MPMI Vol. 15, No. 11, 2002, pp. 1147–1156. Publication no. M-2002-0903-01R. © 2002 The American Phytopathological Society

Induction of Systemic Resistance to *Botrytis cinerea* in Tomato by *Pseudomonas aeruginosa* 7NSK2: Role of Salicylic Acid, Pyochelin, and Pyocyanin

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Submitted 4 February 2002. Accepted 11 July 2002.

The rhizobacterium *Pseudomonas aeruginosa* 7NSK2 produces secondary metabolites such as pyochelin (Pch), its precursor salicylic acid (SA), and the phenazine compound pyocyanin. Both 7NSK2 and mutant KMPCH (Pch-negative, SA-positive) induced resistance to *Botrytis cinerea* in wild-type but not in transgenic *NahG* tomato. SA-negative mutants of both strains lost the capacity to induce resistance. On tomato roots, KMPCH produced SA and induced phenylalanine ammonia lyase activity, while this was not the case for 7NSK2. In 7NSK2, SA is probably very efficiently converted to Pch. However, Pch alone appeared not to be sufficient to induce resistance. In mammalian cells, Fe-Pch and pyocyanin can act synergistically to generate highly reactive hydroxyl radicals that cause cell damage. Reactive oxygen species are known to play an important role in plant defense. To study the role of pyocyanin in induced resistance, a pyocyanin-negative mutant of 7NSK2, PHZ1, was generated. PHZ1 is mutated in the *phzM* gene encoding an *O*-methyltransferase. PHZ1 was unable to induce resistance to *B. cinerea,* whereas complementation for pyocyanin production or co-inoculation with mutant 7NSK2-562 (Pch-negative, SA-negative, pyocyanin-positive) restored induced resistance. These results suggest that pyocyanin and Pch, rather than SA, are the determinants for induced resistance in wild-type *P. aeruginosa* 7NSK2.

Additional keywords: phenazine-1-carboxylate, siderophores.

Induced resistance is a state of enhanced defensive capacity developed by a plant when appropriately stimulated (van Loon et al. 1998). Induced resistance is generally systemic and can be triggered by pathogens, certain chemicals, and nonpathogenic rhizosphere bacteria. The mechanisms involved in rhizobacteria-mediated induced systemic resistance (ISR) appear to vary among bacterial strains and pathosystems. Bacterial determinants of ISR which have been identified are lipopolysaccharides and siderophores (van Loon et al. 1998). Siderophores are high-affinity iron(III)-chelating compounds that are produced by most microorganisms under iron-limiting conditions. The catechol siderophore biosynthesis genes of *Serratia marcescens* are involved in ISR to *Colletotrichum orbiculare* on cucumber (Press et al. 2001). The purified pyoverdine-type siderophore of *Pseudomonas putida* WCS374 induced resistance to Fusarium wilt in radish (Leeman et al. 1996), while a pyoverdine-negative mutant of *P. fluorescens* CHA0 was less effective in inducing resistance to *Tobacco necrosis virus* on tobacco than the wild-type strain (Maurhofer et al. 1994). Another iron-chelating molecule that is well-studied with respect to induced plant defense is salicylic acid (SA). Although the siderophore capacity of SA is rather poor (Chipperfield and Ratledge 2000), it appears to be an important molecule in induced resistance by the rhizobacterium *P. aeruginosa* 7NSK2. This bacterium produces three siderophores under iron-limiting conditions (pyoverdine, pyochelin (Pch), and SA) and can induce resistance to plant diseases caused by *Botrytis cinerea* on bean and tomato (Audenaert et al. 2002; De Meyer et al. 1999b; De Meyer and Höfte 1997), *C. lindemuthianum* on bean (Bigirimana and Höfte 2002), and *Tobacco mosaic virus* (TMV) on tobacco (De Meyer et al. 1999a). In all these systems, the involvement of bacterial SA in induced resistance was shown using siderophore mutants. Under iron limitation, SA-deficient mutants were not able to induce resistance in a pyoverdine-negative or -positive background. For *P. aeruginosa* KMPCH, a Pch^- and SA^+ mutant of *P. aeruginosa* 7NSK2, it was illustrated that bacterial SA induced phenylalanine ammonia lyase (PAL) activity in bean roots (De Meyer et al. 1999b). PAL is a key enzyme in SA biosynthesis and plant defense (Pallas et al. 1996; Smith-Becker et al. 1998). Moreover, SA levels increased in bean leaves upon root colonization with KMPCH. Furthermore, the activation of plant defense by bacterial SA production was mimicked by applying nanogram amounts of exogenous SA to bean roots (De Meyer et al. 1999b). These results clearly demonstrate that the bacterial production of SA by *P. aeruginosa* KMPCH leads to activation of a SA-dependent defense response in plants. In *P. aeruginosa,* SA is produced from chorismate via the shikimate biosynthesis pathway (Serino et al. 1995) and is a direct precursor of Pch. Mutants in SA production also are Pch-negative (Serino et al. 1997); therefore, a role for Pch in induced resistance by the wild-type strain *P. aeruginosa* 7NSK2 cannot be excluded. The resistance-inducing potential of SA was illustrated for other *Pseudomonas* strains in other plant pathosystems as well. When the SA biosynthetic gene cluster *pchDCBA* from *P. aeruginosa* PAO1 was introduced into the non-SA-producing strain *P. fluorescens* P3, the strain was converted into a resistance-inducing strain (Maurhofer et al. 1998). Bac-

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terial SA production, however, does not appear to be involved in disease resistance induced by the rhizobacterium *Serratia marcescens* 90-166 because an SA-negative mutant of this strain induced resistance to the same level as the wild-type strain (Press et al. 1997).

Most likely, rhizobacteria can induce systemic resistance in plants through different, complementary, and additive mechanisms, of which production of SA represents only one (van Loon et al. 1998).

In the present work, we demonstrate that *P. aeruginosa* KMPCH induces resistance in tomato to *B. cinerea* through production of SA and through activation of SA-dependent defense mechanisms in the plant as demonstrated earlier in bean by De Meyer and associates (1999b). However, we provide evidence that for the wild-type (WT) strain *P. aeruginosa* 7NSK2 a combined action of Pch and pyocyanin is more likely to be involved in induced resistance to *B. cinerea* in tomato than is SA production. Pyocyanin (5-methyl-1-hydroxyphenazinium betaine) is a blue phenazine compound that is only produced by *P. aeruginosa* strains (Turner and Messenger 1986) and is considered to be a virulence factor in clinical isolates of *P. aeruginosa* (Britigan et al. 1992, 1997).

RESULTS

Involvement of siderophores in induced resistance to *B. cinerea* in tomato plants by *P. aeruginosa*.

