Iron Availability Affects Induction of Systemic Resistance to Fusarium Wilt of Radish by *Pseudomonas fluorescens*

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ABSTRACT

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A special bioassay on rock wool was used to study the influence of iron availability on the induction of systemic resistance in radish (*Raphanus sativus* L.) against Fusarium wilt mediated by *Pseudomonas fluorescens*. In this bioassay, the pathogen (*Fusarium oxysporum* f. sp. *raphani*) and a strain of *Pseudomonas*, salicylic acid (SA), or a pseudobactin were applied at separate locations on the plant root. Strain WCS374 of *P. fluorescens* and its pseudobactin-minus Tn5 mutant gave greater disease control in the induced systemic resistance bioassay when iron availability in the radish nutrient solution was low than when it was high. Mutants of *P. fluorescens* strains WCS374 and WCS417 lacking the O-antigenic side chain of the lipopolysaccharide induced resistance at low but not at high iron availability. The purified pseudobactin of strain WCS374, but not the pseudobactins of strains WCS358 and WCS417, induced resistance. Gas chromatography and spectrophotometry were used to detect and measure production of SA by these strains. Strains WCS374 and WCS417 produced 47 and 8 µg of SA per milliliter, respectively, at low iron availability in vitro; the production of SA decreased rapidly with increasing iron availability. *P. putida* WCS358 did not induce resistance, either at low or at high iron availability, and did not produce SA in vitro. Commercial SA induced resistance at concentrations as low as 100 fg per root. High concentrations (> 1 mg/ml) of SA reduced growth of hyphae from germinated conidia of *F. oxysporum* f. sp. *raphani*. We hypothesize that the Fe^{3+}-chelating SA, produced by selected *P. fluorescens* strains at low iron availability, is involved in the induction of systemic resistance to Fusarium wilt of radish. The pseudobactin produced by WCS374 may be involved as well. Given these results, it seems appropriate to reevaluate the role of siderophore-mediated competition for iron in the suppression of disease by fluorescent *Pseudomonas* spp.

Different mechanisms are involved in the suppression of soilborne plant pathogens and deleterious microorganisms mediated by plant growth-promoting (PGP) rhizobacteria. Antibiosis, siderophore-mediated competition for iron, competition for carbon, and induction of disease resistance are well-accepted mechanisms of disease suppression (15,49,59). The fluorescent siderophores of *Pseudomonas* spp. (called pseudobactins or pyoverdines) can effectively compete for iron with microorganisms that produce siderophores in lower concentrations or with a lower affinity for iron and that are unable to use the siderophore produced by the suppressing strain (3,8). PGP *Pseudomonas* spp. can suppress pathogenic strains of *Fusarium oxysporum* by siderophore-mediated competition for ferric iron (5,14,33,47,53).

Pseudobactins are produced under iron-limiting conditions in the environment (37). They are composed of a polypeptide chain containing hydroxamate groups linked to a fluorescent quinoline chromophore (23,50). Bakker et al. (5,6) demonstrated that the pseudobactin for the PGP strain WCS358 of *Pseudomonas putida* is highly specific for the uptake of iron. A highly specific outer membrane protein (PupA), which functions as a receptor for the ferrated pseudobactin (7), is also required for the uptake of iron by strain WCS358. Studies of pseudobactin-mediated competition for ferric iron as a mechanism of disease suppression by PGP strains of rhizobacteria have used mutant strains defective in the production of fluorescent siderophores or have changed the availability of iron in the rhizosphere, either by applying iron, artificial iron chelators, or purified pseudobactin or by changing the pH in the environment (3,4,26,47,53).

Some *Pseudomonas* spp. can also produce a nonfluorescent siderophore called pyochelin, consisting of a salicylic-substituted cysteinyl peptide (11). Salicylic acid (SA) is considered to be not only an intermediate in pyochelin synthesis (2), but also an endogenous siderophore for some *Pseudomonas* spp. (43,56). SA is produced by *Pseudomonas* spp. under iron limitation (43,56).

