neither in carnation against *Fusarium oxysporum* f. sp. *dianthi* (Fod) (Duijff et al., 1993), nor in radish against For (Leeman et al., 1995b). Strain WCS374 induces resistance in radish against For (Leeman et al., 1995b), but not in *Arabidopsis* against *Pst* or For (van Wees et al., 1997). Strain WCS417 triggers ISR in carnation (van Peer et al., 1991), radish (Leeman et al., 1995b), *Arabidopsis* (Pieterse et al., 1996), tomato (Duijff et al., 1998), and bean (Bigirimana and

Höfte, 2002) against various pathogens. Bacterial determinants involved in the elicitation of ISR by these strains are the fluorescent siderophore pseudobactin, the outer membrane lipopolysaccharide (LPS), and the flagella of WCS358 (Bakker et al., 2003; Meziane et al., 2005); the pseudobactin, other iron-regulated metabolites and LPS of WCS374 (Leeman et al., 1995b, 1996)), and the LPS and iron-regulated metabolites other than pseudobactin for WCS417 (van Peer and Schippers, 1992; Leeman et al., 1995a, 1996). Notably, strains WCS374 and WCS417 produce salicylic acid (SA) *in vitro* under iron limitation (Leeman et al., 1996), and SA is known to induce systemic resistance in a wide range of plant species (Sticher et al., 1997). However, ISR elicited by WCS417 in *Arabidopsis* is independent of SA accumulation in the plant (Pieterse et al., 1996).

Another PGPR with the ability to induce systemic resistance is *Pseudomonas aeruginosa* 7NSK2. This strain produces pyoverdin, pyochelin, and SA under iron-limiting conditions (Buysens et al., 1996). The production of SA by 7NSK2 seems to be required for the induction of systemic resistance in tobacco against tobacco mosaic virus (TMV) (De Meyer et al., 1999a), and has also been implicated in the systemic resistance induced by this strain against *Botrytis cinerea* in bean (De Meyer and Höfte, 1997). However, Audenaert et al. (2002) described that production of the phenazine compound pyocyanin together with the SA-containing siderophore pyochelin, are required for ISR by 7NSK2 against *B. cinerea* in tomato. Similarly, *P. fluorescens* strain CHA0 is a biocontrol agent of various soilborne diseases (Défago et al., 1990), and induces systemic resistance in tobacco against tobacco necrosis virus (TNV) (Maurhofer et al., 1994). CHA0 also has the ability to produce SA. However, the metabolite of this strain that was suggested as being involved in ISR is pyoverdin (Maurhofer et al., 1994). In *Arabidopsis thaliana* the situation is different and ISR by CHA0 depends on the production of the antibiotic 2,4-diacetylphloroglucinol (Iavicoli et al., 2003). Therefore, the extent to which bacterially produced SA is involved in ISR may vary depending on the biocontrol agent–plant combination.

Eucalypt bacterial wilt has been a severe problem in clonally propagated trees on commercial plantations in south China since the 1980s (Lu and Pan, 1995; Lin et al., 1996; Gan et al., 1998), and no effective control measures are yet available. WCS417r is able to suppress bacterial wilt in *Eucalyptus* when applied by root dip (Ran et al., 2005). Because WCS417r is effective in suppressing diseases in several plant species through the induction of systemic resistance, we questioned whether WCS417r also triggers ISR in *Eucalyptus* and whether bacterially produced SA could play a role. Other strains of fluorescent *Pseudomonas* spp. known to induce systemic resistance were tested for comparison, as was the effect of application of SA.

## Materials and methods

### *Cultivation of* Eucalyptus urophylla

*Eucalyptus urophylla* is highly susceptible to bacterial wilt caused by *Ralstonia solanacearum* (Wu and Liang, 1988; Lin et al., 1996). Seeds were purchased from the Leizhou Forestry Bureau (Leizhou, P.R. China) and germinated in a sand/loam soil mixture. Four-week-old seedlings (average height 1.5 cm) were transferred to pots containing 100 g of a commercial potting soil that had been autoclaved twice for 1 h on alternate days. For each treatment five pots containing four plants each were used. The plants were grown in a growth cabinet for another 7–8 weeks with a 12 h light and 12 h dark cycle at 25–28 °C and 20–23 °C, respectively, and at a relative humidity of 70%. Once a week each pot received 10 ml of half-strength Hoagland nutrient solution (Hoagland and Arnon, 1938), supplemented with 10 $\mu$M FeEDDHA (Fe-ethylenediamine di-*o*-hydroxyphenylacetic acid; CIBA-Geigy, Basel, Switzerland). Tap water was provided whenever necessary.