Detached tomato leaves were infected with *B. cinerea* using the droplet inoculation method described by Audenaert and associates (2002). The inoculation solution that was used (0.01 M glucose, 6.7 mM KH_2PO_4, 10^6 spores per ml), produced a moderate number of spreading lesions in detached leaves from control plants (±60%). This infection allowed us to detect decreased disease severity upon root inoculation with resistance-inducing bacterial strains. Root colonization by bacterial strains was determined and was approximately 10^5 CFU per g of fresh root for all bacterial strains at the time of leaf inoculation (data not shown).

In tomato leaves originating from plants colonized by WT *P. aeruginosa* 7NSK2, KMPCH (SA^+, Pch^-, and pyoverdine$^-$ mutant), and MPFM1 (SA^+, Pch^+, but pyoverdine$^-$ mutant), the number of spreading *B. cinerea* lesions decreased significantly (Fig. 1). Root colonization with the corresponding SA-negative mutants obtained by *pch*A (encodes one of the enzymes catalyzing the formation of SA from chorismate [Serino et al. 1995]) replacement (7NSK2-562, KMPCH-567 and MPFM1-569) no longer induced resistance (Fig. 1), indicating the involvement of bacterial SA in induced resistance. However, 7NSK2 and MPFM1 both produce Pch from SA; therefore, a role for this siderophore could not be excluded.

KMPCH but not 7NSK2 produces SA on tomato roots.

To verify in vivo SA-production by KMPCH and 7NSK2, SA levels on tomato roots were determined. KMPCH root colonization resulted in an increased level of free SA (79.3 ± 24.7 ng of SA per g of fresh weight [FW]) compared to control roots (43.1 ± 8.9 ng of SA per g of FW). The SA-negative mutant of KMPCH (i.e., KMPCH-567) did not result in an increased level of free SA (37.6 ± 13.8 ng of SA per g of FW), indicating that KMPCH produces SA on tomato roots. Surprisingly, root colonization with 7NSK2 did not result in increased free SA levels (45.6 ± 13.6 ng of SA per g of FW), although this strain produces approximately 6 µg of SA per ml when grown on Casamino Acids (CAA) medium (De Meyer and Höfte 1997). No significant differences in bound SA could be observed upon root colonization with the bacterial strains (data not shown).

P. aeruginosa 7NSK2 efficiently converts SA to Pch in the presence of L-cysteine.

We wanted to clarify why SA is produced by 7NSK2 in vitro and not on plant roots. One possibility is that SA is efficiently converted to Pch on plant roots. It was shown by Ankenbauer

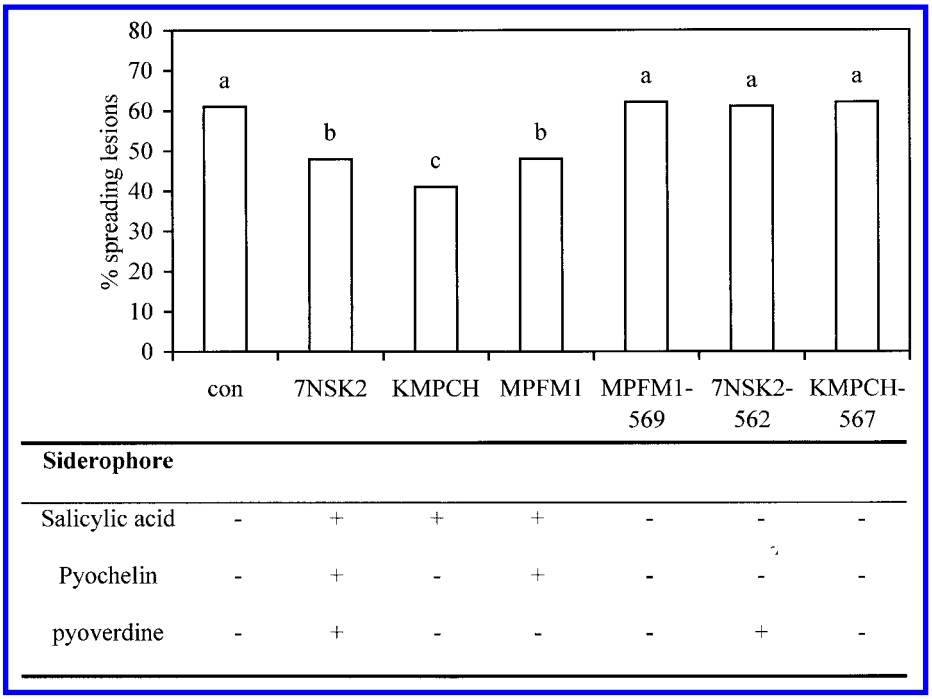


Fig. 1. Influence of tomato root treatment with *Pseudomonas aeruginosa* 7NSK2 and siderophore mutants on the percentage of spreading *Botrytis cinerea* lesions on tomato leaves. All strains were grown on iron-limited King's B medium and applied to tomato seeds and soil. Control plants were treated with water. Tertiary leaves were infected with 10 droplets of 4 µl of spore suspension containing 10^6 spores/ml, 0.01M glucose, and 6.7 mM KH_2PO_4. Infections were evaluated 4 days after inoculation by counting the number of spreading *B. cinerea* lesions on each leaf. Data represented are means for three experiments, each with 12 leaves per treatment. Statistical analysis by a logistic regression was performed on pooled data, because interaction between experiment and treatment was not significant at $P = 0.05$.

and associates (1988) and Reimmann and associates (1998) that Pch is derived from one molecule of salicylate and two molecules of cysteine. In vitro experiments with 7NSK2 grown on M9 succinate amended with L-cysteine in concentrations ranging from 1 to 100 mg/liter clearly demonstrate that high L-cysteine concentrations in the culture medium result in higher Pch production (Fig. 2). It is not unlikely that cysteine concentrations on the tomato root surface are high enough to favor the conversion of SA to Pch, leaving no detectable amounts of free SA on the root surface.

Importance of SA-dependent defense in resistance induced by KMPCH and 7NSK2.

KMPCH produces SA on tomato roots; therefore, the involvement of SA-dependent defense in induced resistance was analyzed. PAL, which was shown to be crucial for activation of SA-dependent defense (Smith-Becker et al. 1998; Pallas et al. 1996), was monitored in control tomato roots and tomato roots colonized by KMPCH and 7NSK2 (Fig. 3). PAL activity in roots of control plants and roots colonized by 7NSK2 was normally distributed and not statistically different. In roots treated with KMPCH, PAL activity was not normally distributed. Colonization of tomato roots by KMPCH increased PAL activity in roots, but only in approximately 50% of the plants. De Meyer and associates (1999b) observed a similar phenomenon in bean roots colonized by KMPCH.