Induced systemic resistance (ISR) (27) against plant pathogens by PGP rhizobacteria is a relatively new topic in studies on disease suppression mediated by fluorescent *Pseudomonas* spp. (49, 51,57). The concept of ISR involves the activation of the plant's defense mechanisms, which lead to systemic protection. Whether fluorescent *Pseudomonas* spp.-mediated ISR has the same characteristics as the classical systemic acquired resistance (SAR) is not yet clear. *Pseudomonas* spp.-mediated ISR was observed in carnation against *F. oxysporum* f. sp. *dianthi* (14,51); in cucumber against *Colletotrichum orbiculare* (57), cucumber mosaic virus (35), and *Pythium aphanidermatum* (62); in bean against *P. syringae* pv. *phaseolicola* (1); in tobacco against tobacco necrosis virus (40); and in radish against *F. oxysporum* f. sp. *raphani* (30,31). In all of these studies, the pathogen and the resistance-inducing pseudomonad were applied at separate locations on the plant, excluding direct antibiosis and competition as mechanisms of disease suppression.

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Lipopolysaccharide (LPS) O-antigenic side chains of *P. fluorescens* strains WCS374 and WCS417 have been implicated as traits involved in ISR against Fusarium wilt in carnation and radish (31,52). Recently, evidence has accumulated that a pyoverdine produced by *P. fluorescens* CHA0 is involved in induced resistance in tobacco against tobacco necrosis virus (40).

The objective of this investigation was to determine whether the induction of systemic resistance against Fusarium wilt of radish by selected *Pseudomonas* spp. is dependent on iron availability. In an ISR rock wool bioassay (30), bacteria were applied on radish roots, which obtained low or high iron availability from a nutrient solution. The in vitro production of siderophores (SA and/or pseudobactin) by the *Pseudomonas* spp. was studied at low and high iron availability, and the involvement of LPS, SA, and pseudobactin in ISR against Fusarium wilt of radish by *Pseudomonas* spp. was evaluated.

MATERIALS AND METHODS

Radish cultivar. Radish (*Raphanus sativus* L.) cultivar SaxaxNova (seed size 2.50 to 2.75 mm) (S&G Seeds B.V., Enkhuizen, The Netherlands), moderately resistant to Fusarium wilt, was used in all experiments.

Microbial cultures and inocula. The wilt pathogen of radish, *F. oxysporum* Schlechtend.:Fr. f. sp. *raphani* J. B. Kendrick & W. C. Snyder (formerly called *F. oxysporum* Schlecht. f. sp. *conglutinans* [(Wollenw.) Snyder & Hansen] race 2 Armstrong & Armstrong) (strain WCS600), was isolated from an infected radish on Komada's agar (28), modified as described by Gams and Van Laar (17). The fungal culture was maintained on modified Komada's agar for short-term storage. Long-term storage was on Microbank beads (Pro-Lab Diagnostics, Wirral, U.K.) at −80°C.

The pathogen was cultured in aerated 2% malt extract. After 7 days of incubation at 22°C, washed microconidia were mixed with gamma-sterilized peat of neutral pH (Agrifutur s.r.l., Alfianello, Italy). The inoculum was incubated for 4 days at 22°C before being used in the bioassays. The number of colony-forming units (CFU) in the peat after incubation was determined by dilution plating on modified Komada's agar. The pathogen inoculum density in the peat (10^6 CFU per g) was adjusted with sterile sand to 4×10^4 CFU per g of peat-sand before inoculation.

P. putida WCS358 and *P. fluorescens* WCS374 were originally isolated from the rhizosphere of potato (18,19), and *P. fluorescens* WCS417 was isolated from the rhizosphere of wheat grown in a field suppressive to take-all disease caused by *Gaeumannomyces graminis* var. *tritici* (29). PGP traits of WCS358, WCS374, and WCS417 have been described in a number of papers (3,4,14,19, 29,30,32,51–53).

LPS mutants LWP74-30b (374OA$^-$) (12) and WCS417B4 (417OA$^-$, isolated by W. Bitter, Department of Molecular Cell Biology, Utrecht University) of strains WCS374 and WCS417, respectively, were used to study the role of LPSs in ISR. Both are phage-resistant mutants lacking the O-antigenic side chain of the LPS but possessing an intact inner core. Pseudobactin-minus mutants JM218 (358PSB$^-$) (39), JM374 (374PSB$^-$) (58), and S680 (417PSB$^-$) (14) of strains WCS358, WCS374, and WCS417, respectively, were used to study the role of pseudobactins in ISR. All pseudobactin-minus mutants are Tn5 derivative strains unable to synthesize their fluorescent siderophore.