### *Bacterial strains*

The sources and relevant characteristics of the bacterial strains used are listed in Table 1. The *Pseudomonas* spp. were routinely cultured on King's medium B (KB) agar plates (King et al., 1954) at 28 °C. Ampicillin (40 $\mu$g ml$^{-1}$), cycloheximide (100 $\mu$g ml$^{-1}$), chloramphenicol (13 $\mu$g ml$^{-1}$), kanamycin (50 $\mu$g ml$^{-1}$), and rifampin (100 $\mu$g ml$^{-1}$) were added when applicable. Cultures grown for 24–30 h were removed from the agar plates, and suspended in 10 mM $MgSO_4$. The

*Table 1*. Microorganisms used in this study

| Strains | Relevant characteristics* | Reference or source |
|---|---|---|
| *Ralstonia solanacearum* Rlz | Isolated from wilted branch of *Eucalyptus urophylla* in Leizhou, China; $amp^r$, $chl^r$; wild type | This study |
| $Rlz^r$ | Spontaneous $rif^r$ mutant of Rlz; $amp^r$, $chl^r$, $rif^r$ | This study |
| *R. solanacearum* Rrp | Isolated from wilted seedling of *E. urophylla* in Raoping, China; $amp^r$, $chl^r$; wild type | This study |
| $Rrp^r$ | Spontaneous $rif^r$ mutant of Rrp; $amp^r$, $chl^r$, $rif^r$ | This study |
| *Pseudomonas aeruginosa* 7NSK2 | Wild type; $Pch^+$, $Pvd^+$, $SA^+$; competes for iron, induces systemic resistance | Buysens et al.(1996); De Meyer and Höfte (1997) |
| *P. fluorescens* CHA0r | Isolated from tobacco rhizosphere; $HCN^+$, $Phl^+$, $Plt^+$, $Pvd^+$, $SA^+$; $amp^r$, $chl^r$, $rif^r$; suppresses disease through antibiotics, induces systemic resistance | Stutz et al. (1986); Keel et al.(1992); Maurhofer et al. (1994) |
| *P. fluorescens* WCS374r | Isolated from potato rhizosphere; $Pvd^+$, $SA^+$; $amp^r$, $chl^r$, $rif^r$; competes for iron, induces systemic resistance | Geels and Schippers (1983); Leeman et al. (1995b) |
| JM374 | Tn5 mutant of WCS374; $sid^-$; $amp^r$, $chl^r$, $Km^r$ | Weisbeek et al. (1986); |
| *P. fluorescens* WCS417r | Isolated from wheat rhizosphere, $Pvd^+$, $SA^+$; $amp^r$, $chl^r$, $rif^r$; competes for iron, induces systemic resistance | Lamers et al. (1988); Duijff et al. (1993); van Wees et al. (1997) |
| *P. putida* WCS358r | Isolated from potato rhizosphere; $Pvd^+$, $SA^-$; $amp^r$, $chl^r$, $rif^r$; competes for iron, induces systemic resistance | Geels and Schippers (1983); Duijff et al. (1994); Pieterse et al. (1996) |
| JM218 | Tn5 mutant of WCS358; $sid^-$; $amp^r$, $chl^r$, $Km^r$, $SA^-$ | Marugg et al. (1985) |
| JM218(pMB374-07) | Tn5 mutant of WCS358 containing plasmid pMB374-07 with the SA biosynthetic genes from *P. fluorescens* WCS374; $sid^-$; $amp^r$, $chl^r$, $Km^r$, $SA^+$ | Mercado-Blanco et al. (2001) |

*Abbreviations: HCN = hydrogen cyanide, Pch = pyochelin, Phl = 2,4-diacetylphloroglucinol, Plt = pyoluteorin, Pvd = pyoverdin, SA = salicylic acid, sid = pseudobactin siderophore; $amp^r$, $chl^r$, $Km^r$, $rif^r$ = resistant to ampicillin, chloramphenicol, kanamycin, and rifampin, respectively.

suspension was centrifuged twice at 7600 × *g* for 10 min. The bacterial pellet was suspended in 10 mM $MgSO_4$, and the concentration adjusted to $10^9$ cfu $ml^{-1}$ based on the absorbance at 660 nm. The pseudobactin siderophores of *P. putida* WCS358 and *P. fluorescens* WCS374 were isolated and purified from cultures in standard succinate medium using chloroform–phenol extraction followed by column chromatography (PD-10 colums, Pharmacia), as described previously (Raaijmakers et al., 1994). For preparation of inoculum of *R. solanacearum*, bacterial cells were streaked onto modified Kelman agar plates [in g $l^{-1}$: proteose peptone (Oxoid) 10, casamino acids (Oxoid) 5, glucose 10, bacto agar (Difco) 10] (Kelman, 1954) containing tetrazolium chloride (50 mg $l^{-1}$), and incubated for 48 h at 30 °C. Single slimy, milky colonies with a pink centre (Fahy and Hayward, 1983) were transferred to modified Kelman agar plates without tetrazolium, and grown for 48 h at 30 °C. Bacterial cells were collected in sterile distilled water, and centrifuged twice at 12,000 × *g* for 10 min. The pellet was suspended in distilled water and bacterial density measured at 660 nm.

### *Bioassay for ISR upon application of SA*

Two strains of *R. solanacearum*, Rrp and Rlz, and their rifampin-resistant derivatives, $Rrp^r$ and $Rlz^r$, were tested for their pathogenicity in a bioassay developed to specifically study induction of systemic resistance. Shoot tips of 12-week-old eucalypt seedlings were cut off and 0.2–0.3 μl of a suspension of the pathogen, containing $10^2$–$10^9$ cfu $ml^{-1}$ was applied to the wound site using a micropipette. The inoculated plants were moved to a growth cabinet with a 12 h day at 30 °C and 12 h night at 25 °C, and a relative humidity of