To study the role of the SA-dependent defense signaling pathway in induced resistance by KMPCH and 7NSK2, transgenic *NahG* tomato plants, which are unable to accumulate SA, were root inoculated with KMPCH, KMPCH-567, and 7NSK2 and infected with *B. cinerea*. All bacterial strains were unable to induce resistance to *B. cinerea* in *NahG* tomato plants (Fig. 4). This result indicates that, although SA is not involved at the induction site of ISR by 7NSK2, the strain needs a functional SA response to induce resistance against *B. cinerea*.

Pch is necessary, but not sufficient to induce resistance in tomato.

A SA^- and Pch^- mutant of 7NSK2 (i.e., 7NSK2-562) no longer induced resistance in tomato to *B. cinerea* (Fig. 1); therefore, we verified if Pch was crucial for induction of resistance in tomato to *B. cinerea*. However, the Pch-producing strain *P. aeruginosa* PNA1 that was used for this purpose was unable to induce resistance (data not shown). Apparently, Pch is necessary but not sufficient to induce resistance in tomato to *B. cinerea.* Interestingly, 7NSK2 and PNA1 differ in the production of phenazines. 7NSK2 produces the blue phenazine pigment pyocyanin, while PNA1 produces both phenazine-1-carboxylate (PCA) and phenazine-1-carboxamide (PCN) (Anjaiah et al. 1998) and is unable to synthesize pyocyanin. It was shown before that high concentrations of purified pyocyanin can induce resistance to *B. cinerea* in bean (Abeysinge 1999); therefore, we wanted to investigate whether pyocyanin production by 7NSK2 is involved in induced resistance. For that purpose, we constructed a pyocyanin-negative mutant in 7NSK2 by miniTn*phoA3* mutagenesis. In the white mutant PHZ1, the miniTn*phoA3* had inserted at position 4712706 in gene PA4209 (98% sequence identity), which corresponds to *phzM* (Mavrodi et al. 2001). The product of this gene is an *O*-methyl transferase, which is necessary for the conversion of PCA to 5-methylphenazine-1-carboxylic acid betaine (Mavrodi et al. 2001).

7NSK2 may induce resistance in tomato through production of pyocyanin and Pch.

The lack of pyocyanin production by PHZ1 was confirmed in in vitro experiments monitoring production of pyocyanin by the wild-type strain 7NSK2, PHZ1, and 7NSK2-562 (Fig. 5). PHZ1 was completely unable to produce pyocyanin whereas 7NSK2-562 appeared to overproduce this phenazine.

In induced resistance infection assays, the pyocyanin-negative mutant PHZ1 was not able to induce resistance in tomato to *B. cinerea* (Fig. 6), indicating that pyocyanin is necessary for 7NSK2 to induce resistance. We applied 7NSK2-562 and PHZ1 together on tomato roots and observed an induction of resistance to a level comparable to that observed in plants colonized by the WT strain 7NSK2 or KMPCH, where PHZ1 (Pch^+, SA^+, $pyocyanin^-$) and 7NSK2-562 (Pch^-, SA^-, $pyocyanin^+$) alone were not sufficient to induce resistance (Fig. 6). These results indicate that 7NSK2 induces resistance by means of a synergistic action of Pch and pyocyanin, although a role of SA cannot be excluded. How a combined action of Pch and pyocyanin can lead to induced resistance remains to be investigated. However, in mammalian cells it is known that pyocyanin can be redox-cycled, generating OH radicals in the presence of Fe-Pch. It is not unlikely that a similar mechanism occurs on plant roots and that reactive oxygen species (ROS) are involved in the induction of resistance (Mehdy 1994). Although we analyzed several ROS scavenging enzymes (i.e., peroxidases and catalases), we were unable to detect changes in enzyme activity in roots or leaves upon root colonization by *P. aeruginosa* 7NSK2 (data not shown).

In-trans complementation of PHZ1 for pyocyanin production restores ability to induce resistance in tomato to *B. cinerea.*

With the aid of the *Pseudomonas* genome-sequencing project, the complete functional *phzM* gene of 7NSK2 was polymerase chain reaction (PCR)-amplified and cloned into pBBR1MCS. The complemented PHZ1-C strain thus obtained induced resistance in tomato to B. cinerea (Fig. 7) to a level

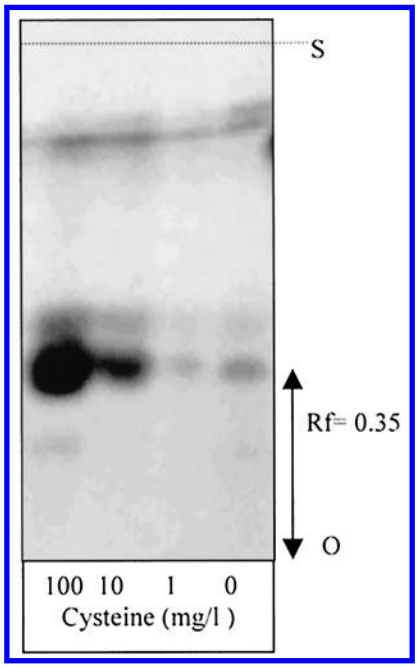


Fig. 2. Effect of L-cysteine added to M9-succinate culture medium on the production of pyochelin by *Pseudomonas aeruginosa* 7NSK2. L-cysteine was added to the medium in concentrations ranging from 1 to 100 mg/liter. Pyochelin was extracted from 25 ml of supernatant with ethyl acetate and thin-layer chromatography (TLC) plates were developed in chloroform:acetic acid:ethanol (90:5:2.5) and analyzed under white light after spraying with 2 M $FeCl_3$. Pyochelin migrates on TLC plates with an R_f-value of 0.35 to 0.4 (Visca et al. 1993). O = origin of migration; S = solvent front.

comparable with other resistance-inducing strains, such as KMPCH. These results demonstrate unambiguously the necessity of pyocyanin production in the 7NSK2-type of induced resistance.

DISCUSSION

In the present work, we demonstrate that *P. aeruginosa* KMPCH induces resistance to *B. cinerea* in tomato. KMPCH root colonization resulted in increased SA levels on tomato roots; therefore, we suggest that this increase in free SA is attributed to the production of bacterial SA, which apparently stimulates PAL activity in tomato roots. Similar increases in free SA levels were observed on bean roots colonized by *P. aeruginosa* KMPCH (11.5 ng/g of root) (De Meyer et al. 1999b) and on tobacco roots colonized by *P. fluorescens* CHA0 (45.3 ng/plant), a resistance-inducing strain, which produces SA (Maurhofer et al. 1998). It cannot be excluded that increases in free SA on tomato roots colonized by KMPCH are due to in planta SA biosynthesis. De Meyer and associates (1999b), however, demonstrated that nanogram amounts of exogenous SA, applied to bean roots in a hydrophonic system, do not affect basal levels of free SA in bean roots 24 or 48 h after application, although these low SA amounts do induce PAL-activity.