To prepare bacterial suspensions for the bioassays, overnight cultures (27°C) were scraped from plates of King's medium B agar (25) in 0.01 M $MgSO_4$. These suspensions were washed twice in $MgSO_4$, diluted (to approximately 10^8 CFU per ml), and mixed with talcum (1:1, w/w). The bacterial inoculum densities in the talcum, as determined by dilution plating on King's B agar, were approximately 6×10^7 CFU per g of talcum.

Purification of pseudobactins. Pseudobactins of strains WCS358, WCS374, and WCS417 were isolated from stationary phase cultures in standard succinate medium (SSM) (pH 7.0, 48 h, 27°C) (42). Cell-free culture supernatants were lyophilized, and the unferrated pseudobactins were isolated by the method of Van der Hofstad et al. (50) and further purified as described by Raaijmakers et al. (45). The purity of the pseudobactins was greater than 95%, based on the molar extinction coefficient of pseudobactin 358 of WCS358, which is 13,670/M/cm at 400 nm and pH 7.1 (P. J. Weisbeek, *personal communication*).

ISR rock wool bioassay. Radish seeds were sown in sand; after 5 days, seedlings were transferred to MM40/40S rock wool growth cubes (Grodan B.V., Roermond, The Netherlands). This system is described by Leeman et al. (30). In this experimental design, the bacteria, SA (Baker, 100 fg to 100 μg per root, pH 6.5), or purified pseudobactins (70 μg per root) were applied in talcum (1 g per root) on the root tips. Two days later, the pathogen (1 g of peat-sand mixture per root) was applied on the root base. Each treatment was replicated 12 times, and each replicate consisted of three plants.

Plants were maintained in the greenhouse as described before (30). Half-strength Hoagland's nutrient solution (21) at pH 7 was applied to the root base compartment (zone of pathogen inoculation). The iron nutrition of the nutrient solution was modified by using 10 μM Fe-EDDHA (Sequestrene, Ciba-Geigy) (5% ferric iron, 80% of which is bound as Fe-ethylenediamine di(o-hydroxyphenylacetic acid) as the iron source. To study the effect of iron availability on *Pseudomonas* spp.-mediated ISR, 10 μM EDDHA (Sigma) (low iron availability) or 10 μM Fe^{3+}-saturated EDDHA (12 μM $FeCl_3$ + 10 μM EDDHA) (high iron availability) were substituted for the iron source (Sequestrene) of the radish nutrient solution, and this nutrient solution was applied to the root tip compartment (zone of bacterization).

Three weeks after the roots were inoculated with the pathogen, the plants were harvested and the percentage of plants with Fusarium wilt symptoms was recorded. Symptoms consist of browning and/or blackening of the xylem tissue in root and radish and yellowing and/or browning of the leaves, which turn brown and brittle.

In vitro growth and production of pseudobactins and SA by *Pseudomonas* strains. In vitro growth and production of pseudobactins and SA by the *Pseudomonas* strains were measured in a stationary phase shake culture (48 h, 27°C) in SSM, pH 7.0 (42), which was inoculated with washed cells from a 48-h-old SSM shake culture. To study the effect of iron on these parameters, we added filter-sterilized $FeCl_3$ to the SSM in a linear concentration range (0 to 30 μM).

Bacterial growth was measured turbidimetrically at 650 nm with a Beckman Du-64 spectrophotometer. A standard curve was used for quantification. The cell-free culture supernatants were used to measure pseudobactins and SA. To quantify the pseudobactins, absorbance at 400 nm (pH 7.1) was measured and the molar extinction coefficient of *P. putida* WCS358 (13,670/M/cm) was used.