over 93%. Disease was scored 10 days later. To test whether SA is active in inducing systemic resistance in *Eucalyptus*, 10 ml of solution containing SA at different concentrations was poured onto the soil of each pot with four seedlings, at different times with respect to challenge inoculation with the pathogen. Challenge inoculation was performed by decapitating the seedlings and putting a 0.2–0.3 $\mu$l droplet containing $10^5$ cfu $Rrp^r$ $ml^{-1}$ on the wound. Alternatively, SA solutions at different concentrations were pressure-infiltrated into two lower leaves (approximately 5–10 $\mu$l per 10–20 mg leaves) of 12-week-old seedlings 7 days before challenge inoculation. To study possible direct effects of SA on growth of *R. solanacearum*, a modification of methods described earlier (Loo, 1945; Johnson et al., 1972) was used. One hundred microlitre of a suspension of $Rrp^r$ ($10^8$ cfu $ml^{-1}$) was spread evenly onto modified Kelman agar medium. Immediately afterwards, sterile filter paper discs (7 mm diam) were dipped in a sterile solution of 1, 5, 10 or 20 mM SA, and placed in the centre of the agar plates seeded with $Rrp^r$. After incubation for 48 h at 30 °C, plates were inspected for zones of growth inhibition around the paper discs.

*Bioassay for ISR upon soil bacterization*

The bioassay was performed by adding 50 ml solution containing biocontrol bacteria at $10^9$ cfu $ml^{-1}$ to 1 kg of soil, mixing evenly, and planting 4-week-old seedlings, four plants per pot and five pots for each treatment. The seedlings were allowed to grow for 7–8 weeks to an average height of 10–15 cm before shoot tip inoculation with $Rrp^r$ at $10^5$ cfu $ml^{-1}$. Disease development was scored up to 15 days after challenge inoculation. Bacterial colonization of the roots was checked at 7, 8 and 10 weeks after seedlings were transplanted into the treated soil. At each time point five pots per treatment were sampled and for each pot bacterial population densities were determined.

*Bioassay for ISR upon bacterization by leaf infiltration*

In a different type of bioassay, biocontrol bacteria were pressure-infiltrated into the fourth pair of leaves from the top of 11- to 12-week-old seedlings using a syringe without a needle. Eight to 10 h before infiltration, pots with four seedlings were placed in containers covered with transparent plastic film to increase relative humidity to more than 93%. Aliquots of 5–10 $\mu$l of bacterial suspension ($10^9$ cfu $ml^{-1}$) or purified siderophore (10–100 $\mu$g $ml^{-1}$ in 10 mM $MgSO_4$) were pressure infiltrated into the abaxial surface of the leaves. In the control treatment a sterile 10 mM $MgSO_4$ solution was pressure infiltrated. At different time intervals after treatment plants were challenge inoculated as described above, and thereupon, seedlings were kept at high humidity for 24 h. For each treatment five pots, each containing four plants was used.

*Bacterial colonization of plant tissues*

Roots of five plants per treatment were collected, weighed, and shaken vigorously for 1 min in glass tubes containing a sterile 10 mM $MgSO_4$ solution. Aliquots of 100 $\mu$l of appropriate dilutions from the resulting suspensions were transferred to 24-well microtiter plates, followed by the addition of 400 $\mu$l of KB agar supplemented with cycloheximide (100 $\mu$g $ml^{-1}$), ampicillin (40 $\mu$g $ml^{-1}$), chloramphenicol (13 $\mu$g $ml^{-1}$), and rifampin (150 $\mu$g $ml^{-1}$) for the rifampin-resistant bacterial strains. For 7NSK2 selective conditions were used as described by De Meyer and Höfte (1997). After incubation at 28 °C for 20–36 h the numbers of cfu $g^{-1}$ root fresh weight were determined by counting colonies under a microscope. To determine in how far biocontrol bacteria are transported within the vascular system of the plants, five shoot tips, infiltrated leaves, and two stem segments of 2 cm length above and below the infiltrated leaf pair were harvested at the time of challenge inoculation. Tissues were rinsed with sterile water containing 0.01% detergent for 5 min, washed in sterilized water three times, and homogenized in sterile 10 mM $MgSO_4$. The number of cfu $g^{-1}$ fresh weight of tissue was determined as described above.

*Disease ratings and data analysis*

Bacterial ooze was apparent on the cut surface of the decapitated shoots 5 days after challenge inoculation, and the shoot below the tip became

brown or black with time. Diseased seedlings were scored in classes at different time points after challenge, based on the length of the blackened shoot tip zone:

0 – no symptoms;
1 – blackened zone shorter than 0.5 cm;
2 – blackened zone 0.5–2.0 cm;
3 – blackened zone over 2.0 cm or seedling completely wilted and dead.

The disease index (DI) was calculated as $DI = 100 \times \sum(N_i \times X_i)/3 \times \sum N_i$, where $N_i$ indicates the number of seedlings in each class, and $X_i$ is the class number. When the DI in the control treatment was over 80, the experiment was terminated. Disease indices were statistically analyzed for significant differences using one-way analysis of variance (ANOVA), followed by Fisher's least significant difference test ($\alpha = 0.05$), using SPSS 8.0 (SPSS for Windows). All experiments were repeated at least twice.