Surprisingly, the WT strain 7NSK2, which also produces SA in vitro (De Meyer and Höfte 1997), does not produce detectable amounts of SA on tomato root surface and does not induce PAL activity (Fig. 3). However, a functional SA response in the plant is needed to induce resistance because 7NSK2 no longer induced resistance in transgenic *NahG* tomato plants in which SA is converted to catechol (Brading et al. 2000). We have shown before that 7NSK2 also is unable to induce resistance to TMV in transgenic *NahG* tobacco (De Meyer et al. 1999a).

7NSK2 produces SA in vitro (De Meyer and Höfte 1997), whereas in vivo SA production could not be demonstrated. It cannot be excluded that 7NSK2 still produces low levels of SA on tomato roots which cannot be detected with our extraction method. Alternatively, it is possible that SA is efficiently converted to Pch on tomato roots. Pch is derived from one molecule of SA and two molecules of cysteine (Ankenbauer et al. 1988; Reimmann et al. 1998). In vitro experiments have shown that Pch production by 7NSK2 increases in the presence of L-cysteine (Fig. 2). Cysteine is one of the amino acids exudated by tomato roots (Gamliel and Katan 1992), so it is likely that SA is efficiently converted to Pch in the rhizosphere of tomato in the presence of L-cysteine. A similar phenomenon has been described for the biocontrol strain *P. fluorescens* WCS374, which produces the SA-based siderophore pseudomonine (Mercado-Blanco et al. 2001). This bacterium secretes large amounts of SA under iron-limiting conditions in culture. However, this is likely to be an artifact resulting from the lack of substrate (in this case histamine) required for pseudomonine biosynthesis (van Loon et al. 1998). Although we have shown, by using our SA-producing mutant KMPCH, that bacterial SA can induce resistance in various plants, it is possible that this is not an important bacterial determinant in induced resistance in wild-type bacteria that produce SA as a precursor for other siderophores.

7NSK2 root colonization does not lead to detectable amounts of SA on tomato roots, but SA^- mutants (which are automatically Pch^-) of 7NSK2 loose their capacity to induce resistance; therefore, we investigated the role of Pch in induced resistance. The Pch-producing rhizobacterial strain *P. aeruginosa* PNA1 (Anjaiah et al. 1998), however, did not induce resistance to *B. cinerea* in tomato. We also were unable to induce resistance in tomato with purified Pch (data not shown). In addition, the mutant PHZ1 ($pyocyanin^-$) which still produces SA and Pch lost the ability to induce resistance in tomato to *B. cinerea*. Therefore, we concluded that Pch alone was not sufficient to induce resistance in tomato to *B. cinerea.* PNA1 and PHZ1 differ from 7NSK2 in the production of their phenazine antibiotics. PNA1 produces PCA and PCN and is a strong antagonist of *Pythium* and *Fusarium* spp. (Anjaiah et al. 1998; Tambong and Höfte 2001). PNA1, however, is unable to produce pyocyanin, whereas this blue phenazine compound is produced by 7NSK2. Abeysinghe (1999) has shown that high concentrations of purified pyocyanin (0.1 mM) can induce resistance to *B. cinerea* in bean. Mutant PHZ1 is not able to produce pyocyanin due to an insertion in the p*hzM* gene that encodes an *O*-methyltransferase. In their study, Mavrodi and

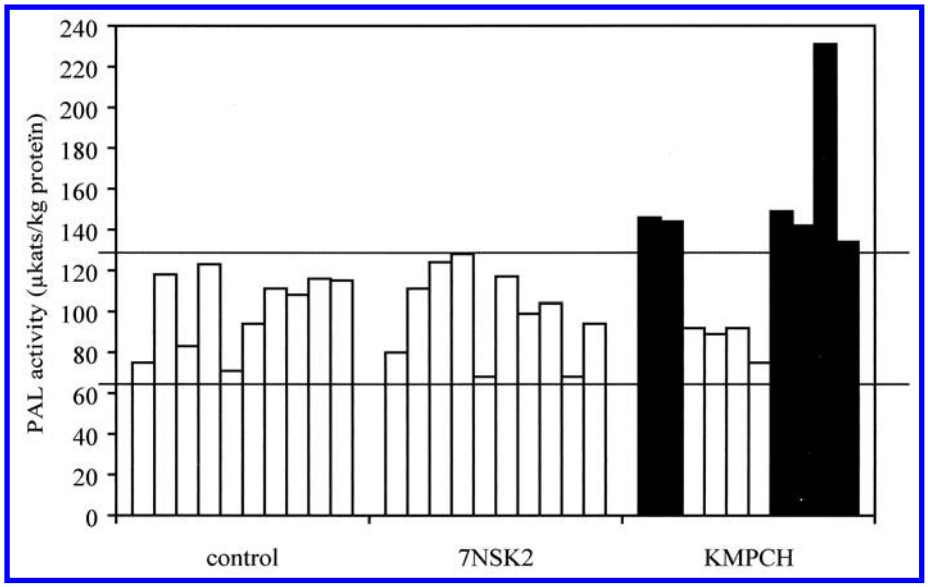


Fig. 3. Phenylalanine ammonia lyase (PAL) activity in roots of 5-week-old soil-grown tomato plants treated with *Pseudomonas aeruginosa* KMPCH and 7NSK2. Bars represent the average of two experiments, each consisting of five replicates. PAL data of KMPCH-colonized roots were not normally distributed after analysis using the Kolmogorov-Smirnov test. PAL activity was not induced in all KMPCH-treated roots. Horizontal lines represent the 95% confidence intercept of the normally distributed PAL activity in control roots. Shaded bars exceed the 95% confidence intercept.