For qualitative detection of SA, the culture supernatants (4 ml) were acidified with HCl to pH 2, and the SA was extracted into $CHCl_3$ (2 × 2 ml). An absorbance spectrum was made of the pooled $CHCl_3$ phases for the wavelength interval from 220 to 450 nm. For quantitative measurements with the spectrophotometer, 5 μl of 2 M $FeCl_3$ and 4 ml of water were added to the pooled $CHCl_3$ phases, according to the method of Meyer et al. (43). The absorbance of the purple iron-SA complex, which developed in the aqueous phase, was measured at 527 nm. For quantification, a standard curve (0 to 0.3 mg of SA per milliliter, Baker) was dissolved in SSM and treated as above.

Further qualitative analysis of the production of SA in culture supernatants was done with a Hewlett-Packard 5890 gas chromatograph (GC) with a CP-Sil 5CB (25 m) column. Following the method of Groeneveld et al. (20), 30 ml of culture supernatant was acidified (pH 2) with H_2SO_4 and extracted two times with

100 ml of ethyl acetate. The organic layers were pooled and dried under vacuum, and the residue was derivatized (propyl esters) for 9 min at 100°C with 1.5 ml of BF_3-propanol (Aldrich) and cooled. Two milliliters of $CHCl_3$ and 3 ml of 50% saturated $(NH_4)_2SO_2$ were then added, and the mixture was shaken vigorously. The top layer was then discarded, 4 ml of demineralized water and about 1 g of Na_2SO_4 were added to the $CHCl_3$ layer, and the mixture was again shaken. The dried $CHCl_3$ fraction was then passed over a silica gel column (1 g of silica gel G), which was eluted with 7 ml of fresh $CHCl_3$. The eluent containing the propyl esters was concentrated to approximately 100 µl.

Using the same protocol, we made propyl esters of an organic acid mixture (oxalic, malonic, maleic, succinic, fumaric, malic, tartaric, adipic, pimelic, suberic, cis-aconitic, and citric acids) and an aromatic acid mixture (benzoic, 2-OH-benzoic [=salicylic], cinnamic, p-OH-benzoic, 3-OH-phenylacetic, 4-OH-phenylacetic, 4-OH-3-methoxy-benzoic [=vanillic], 2,5-diOH-benzoic [=gentisic], 2-OH-cinnamic [=o-coumaric], 4-OH-3,5-dimethoxy-benzoic [=syringic], p-OH-cinnamic [=p-coumaric], 4-OH-3-methoxy-cinnamic [=ferulic], 3-OH-4-methoxy-cinnamic, 3,4-diOH-cinnamic [=caffeic], and 3,5-dimethoxy-4-OH-cinnamic [=sinapic] acids). Relative GC retention times (temperature program: 70 to 210°C, 4°C/min) of the SA in the culture supernatants were compared with those of the mixtures of organic acids and aromatic acids.

All in vitro measurements were done in triplicate, and experiments were repeated three times.

Effects of SA on hyphal growth of *F. oxysporum* f. sp. *raphani*. Growth of hyphae of *F. oxysporum* f. sp. *raphani* from germinated microconidia in SA was measured by the method of Woloshuk et al. (60), in which 50 µl of a suspension containing 1×10^4 conidia per milliliter is placed on potato-dextrose agar (250 µl) in 24-well macrowell plates (Greiner). After germination (6 h, light, 21°C), the conidia were incubated (2 days, dark, 4°C, followed by 1 day, light, 21°C) in solutions of SA in sterile demineralized water (100 µl, pH 7.0, NaOH). To make growth visible for quantification with interactive image analyzing equip-ment (IBAS, Zeiss), the hyphae were colored (1 h) with lacto-phenol cotton blue (20% phenol and 0.05% cotton blue in lactic acid/glycerol/ 96% ethanol [1:2:2, v/v/v]), then destained with 70% ethanol until the agar was colorless but the hyphae were still blue.

Microbial root colonization. Colonization of the root base (zone of pathogen inoculation) and of the root tips (zone of bacterization) of radish in the rock wool bioassay was checked at harvest (30). The pathogen population was determined by dilution plating on modified Komada's agar, and the fluorescent pseudomonads and introduced *Pseudomonas* strains were monitored by immunofluorescence colony-staining (54), as modified by Leeman et al. (30).