## Results

*Pathogenicity of* R. solanacearum *to* E. urophylla

When inoculated onto decapitated shoots, *R. solanacearum* strains Rlz and Rrp, and their spontaneous rifampin-resistant mutants, $Rlz^r$ and $Rrp^r$, induced disease in a clear density-dependent manner (Figure 1). No significant difference in pathogenicity was observed between the two wild-type strains, or between strain Rrp and its rifampin-resistant mutant $Rrp^r$. In contrast, the rifampin-resistant mutant $Rlz^r$ was significantly less pathogenic than its parental strain, particularly at lower inoculum densities. At pathogen inoculum densities exceeding $10^8$ cfu $ml^{-1}$, most of the plants were completely wilted 1 week later, whereas if the inoculum density was lower than $10^4$ cfu $ml^{-1}$, some seedlings were not diseased even after 10 days. An inoculation density of $10^5$ cfu $ml^{-1}$ was adopted for further experiments, using the equally pathogenic strain Rrp and its rifampin-resistant mutant, $Rrp^r$.

*Induction of systemic resistance by treatment with SA*

Strain Rrp rapidly colonized decapitated seedlings, causing a DI of 76 by day 10 and 85 by day 15. Application of 5 mM SA as a soil drench reduced disease development significantly whereas lower concentrations of SA were not effective (Figure 2). Higher concentrations of SA (10 and 20 mM), while strongly suppressing disease development after challenge inoculation, proved toxic and at least half of the plants died within 2–5 days after SA treatment (data not shown). To determine the optimum interval for systemic resistance to develop, the time between application of SA and

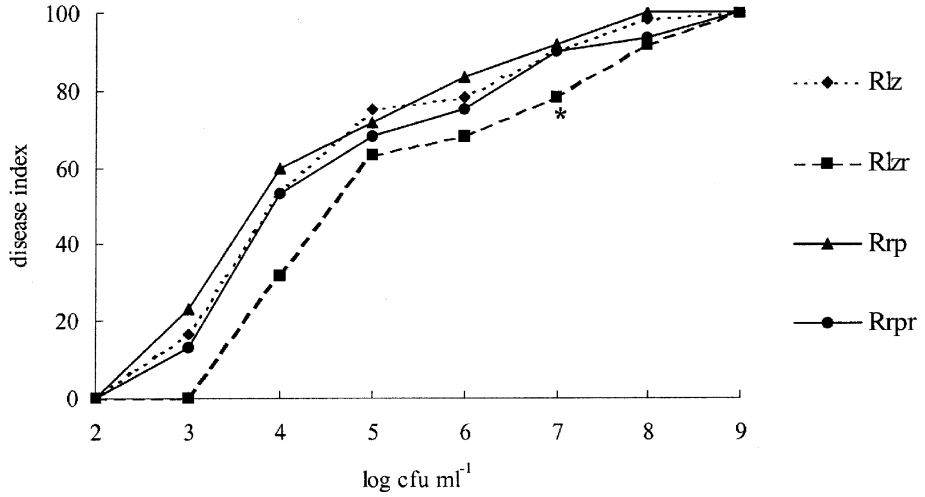


*Figure 1.* Pathogenicity of *Ralstonia solanacearum* wild-type strains, Rlz and Rrp, and their rifampin-resistant derivatives, $Rlz^r$ and $Rrp^r$, to 10–15 cm high *Eucalyptus urophylla* seedlings when inoculated at different densities onto the shoot tips. Disease was scored 10 days after inoculation. *Significantly different from the other treatments ($P = 0.05$).

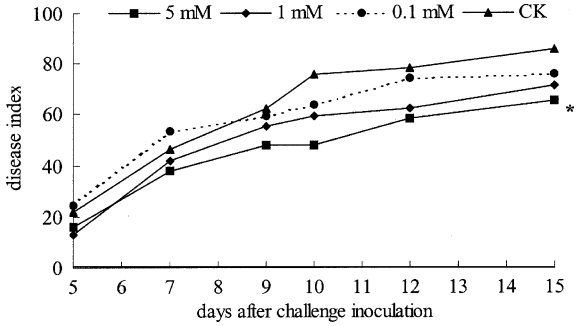


*Figure 2.* Induction of systemic resistance against bacterial wilt in *Eucalyptus urophylla* by pouring different amounts of SA on the soil. 7 days after SA treatment plants were challenge inoculated by applying 20–30 cfu of strain Rrp$^r$ to the cut surface of decapitated seedlings. CK = water-treated control. *Significantly different from the control treatments ($P = 0.05$).

challenge inoculation was varied. As can be seen from Figure 3, DI was significantly reduced already at an interval of 1 day ($\alpha = 0.05$), and tended to decrease further when the time interval increased up to 7 days. Thereafter, disease severity increased again (Figure 3). These results indicated that a time interval of 7 days between a soil drench of SA and challenge inoculation of the shoot tip was optimal for disease suppression. Infiltration of solutions of SA at concentrations ranging from 1 $\mu$M to 5 mM into the fourth pair of leaves did not induce resistance. In several repeated experiments no reduction of disease caused by tip-inoculated Rrp$^r$ was evident (data not shown).

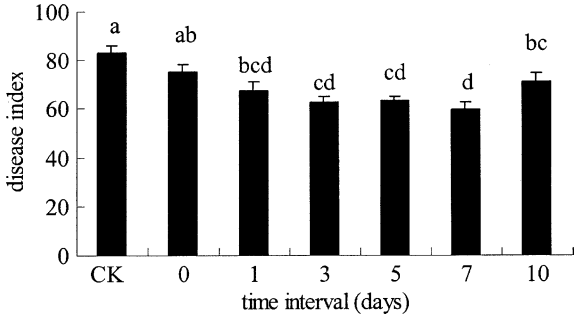


*Figure 3.* Effect of time interval between applying applying SA (5 mM) on the soil and challenge inoculation with *Ralstonia solanacearum* Rrp$^r$ on disease development in *Eucalyptus urophylla*. For challenge inoculation 20–30 cfu of the pathogen were applied to the cut surface of decapitated seedlings. Disease was scored 10 days after inoculation. CK = water-treated control. Bars with different letters are significantly different ($P = 0.05$).