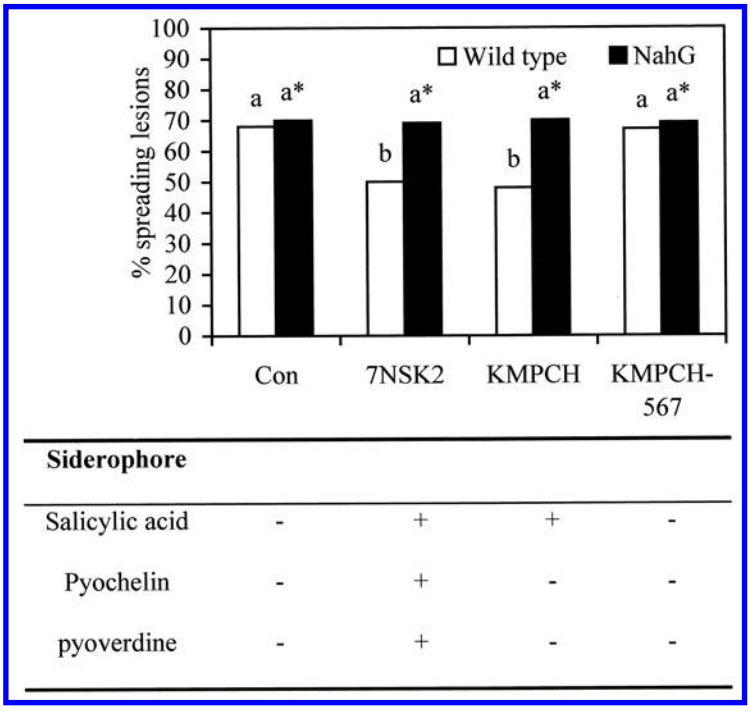
associates (2001) mentioned the fact that a *phzM* mutant of PAO1 produces PCA, 1-hydroxyphenazine (1-OH-PHZ), and PCN. Ethyl acetate extracts of the *phzM* mutant in 7NSK2 revealed the production of PCA and a yellow compound that was not migrating at the same position as PCA or PCN. We are currently investigating whether this compound is 1-OH-PHZ or another phenazine-derivative. It is suggested that, in a *phzM* mutant of PAO1, 1-OH-PHZ is formed from PCA via enzymatic synthesis, involving the product of the *phzS* gene (Mavrodi et al. 2001). *PhzS* encodes a flavine monooxygenase and probably is responsible for the oxidative decarboxylation of the precursor 5-methylphenazine-1-carboxylate betaine. In the absence of the SAM-dependent methylase PhzM, the conversion of PCA to 5-methylphenazine-1-carboxylate betaine does not take place.

Spectrophotometrical analysis clearly showed no production of pyocyanin by PHZ1. In infection experiments, PHZ1 did not induce resistance in tomato to *B. cinerea*. In addition, the Pch^- and SA^- mutant 7NSK2-562 did not induce resistance either, although it overproduced pyocyanin. Overproduction of pyocyanin by this strain might be explained by the fact that SA, Pch, and pyocyanin are all produced via the shikimate pathway with chorismate as a precursor (Mavrodi et al. 2001; Serino et al. 1997). When 7NSK2-562 and PHZ1 were applied together on tomato roots, they induced resistance in tomato to *B. cinerea,* indicating that both Pch or SA and pyocyanin are needed in induced resistance by 7NSK2.

An attractive hypothesis for the possible synergistic action of pyocyanin and Pch in induced resistance by 7NSK2 can come from findings in the infection process of the opportunistic human pathogen *P. aeruginosa*. Pyocyanin is considered to be a pathogenicity factor in the infection process of pulmonary epithelial cells. It can undergo redox-cycling, resulting in the generation of superoxide and H_2O_2 (Hassan and Fridovich 1979, 1980; Muller and Sorrell 1997). These ROS are converted to the very reactive OH-radical through the Haber-Weiss reaction in the presence of Fe-Pch (Britigan et al. 1997). Ultimately, pyocyanin in the presence of Fe-Pch leads to an inactivation of α1-protease inhibitor, resulting in higher human neutrophil elastase activity, contributing to the progress of lung inflammation in cystic fibrosis patients (Britigan et al. 1999). ROS also are important in plants where they play a role in the activation of plant defense mechanisms (Mehdy 1994).

Abeysinghe (1999) has shown that it is possible to generate ROS in bean leaves when pyocyanin was fed to the leaf petiole. However, the amount of pyocyanin produced by 7NSK2 and 7NSK2-562 on tomato roots (which is in the nanogram range) probably is not sufficiently high to generate ROS by itself. It is possible, however, that pyocyanin and Fe-Pch act synergistically on tomato roots producing the very reactive OH-radical, which in turn triggers induced resistance. Upon root colonization with 7NSK2, however, we could not observe changes in peroxidase or catalase. It is possible that changes in ROS are just too small to affect the activity of ROS-associated enzymes, or changes are localized in specific cells or on specific sites on the root surface and not present ubiquitously.

We showed that the *P. aeruginosa* strain PNA1 (which produces SA, Pch, and the phenazine compounds PCA and PCN) and the 7NSK2-derived mutant PHZ1 (which is still able to produce SA, Pch, PCA, and an unidentified yellow phenazine-compound) are not able to induce resistance. Various phenazine compounds, including PCA and 1-OH-PHZ, are known to be redox active (Kerr 2000; Muller 1995). However, it is possible that the ability to reduce molecular oxygen to superoxide is unique for pyocyanin. Muller (1995) showed that 1-OH-PHZ is unable to reduce molecular oxygen to superoxide but, to our knowledge, this has not been investigated for PCA. In addition, it has been shown that pyocyanin, but not 1-



Siderophore				
Salicylic acid	-	+	+	-
Pyochelin	-	+	-	-
pyoverdine	-	+	-	-

Fig. 4. Induced resistance by *Pseudomonas aeruginosa* 7NSK2 and its siderophore mutants in wild-type and *NahG* tomato plants, containing the bacterial salicylic acid (SA) hydroxylase converting SA to catechol. Plants were grown under greenhouse conditions at 24 ± 3°C. All strains were grown on iron-limited King's B medium and control plants were treated with water. Tertiary leaves were infected with 10 droplets of 4 µl of spore suspension containing 10^6 spores/ml, 0.01M glucose, and 6.7 mM KH_2PO_4. Infections were evaluated 4 days after inoculation by counting the number of spreading *Botrytis cinerea* lesions on each leaf. Data represented are means for three experiments each with 12 leaves per treatment. Infection was evaluated 4 days after infection by counting the number of spreading lesions. Statistical analysis by a logistic regression was performed on pooled data, because interaction between experiment and treatment was not significant at $P = 0.05$.

OH-PHZ, increased the rate and duration of oxygen uptake by neutrophils (Ras et al. 1990). In the same study, the authors also mentioned other differences in the proinflammatory interactions of 1-OH-PHZ and pyocyanin with human phagocytes. It is not unlikely that the generation of ROS by the Fe-Pch–pyocyanin interaction is the basis for induced resistance by 7NSK2.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and plasmids.