Data analysis. Data were analyzed for significance by analysis of variance, followed by Fisher's least significant difference test ($\alpha = 0.05$), with SAS software (SAS Institute, Cary, NC). Normal distributions and homogeneity of variances were checked beforehand. In all cases normal distributions and homogeneity of variances were obtained after arcsine square root transformations. Repeated experiments demonstrated the same significant differences among treatments.

RESULTS

ISR rock wool bioassay. The introduced *Pseudomonas* strains and the pathogen stayed at separate locations on the root. No contamination of the root base or root tips in the opposite rock wool cubes with the *Pseudomonas* strain or the wilt pathogen, respectively, was ever detected in the root macerates (detection limits are 1.3×10^2 and 5.6×10^2 CFU per g for applied *Pseudomonas* strains and *F. oxysporum,* respectively).

Growth of the radish plants was similar in nutrient solutions with low and high iron availability. At the low iron availability (10 µM EDDHA), plants did not show any chlorotic leaves, so it can be assumed that the roots took up sufficient iron in the rock wool compartment containing the pathogen, which received a nutrient solution containing Sequestrene iron.

Role of iron availability in ISR. At high iron availability in the growth medium of radish (10 µM Fe^{3+}-saturated EDDHA), strains WCS374 and WCS417 and their pseudobactin-minus mutants delivered in talcum significantly reduced Fusarium wilt (Fig. 1). Neither strain WCS358 and its pseudobactin-minus mutant nor the O-antigen-minus mutants 374OA⁻ and 417OA⁻ suppressed the disease significantly.

Strain WCS374 and its pseudobactin-minus mutant 374PSB⁻ reduced the disease significantly more at low iron availability (10 µM EDDHA) than under the high iron condition (Fig. 1). At the low iron condition, the LPS mutants 374OA⁻ and 417OA⁻ also induced significant systemic resistance and to the same level as their respective wild types and pseudobactin-minus mutants. Iron availability had no significant effect on the suppression of Fusarium wilt of radish by strain WCS417 or its pseudobactin-minus mutant 417PSB⁻. Strain WCS358 and its pseudobactin-minus mutant 358PSB⁻ did not suppress the disease either at high or at low iron availability.

Bacterial root colonization. No differences in root colonization were observed among the treatments at harvest; the total pseudomonad populations were 2.0 to 3.5×10^7 CFU per g of root fresh weight at low iron availability and 1.2 to 3.4×10^7 CFU per g at high iron availability. The population densities of the

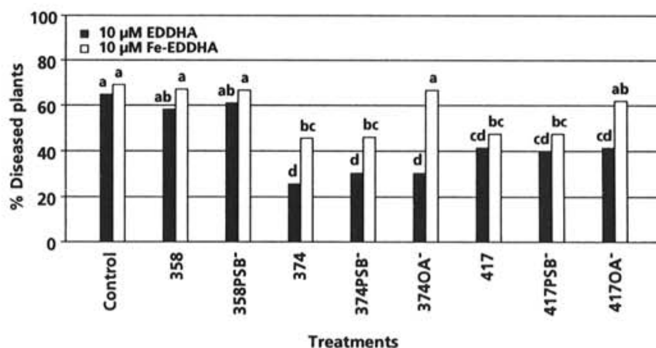

Fig. 1. Percentage of radish plants (cultivar Saxa×Nova) with Fusarium wilt in the ISR rock wool bioassay at low (10 µM EDDHA) and high (10 µM Fe-EDDHA) iron availability. The root tips were treated with *Pseudomonas putida* WCS358, 358PSB⁻ (pseudobactin-minus), *P. fluorescens* WCS374, 374PSB⁻, 374OA⁻ (O-antigen-minus), WCS417, 417PSB⁻, or 417OA⁻, in talcum, 2 days before inoculation with the pathogen. The pathogen was inoculated on the root base in peat. The populations of the bacteria and the pathogen were inoculated at separate locations on the roots and remained there during the experiment. Bars with the same letter are not significantly different at $P \leq 0.05$ according to Fisher's least significant difference test (unpooled data).