Moreover, all leaves infiltrated with 5 mM SA and about 30% of the leaves infiltrated with 1 mM SA became curled and necrotic. At concentrations up to 20 mM, SA did not have any inhibitory effect on the growth of *R. solanacearum in vitro* (data not shown). Thus, disease suppression by application of SA to the soil cannot be due to a direct inhibitory effect on the pathogen, and induced resistance appears to be involved.

### *Influence of soil application of fluorescent* Pseudomonas *spp. on bacterial wilt*

Five ISR-inducing fluorescent *Pseudomonas* spp. strains were tested for their ability to induce resistance when mixed through the soil. Upon inoculation of seedling shoot tips with the pathogen, strains WCS358r, WCS374r and WCS417r had no significant effect on disease development (Figure 4). All strains established significant populations on the *Eucalyptus* roots as measured 7, 8 and 10 weeks after seedling transplant, and their numbers remained well above $10^5$ cfu per gram root (Table 2). In *Arabidopsis*, strain WCS374r did not trigger ISR when grown at 28 °C, but it did when grown at elevated temperatures (Ran et al., 2000). When grown at low iron availability WCS374r produces SA *in vitro*, and at elevated temperatures SA production is increased in this strain. Nevertheless, WCS374r grown at different temperatures and iron availabilities never induced resistance in *Eucalyptus*, although rhizosphere population densities were well above $10^5$ cfu $g^{-1}$

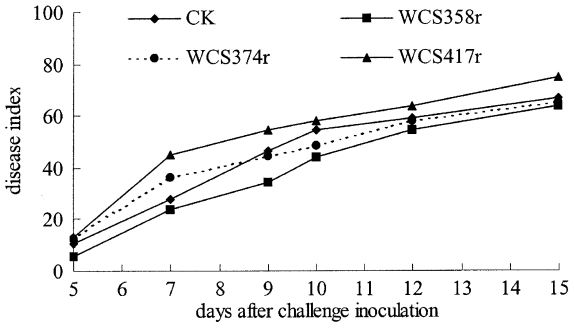


*Figure 4.* Effects of mixing *Pseudomonas* strains into the soil on bacterial wilt in *Eucalyptus urophylla*. Seedlings were challenge inoculated by applying 20–30 cfu of *Ralstonia solanacearum* Rrp$^r$ to the cut surface of decapitated seedlings. CK = treated with 10 mM $MgSO_4$. No significant differences between treatments were observed.

*Table 2*. Population densities of *Pseudomonas* spp strains in the rhizosphere of *Eucalyptus urophylla*, at different time intervals after transplanting seedlings into soil into which the bacterial strains were mixed.

| Log cfu/g-1 root | Weeks after seedling transplant | | |
|---|---|---|---|
| | 7 | 8 | 10 |
| WCS358r | 6.1 | 6.6 | 5.9 |
| WCS374r | 5.8 | 6.6 | 5.6 |
| WCS417r | 6.0 | 6.1 | 6.0 |

No significant differences between the strains were observed.

root (data not shown). Likewise, strains 7NSK2 and CHA0r did not reduce bacterial wilt in *Eucalyptus* (data not shown).

*Leaf infiltration with fluorescent* Pseudomonas *spp.*

Possible effects of the five strains were tested further by infiltrating them into the fourth pair of leaves and challenging decapitated shoot tips 7 days later. Compared to the water control, strains CHA0r, WCS417r and 7NSK2 were ineffective in systemic disease suppression (Figure 5). In contrast, both WCS358r and WCS374r significantly decreased bacterial wilt (Figure 5), indicating development of ISR. Different time intervals between infiltration of the bacterial cells into the leaves and challenge inoculation of the shoot tips with the pathogen were applied to determine the time course required for development of ISR. As

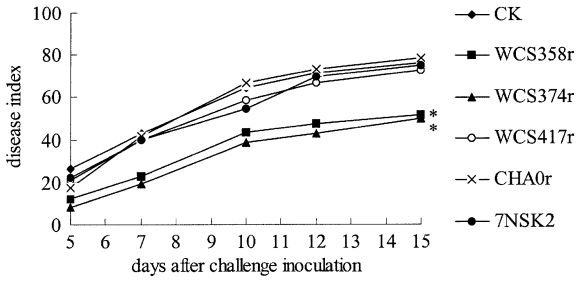


*Figure 5*. Induction of systemic resistance against eucalypt bacterial wilt by infiltration of suspensions of fluorescent pseudomonads at a density of $10^9$ cfu $ml^{-1}$ into the fourth pair of leaves 7 days before challenging decapitated shoot tips with 20–30 cfu of *Ralstonia solanacearum* strain $Rrp^r$. CK = infiltrated with 10 mM $MgSO_4$. *Significantly different from the other treatments ($P = 0.05$).

seen in Figure 6a, an interval of 5 days was sufficient to reach significant protection by strain WCS358. For strain WCS374, an interval of three days seemed sufficient for ISR to develop (Figure 6b). Thus, for both strains 3–5 days between induction and challenge seems to be required for ISR. To determine the dose of bacteria required to significantly suppress disease development in the leaf infiltration assay, WCS358r and WCS374r were infiltrated at different cell densities. For strain WCS358 densities of $10^9$ cfu $ml^{-1}$ significantly suppressed disease, whereas lower densities were not effective (Figure 7a). Strain WCS374r was effective at $10^7$ cfu $ml^{-1}$ and higher, but not at $10^6$ cfu $ml^{-1}$ (Figure 7b).