The bacterial strains used in this study are listed in Table 1. For mutation and cloning experiments, *P. aeruginosa* and *Escherichia coli* strains were grown overnight at 37°C in liquid or solid Luria-Bertani medium (LB) (Miller 1972). *P. aeruginosa* strains were differentiated from *E. coli* on solid CAA medium (Difco Laboratories, Detroit), on which *P. aeruginosa* strains produce fluorescent yellow colonies due to the production of pyoverdine. Pyocyanin production of the different *P. aeruginosa* strains was observed as a diffusing deep-blue color in *Pseudomonas* P broth or solid *Pseudomonas* P agar (Difco Laboratories) at 28°C. For inoculation experiments on tomato plants, *P. aeruginosa* was grown overnight on iron-limiting King's B (KB) medium (King et al. 1954) at 37°C.

X-gal was used at a concentration of 40 µg/ml and IPTG (isopropyl-β-D-thiogalactoside) at a concentration of 100 mM. Antibiotics were added at the following concentrations: ampicillin (100 µg/ml), chloramphenicol (25 µg/ml for *E. coli* and 200 µg/ml for *P. aeruginosa*), tetracycline (15 µg/ml), gentamicin (20 µg/ml for *E. coli* and 100 µg/ml for *P. aeruginosa*), and spectinomycin (50 µg/ml), unless otherwise stated.

Mobilization of plasmids from *E. coli* to *P. aeruginosa* was done by spreading 0.1 ml of saturated cultures in LB medium of donor and recipient on solid LB and incubating overnight at 37°C.

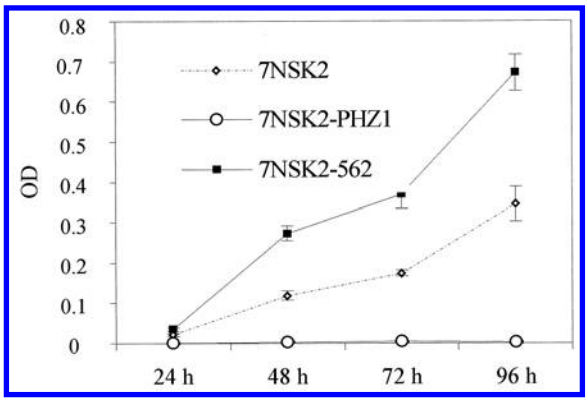
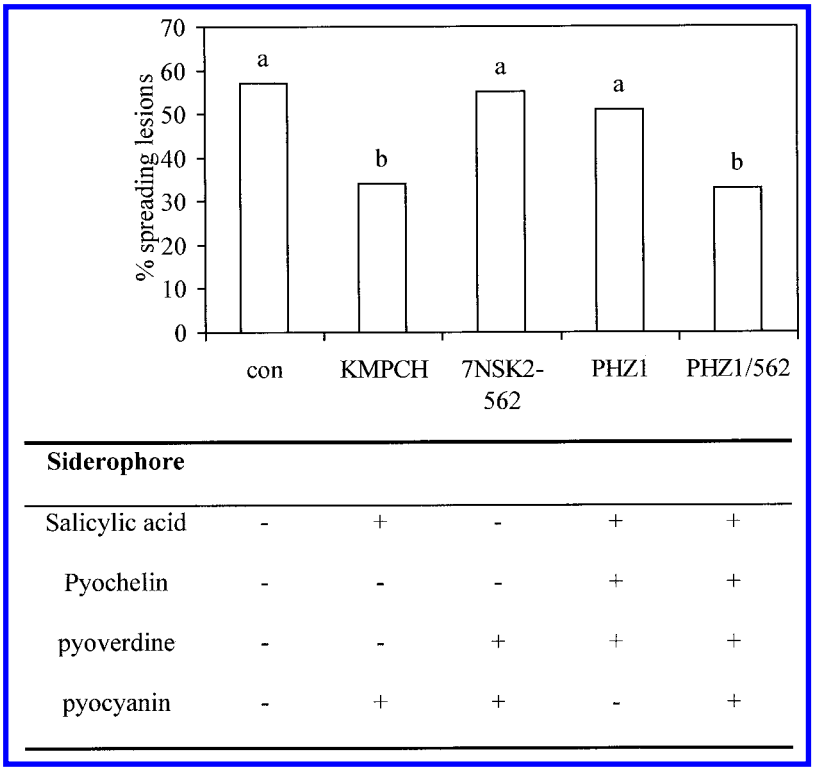


Fig. 5. In vitro production of pyocyanin by *Pseudomonas aeruginosa* 7NSK2, 7NSK2-562, and the pyocyanin-negative mutant PHZ1 determined spectrophotometrically at 510 nm in the presence of HCl. Production was monitored at 24, 48, 72, and 96 h after inoculation. Results are the mean of two experiments, each consisting of three replicates.

Construction of a pyocyanin-negative mutant in *P. aeruginosa* 7NSK2.

MiniTn*phoA3* was constructed from mini*TnphoA2* (Pattery et al. 1999) by replacing the kanamycin cassette at the *Not*I site with a gentamicin resistance cassette. The gentamicin cassette used for construction of miniTn*phoA3* was obtained as a *Not*I fragment (803 bp) from pGM (C. Baysse, personal communication).

Mutagenesis of the wild type 7NSK2 *P. aeruginosa* strain was carried out by mobilization of the suicide vector pUTmini*TnphoA2* from *E. coli* S17-1(λ*pir*). Transconjugants



Siderophore					
Salicylic acid	-	+	-	+	+
Pyochelin	-	-	-	+	+
pyoverdine	-	-	+	+	+
pyocyanin	-	+	+	-	+

Fig. 6. Influence of tomato root treatment with *Pseudomonas aeruginosa* 7NSK2, PHZ1, 7NSK2-562, and a combined application of 7NSK2-562 and PHZ1 on the percentage of spreading *Botrytis cinerea* lesions on tomato leaves. All strains were grown on iron-limited King's B medium and control plants were treated with water. Tertiary leaves were infected with 10 droplets of 4 µl of spore suspension containing 10^6 spores/ml, 0.01M glucose, and 6.7 mM KH_2PO_4. Infections were evaluated 4 days after inoculation by counting the number of spreading *B. cinerea* lesions on each leaf. Data represented are means for three experiments each with 12 leaves per treatment. Statistical analysis by a logistic regression was performed on pooled data, because interaction between experiment and treatment was not significant at $P = 0.05$.

initially were selected on solid CAA medium with gentamicin and tetracycline in order to counterselect the *E. coli* strain (Cornelis et al. 1992). All mutants were then individually toothpicked into microtiter wells that contained solid P-agar with gentamicin. This facilitated the identification of white-colored mutants that failed to produce any pyocyanin from the pyocyanin producers (blue-colored) because there was no diffusion of the pigment. A total of three white mutants were obtained, out of which one was found to be completely white (PHZ1), while the other two (PHZ2 and PHZ3) produced traces of pyocyanin (light-blue) after 2 days.

Molecular characterization of PHZ1 and in trans complementation of PHZ1 for pyocyanin production.