*Involvement of siderophores of WCS358 and WCS374 in induction of systemic resistance against bacterial wilt*

The possible involvement of the fluorescent pseudobactin-type siderophores of WCS358 and WCS374 in the induction of resistance after leaf infiltration were investigated using pseudobactin-minus mutants and purified siderophores. Whereas the parental strain WCS358r or its purified siderophore did significantly reduce disease, the pseudobactin mutant JM218 did not. This result suggests that pseudobactin358 is the determinant of WCS358r responsible for induction of systemic resistance in *E. urophylla* (Figure 8a). As can be seen in Figure 8b, JM374, the siderophore-minus mutant of WCS374r, reduced disease severity almost to the same level as its parental strain WCS374r. However, at concentrations of 10 or 100 $\mu g\ ml^{-1}$ the purified pseudobactin siderophore of WCS374r also induced resistance against bacterial wilt. These results indicate that this siderophore is active in triggering ISR, but another bacterial determinant of WCS374r also induces systemic resistance. The possible role of SA and the SA-containing siderophore pseudomonine, that are likewise produced by WCS374 under conditions of iron limitation, was studied using JM218 transformed with cosmid pMB374-07. Transformant JM218 (pMB374-07) produces both SA and pseudomonine *in vitro* (Mercado-Blanco et al., 2001). However, no obvious disease reduction was observed when JM218 (pMB374-07) was tested in this type of bioassay (Figure 8a).

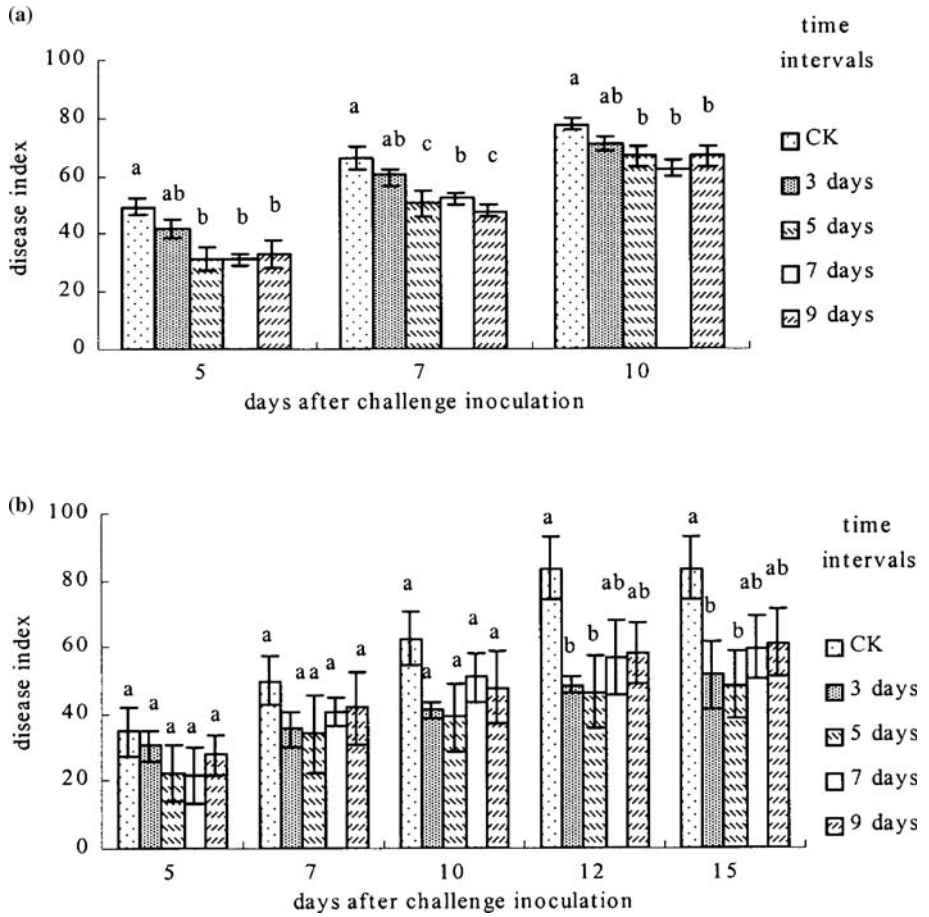


*Figure 6*. Effects of time intervals between infiltration of $10^9$ cfu $ml^{-1}$ of WCS358r (a) or WCS374r (b) into the fourth pair of leaves and challenge inoculation of decapitated shoot tips with 20–30 cfu of *Ralstonia solanacearum* $Rrp^r$ on development of systemic resistance against Eucalyptus bacterial wilt. CK = infiltrated with 10 mM $MgSO_4$. For each time point bars with different letters are significantly different ($P = 0.05$).