The DNA sequence adjacent to the miniTn*phoA3* insertion in PHZ1 was isolated by inverse PCR (IPCR). PCRs and IPCRs were performed either with ExTaq enzyme (Takara) or Proofstart DNA polymerase (Qiagen, Leusden, The Netherlands). Isolation of total DNA from *P. aeruginosa* strains was carried out as described by Wilson (1990). Elution of DNA fragments from 0.8% agarose gels was carried out using the Qiaquick gel extraction kit (Qiagen).

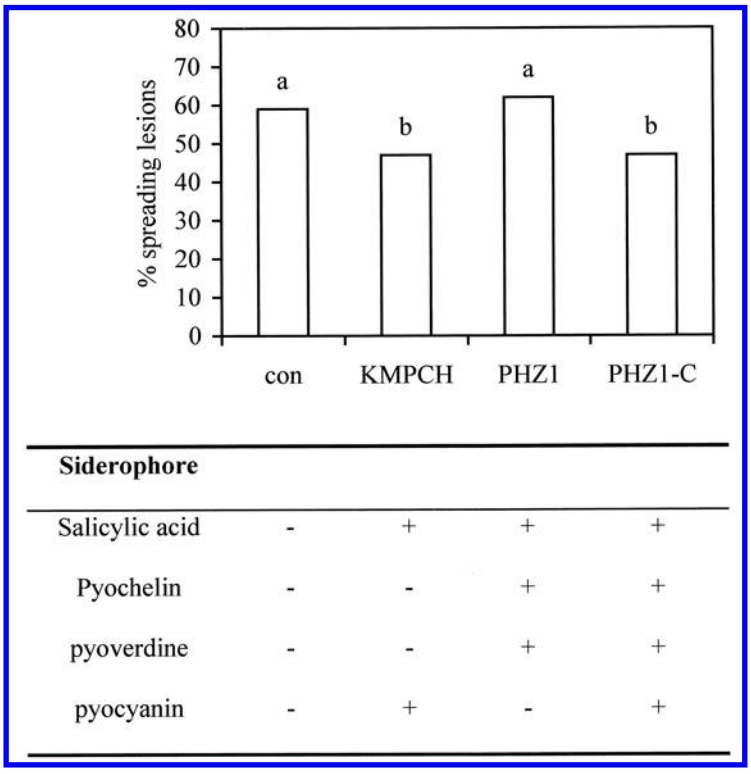
Total DNA of 7NSK2 and PHZ1 was digested with *Sma*I and ligated under conditions favoring intramolecular circularization. Amplification of the ligation mixture was carried out using the primer pair PhoA5 (5′-GCGGCAGTCTGATCACCCGTTA-3′) located at position 443-422 (Chang et al. 1986) and Gm1 (5′-TGGACCAGTTGCGTGAGCGCATA-3′). Reamplification of the IPCR fragment using nested primers PhoA4 (5′-GCACCGCCGGGTGCAGTAATAT-3′) located at position 413-392 (Chang et al. 1986) and Gm2 (5′-TGTCAACTGGGTTCGTGCCTTC-3′) showed a 1-kb amplified fragment for strain PHZ1, while the control 7NSK2 showed no amplification. Subsequently, the PCR-amplified product was cloned into pCR2.1 using the TA cloning kit (Invitrogen, Merelbeke, Belgium). The resulting plasmid pMI1 was completely sequenced. The *Pseudomonas* DNA and protein database was searched for similarities and also for obtaining the complete sequence of *phz*M (PA4209) and *phz*S (PA4217).

The complete functional *phz*M gene of 7NSK2 was PCR-amplified along with the surrounding flanking sequences using the primers 4209A (5′-CGGCAACGCGCTCAACCAACT-3′) and 4209B (5′-TCGAGGGGTGTTTCCCTGTAC-3′) which correspond to positions 4711777-4711797 and 4713829-4713809 on the *Pseudomonas* genome sequencing project. A fragment of 2,054 bp was obtained which was subsequently cloned blunt into the broad-host range vector pBBR1MCS at the *EcoRV* site to yield pPHZM.

This plasmid was mobilized from *E. coli* S17-1(λ*pir*) into PHZ1 by conjugation and the transconjugants were selected on solid CAA medium containing chloramphenicol and tetracycline. In order to observe pyocyanin production, 50 transconjugants were toothpicked onto gridded solid P-agar plates containing chloramphenicol. All the clones (PHZ1-C) turned deep blue, indicating that the mutation in strain PHZ1 was indeed complemented in trans. These findings are in accordance with Mavrodi and associates (2001).

Plant material and assay for induced resistance.

Experiments were performed with tomato plants (*Lycopersicon esculentum* Mill. cv. Moneymaker) and corresponding transgenic *NahG* plants in which SA is converted to catechol (Brading et al. 2000). Plants were grown under nonsterile greenhouse conditions in potting soil (Klasmann, substrat no 4, Otrebusy, Germany). The bacterial inoculum for root colonization was prepared from KB plates. The bacteria were washed from the plates with physiological solution and added to the soil in a concentration of 10^7 bacteria per gram of soil.



Siderophore				
Salicylic acid	-	+	+	+
Pyochelin	-	-	+	+
pyoverdine	-	-	+	+
pyocyanin	-	+	-	+

Fig. 7. Influence of complemented pyocyanin production in *Pseudomonas aeruginosa* PHZ1 on induced resistance in tomato to *Botrytis cinerea*. All strains were grown on iron-limited King's B medium and control plants were treated with water. Tertiary leaves were infected with 10 droplets of 4 µl of spore suspension containing 10^6 spores/ml, 0.01M glucose, and 6.7 mM KH_2PO_4. Infections were evaluated 4 days after inoculation by counting the number of spreading *B. cinerea* lesions on each leaf. Data represented are means for three experiments each with 12 leaves per treatment. Statistical analysis by a logistic regression was performed on pooled data, because interaction between experiment and treatment was not significant at $P = 0.05$.