*Spatial separation between fluorescent* Pseudomonas *spp. and* R. solanacearum

To check whether the disease suppression by WCS358 and WCS374 is plant-mediated and, therefore, can be ascribed solely to induced resistance, it was tested whether the infiltrated *Pseudomonas* strains remained spatially separated from the challenge-inoculated *R. solanacearum*. At the time of challenge inoculation around $10^7$ cfu $g^{-1}$ tissue were present in the infiltrated leaves (Table 3). The two 2-cm stem segments below the infiltrated fourth leaf pair were colonized to substantial levels, but colonization of the stem above the infiltrated leaves was only slightly above the detection limit and confined to the first 2 cm only. No *Pseudomonas* spp. cells could be recovered from the shoot tip region (Table 3). These observations indicate that the infiltrated bacterial cells move mainly downwards through the vascular tissues.

## Discussion

Bacterial wilt of *Eucalyptus*, caused by *R solanacearum*, is a serious problem in production plantations in south China. Possibilities for employing fluorescent *Pseudomonas* spp. strains for control of this disease are being investigated (Ran et al., 2005). Several of these biocontrol strains have the ability to trigger ISR in plants. For example,

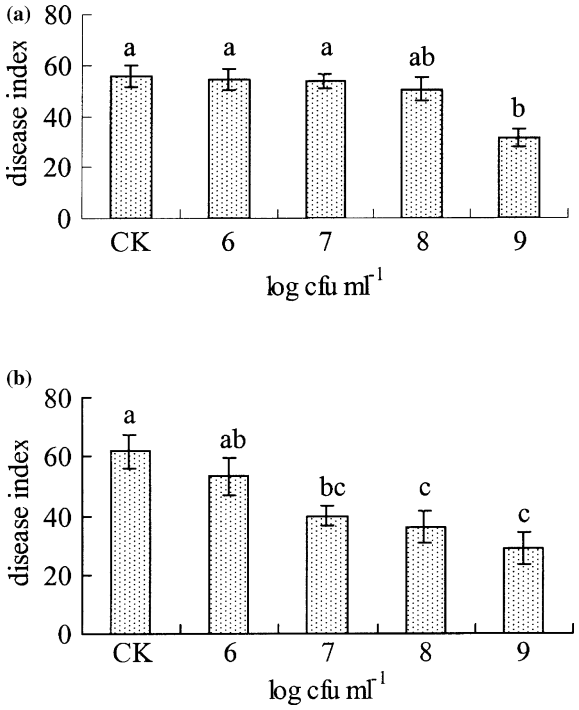


*Figure 7*. Effects of population density of WCS358r (a) and WCS374r (b) on induction of systemic resistance when infiltrated into the fourth pair of leaves. Decapitated seedlings were challenge inoculated with 20–30 cfu of *Ralstonia solanacearum* $Rrp^r$ 7 days after leaf infiltration with the *Pseudomonas* strains, and disease was scored 10 days after inoculation. CK = infiltrated with 10 mM $MgSO_4$. Bars with different letters are significantly different ($P = 0.05$).

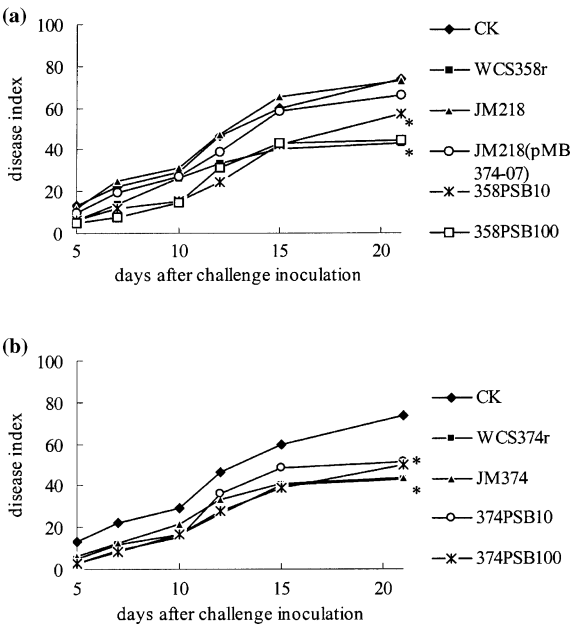


*Figure 8*. Effects of the pseudobactin siderophore of WCS358r (a) and WCS374r (b) on induction of systemic resistance in *Eucalyptus urophylla*. Bacterial suspensions ($10^9$ cfu $ml^{-1}$), or purified siderophore (10 or 100 $\mu g\ ml^{-1}$) were pressure infiltrated into the fourth pair of leaves 7 days before challenge inoculation of decapitated shoot tips with 20–30 cfu of *Ralstonia solanacearum* $Rrp^r$. Disease was scored at different time points after the challenge inoculation. JM218 is a pseudobactin mutant of WCS358, and JM218(pMB374–07) is a SA producing derivative of JM218. 358PSB10, 358PSB100, 374PSB10 and 374 PSB100 are treatments with the purified siderophores of the strains at 10 respectively 100 $\mu g\ ml^{-1}$. *Significantly different from the other treatments ($P = 0.05$).

*P. fluorescens* WCS417r can induce systemic resistance in several plant–pathogen systems, (van Peer et al., 1991; Leeman et al., 1995b; Pieterse et al., 1996; Duijff et al., 1998; Bigirimana and Höfte, 2002). Therefore, the possible involvement of ISR in suppression of bacterial wilt of *Eucalyptus* by a selection of biocontrol strains was investigated.