Table 1. Plasmids and bacteria used in this study with their relevant characteristics

Strains or plasmids	Relevant characteristics[a]	Reference or source
Pseudomonas aeruginosa		
7NSK2	Pvd$^+$, Pch$^+$, SA$^+$, wild type	Iswandi et al. 1987
MPFM1	*Tn5* mutant of 7NSK2, Pvd$^-$, Pch$^+$, SA$^+$	Höfte et al. 1991
KMPCH	Pvd$^-$, Pch$^-$, SA$^+$, Chemical mutant of MPFM1; Km^r	Höfte et al. 1993
7NSK2-562	Pvd$^+$, Pch$^-$, SA$^-$, *pchA* replacement mutant of 7NSK2	De Meyer et al. 1999b
MPFM1-569	Pvd$^-$, Pch$^-$, SA$^-$, *pchA* replacement mutant of MPFM1	De Meyer and Höfte 1997
KMPCH-567	Pvd$^-$, Pch$^-$, SA$^-$, *pchA* replacement mutant of KMPCH; Km^r	De Meyer et al. 1999b
PHZ1	Pvd$^+$, Pch$^+$, SA$^+$, pyocyanin$^-$, *phzM* : :miniTn*phoA*3 derivative of 7NSK2 that is pyocyanin negative, Gm^r	This work
PHZ1-C	Pvd$^+$, Pch$^+$, SA$^+$, PHZ1 containing pHZM (functional *phz*M gene on plasmid pBBR1MCS) that restores pyocyanin production, Gm^r, Cm^r	This work
PNA1	Pvd$^+$, Pch$^+$, SA$^+$, produces phenazine-1-carboxylate (PCA), wild type	Anjaiah et al. 1998
Escherichia coli		
DH5α	*end*A1 *hsd*R17 *sup*E44 *thi*-1 *rec*A1 *gyr*A1 Δ(*lac*IZA-*arg*F)U169 *deo*R	Woodcock et al. 1989
GJ23	JC2692 (pGJ28) (R64*drd*11), Km^r/Sm^r/Tc^r	Van Haute et al. 1983
S17-1 (λ*pir*)	*thi pro hsd*R *rec*A::RP4-2-Tc^r::Mu Km^r::Tn7 (λ*pir*), Sm^r	Simon et al. 1983
Plasmids		
pUTminiTn*pho*A3	MiniTn*pho*A3 in pUT, Gm^r/Cb^r, Derivative of miniTn*PhoA*	Pattery et al. 1999
pUC19	Cloning and sequencing vector, Cb^r	Yanisch-Perron et al. 1985
pCR2.1	TA cloning vector, Cb^r/Km^r	Invitrogen
pBBR1MCS	Broad host-range cloning vector for *Pseudomonas*, Cm^r	Kovach et al. 1994
pBR325	Suicide vector in *Pseudomonas*, Cm^r/Cb^r/Tc^r	Bolivar 1978
pMI1	1-kb *Sma*I IPCR fragment of PHZ1 in pCR2.1, Cb^r/Km^r	This work
pHZM	A 2,054-bp PCR-amplified fragment of primer pair 4209A-B (*phz*M) of 7NSK2 cloned in the *Eco*RV site of pBBR1MCS, Cm^r	This work

[a] Pvd = Pyoverdine, Pch = Pyochelin, SA = Salicylic acid, Km = Kanamycin, Gm = Gentamicin; Tc = Tetracycline; Cm = Chloramphenicol, Cb = Carbenicilin, Sm = Spectinomycin, IPCR = inverse polymerase chain reaction.

In addition, seeds were dipped prior to sowing in a bacterial suspension of 10^7 bacteria per ml of water. After 10 days, seedlings were transplanted and grown for 4 weeks under greenhouse conditions (24°C ± 3). Four weeks after seedling transfer, plants were infected with *B. cinerea* R16 (Faretra and Pollastro 1991) in a detached leaf assay as described by Audenaert and associates (2002). Each tertiary leaf composed of five leaflets was inoculated with 10 4-µl drops of a suspension of 10^6 *B. cinerea* spores per ml, obtained as previously described (Audenaert et al. 2002), in 0.01 M glucose and 6.7 mM KH_2PO_4 (pH 5). Each experiment was carried out three times and contained 12 infected leaves per treatment. Four days after inoculation, *B. cinerea* infections were scored as a spreading lesion if the fungus had developed beyond the inoculum drop. Results were categorized in a dichotomous variable (spreading or nonspreading) and analyzed by logistic regression analysis (Agresti 1990). Data for experiments with the same set-up were pooled for statistical analysis if a two-way analysis of variance (ANOVA) revealed that interaction between treatment and experiment was not significant at $P = 0.05$.

Enzymatic assay.

PAL activity was determined in roots of 5 week-old tomato plants as described by Edwards and Kessmann (1992) and Audenaert and associates (2002). Roots were ground in liquid N_2 and extracted with 50 mM Tris-HCl (pH 8.5). Five plants per treatment were analyzed and experiments were repeated two times. Results were statistically analyzed by ANOVA and means were compared with a post hoc Duncan test at $P = 0.05$.

Determination of SA on roots of tomato plants.

Free SA and bound SA was measured as described by Meuwly and Métraux (1993) with *O*-anisic acid as an internal standard. Samples were taken from control roots and roots colonized by bacteria, 4 weeks after seedling transfer. Each sample consisted of 2.5 g of root material pooled from five individual plants. Five samples were taken per treatment. Results were statistically analyzed by ANOVA and means were compared with a post hoc Duncan test at $P = 0.05$.

In vitro production of SA, pyocyanin, and Pch by bacterial strains.

To monitor in vitro production of pyocyanin by the distinct strains, bacteria were grown for 96 h in 100-ml Erlenmeyers containing 25 ml of *Pseudomonas P* broth at 28°C. Pyocyanin present in the supernatant was extracted twice with chloroform and determined spectrophotometrically in the presence of 0.1 M HCl (optical density at 510 nm) as described by Essar and associates (1990). This experiment was set up in three replicates.

To determine SA and Pch production in the presence of L-cysteine, bacteria were grown in 100-ml Erlenmeyer flasks containing 25 ml of M9 succinate medium (Anjaiah et al. 1998) with L-cysteine at 0, 1, 10, and 100 mg per liter (Sigma, Bornem, Belgium) and were put for 2 days at 28°C and 150 rpm. Bacterial cultures were centrifuged and the supernatant was adjusted to pH 1.5. Supernatant was extracted twice with ethylacetate and organic layers were dried under vacuum. Pellets were dissolved in 20 µl of methanol and were spotted on a thin-layer chromatography (TLC) plate that was developed in chloroform:acetic acid:ethanol (90:5:2.5) as solvent (Visca et al. 1993). TLC plates were analyzed under UV light and under white light after spraying with 2M $FeCl_3$ dissolved in 0.1 M HCl.

ACKNOWLEDGMENTS

This project was supported by a specialization fellowship of the Flemish Institute for the Stimulation of Scientific-Technological Research in Industry (IWT, Belgium) given to K. Audenaert and by a grant from the Fund for Scientific Research-Flanders (FWO, Belgium). We thank J. Jones for providing *NahG* tomato seeds, A. Buchala for carrying out the SA-determinations, and I. Delaere and A. Van Damme for their technical assistance and help during the plant experiments.

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AUTHOR-RECOMMENDED INTERNET RESOURCE

The *Pseudomonas* DNA and protein database: www.pseudomonas.com