Application of SA induces systemic resistance in plants (Sticher et al., 1997) and for root-colonizing *P. aeruginosa* 7NSK2 production of SA seems to be important in triggering ISR (De Meyer and Höfte, 1997; De Meyer et al., 1999a). In this study application of SA as a soil drench induced resistance in *Eucalyptus* against *R. solanacearum*. However, when pressure-infiltrated into lower leaves, SA did not induce systemic resistance against bacterial wilt, confirming that the effect of SA is dependent on the site of application. Application of SA on plant roots results in triggering of systemic acquired resistance in tobacco (Van Loon and Antoniw, 1982), radish (Leeman et al., 1996), *Arabidopsis* (Van Wees et al., 1997), and bean (De Meyer et al., 1999b). In contrast, infiltration of leaves with SA only occasionally resulted in systemic resistance in tobacco (Van Loon and Antoniw, 1982), whereas it did induce systemic resistance in *Arabidopsis* (Pieterse et al., 1996).

Whereas strains 7NSK2, CHA0, WCS374r, and WCS417r all produce SA under iron-limiting conditions *in vitro*, application of these strains to roots of *Eucalyptus* was not effective against bacterial wilt. Either the strains did not produce SA under the conditions employed, or the amounts produced were insufficient for triggering systemic resistance. Whereas 5 mM of SA was required for triggering

*Table 3*. Movement of bacterial cells in *Eucalyptus urophylla* seedlings 1 week after infiltration of the fourth pair of leaves with $5 \times 10^8$ cfu $g^{-1}$ tissue

| Strains | Below [b] | | | Above[b] | | |
|---|---|---|---|---|---|---|
| | Infiltrated leaves | 0–2 cm | 2–4 cm | 0–2 cm | 2–4 cm | Shoot tips |
| WCS358r | 7.59 | 4.81 | 3.39 | 2.40 | n.d.[a] | n.d. |
| WCS374r | 6.72 | 4.25 | 2.92 | 2.17 | n.d. | n.d. |

[a]n.d. = not detected, the detection limit is 100 cfu $g^{-1}$ tissue.
[b]Stem segments below or above the infiltrated leaf pair.

ISR in Eucalyptus (Figure 2), the bacterial strains produce SA in the $\mu$M range under optimal conditions *in vitro* (Leeman et al., 1996). Strain WCS358r does not produce SA *in vitro* (Leeman et al., 1996), but it does induce resistance in *Arabidopsis* (Van Wees et al., 1997). This strain was also unable to trigger ISR when applied to *Eucalyptus* roots. All strains were applied in several ways to the soil, including booster applications at different time intervals after planting (data not shown). In none of the experiments application of bacteria to the roots resulted in significant disease suppression. The lack of disease suppression was not due to poor root colonization, because the population densities of all strains were well above $10^5$ cfu $g^{-1}$ root, the threshold level for triggering ISR in radish reported by Raaijmakers et al. (1995). However, when infiltrated into the leaf, strains WCS358r and WCS374r did induce systemic resistance in *Eucalyptus*, whereas WCS417r, 7NSK2 and CHA0r did not. Since no bacterial cells of either WCS358r or WCS374r were detected in the shoot tips of seedlings that had been infiltrated with these strains in the fourth pair of leaves, the protective effects were plant-mediated and, hence, have to be ascribed to ISR (Van Loon et al., 1998). Infiltrating bacteria into Eucalyptus leaves to protect them from bacterial wilt may be too laborious to be applied in practice; however it might be possible to spray the bacteria onto the leaves and also gain control of the disease.

For WCS358r, the fluorescent siderophore pseudobactin is the determinant that triggers ISR in *Eucalyptus*, because a transposon insertion mutant defective in the biosynthesis of this siderophore, JM218, did not induce ISR, and the purified pseudobactin did. This is different from the situation in *Arabidopsis thaliana* where pseudobactin, LPS, and flagella are all involved in ISR by WCS358 (Bakker et al., 2003; Meziane et al., 2005). The siderophore-minus mutant, JM374 of WCS374r, induced the same level of disease resistance as its parental strain; its purified pseudobactin siderophore also induced disease resistance, indicating that this siderophore can act as an inducing factor but is not the only bacterial determinant responsible for disease reduction in *Eucalyptus*. In radish the pseudobactin siderophore of WCS374r triggered ISR (Leeman et al., 1996), but also the 0-antigen of the LPS is an important determinant (Leeman et al., 1995a). Expression of the SA and pseudomonine biosynthetic genes of WCS374 (Mercado-Blanco et al., 2001) in JM218 did not lead to ISR, making it unlikely that SA or pseudomonine act as inducing determinants.

Whereas WCS417r induces systemic resistance in various plant species, it did not trigger ISR in *Eucalyptus*. Because WCS417r did colonize the roots of *E. urophylla* as well as WCS358r or WCS374r, the bacterial determinants involved in the induction of resistance by WCS417r in other plant species, are apparently not perceived by *E. urophylla*. Whether this lack of perception is restricted to *E. urophylla* or *Eucalyptus* in general remains to be investigated. However, these results again illustrate the host specificity in the elicitation of ISR by fluorescent *Pseudomonas* spp.

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