TABLE 1. Production of salicylic acid (SA) and pseudobactins by *Pseudomonas putida* WCS358 and *P. fluorescens* WCS374 and WCS417 and their derivative pseudobactin-minus (PSB⁻) and O-antigen–minus mutants (OA⁻) at low iron availability, determined by spectrophotometric detection of the purple iron-SA complex (527 nm) and the pseudobactins (400 nm)

Pseudomonas strain	SA[y] (µg/ml, 527 nm)	Pseudobactins[y] (µM, 400 nm)
WCS358	ND[z]	81.4 b
358PSB⁻	ND	ND
WCS374	54.6 a	59.0 c
374PSB⁻	39.4 b	ND
374OA⁻	47.6 a	51.3 c
WCS417	7.8 c	182.4 a
417PSB⁻	10.0 c	ND
417OA⁻	7.6 c	187.1 a

[y] Means followed by the same letter are not significantly different at $P \leq 0.05$ according to Fisher's least significant difference test (unpooled data).
[z] ND = not detected.

introduced *Pseudomonas* strains and their mutants did not differ (1.6 to 2.7 × 10^6 CFU per g of root fresh weight at low iron availability, 1.2 to 2.8 × 10^6 CFU per g at high iron availability). Introduced bacteria were never detected at the root base (zone of pathogen inoculation).

In vitro growth and production of SA and pseudobactin by *Pseudomonas* strains. In the SSM shake cultures, the cell yield of the different strains after 48 h (stationary phase) fluctuated between 9 × 10^8 and 2 × 10^9 CFU per ml of SSM, which resulted in absorbance readings of approximately 1 at 650 nm.

The production of SA by strains WCS374 and WCS417 of *P. fluorescens* and their pseudobactin-minus and O-antigen-minus mutants was qualitatively demonstrated by GC and spectrophotometric detection. With GC, the retention time for succinic acid, the carbon source in the SSM, was 9.80 min. In culture supernatants of the *P. fluorescens* strains grown in SSM at low iron availability, a clear separation of succinic acid and SA (retention time 9.60 min) was established. SA was not detectable in culture supernatants of these strains grown in SSM with 25 μM $FeCl_3$. No other organic or aromatic acids were detected in the supernatant of the strains tested. In the absorbance spectrum of commercial SA, the characteristic peaks were found at 244 and 309 nm. The spectrum of extracted culture supernatant of WCS374 was identical to that of commercial SA (43). SA could not be detected in culture supernatant of strain WCS358 or its pseudobactin-minus mutant 358PSB⁻.

Strain WCS374 and its O-antigen-minus mutant 374OA⁻ produced significantly more SA than the pseudobactin-minus mutant 374PSB⁻, as detected at 527 nm (Table 1). Strain WCS417 and derivatives produced significantly less SA than strain WCS374 and derivatives.

Pseudobactins were produced by all wild-type strains and their O-antigen-minus mutants, but not by their pseudobactin-minus mutants, as detected at 400 nm (Table 1). Strain WCS417 and derivatives appeared to produce more pseudobactins than the other strains, and strain WCS358 appeared to produce more than strain WCS374 and derivatives.

The production of pseudobactins and SA by strains WCS374 and WCS417 and their derivatives decreased with increasing iron availability (Fig. 2). At an $FeCl_3$ concentration of 2 μM, strain WCS374 and derivatives produced considerably less pseudobactin and SA than they did when no iron was added. For strain WCS417 and derivatives, the rapid decrease in pseudobactin and SA production was observed between concentrations of $FeCl_3$ of 5 and 10 μM. The production of both pseudobactins and SA fell below the detection level at iron concentrations between 20 and 30 μM.

SA and pseudobactins in ISR. At low iron availability, SA induced systemic resistance in radish at concentrations ranging from 100 fg to 100 μg per root system (Fig. 3). Within this range, SA had no observable effect on the development of the radish plants. The pseudobactin of WCS374, but not those of WCS358 and WCS417, induced resistance when applied at a concentration of 70 μg per root system (Fig. 4).

Effects of SA on hyphal growth of *F. oxysporum* f. sp. *raphani*. Effects of SA on growth of hyphae from germinated microconidia of the Fusarium wilt pathogen were concentration-dependent. At 0.1 mg/ml, SA had no significant effect; 1 mg of SA per milliliter significantly reduced growth; and at 10 mg/ml, hyphal growth was completely inhibited (Fig. 5).

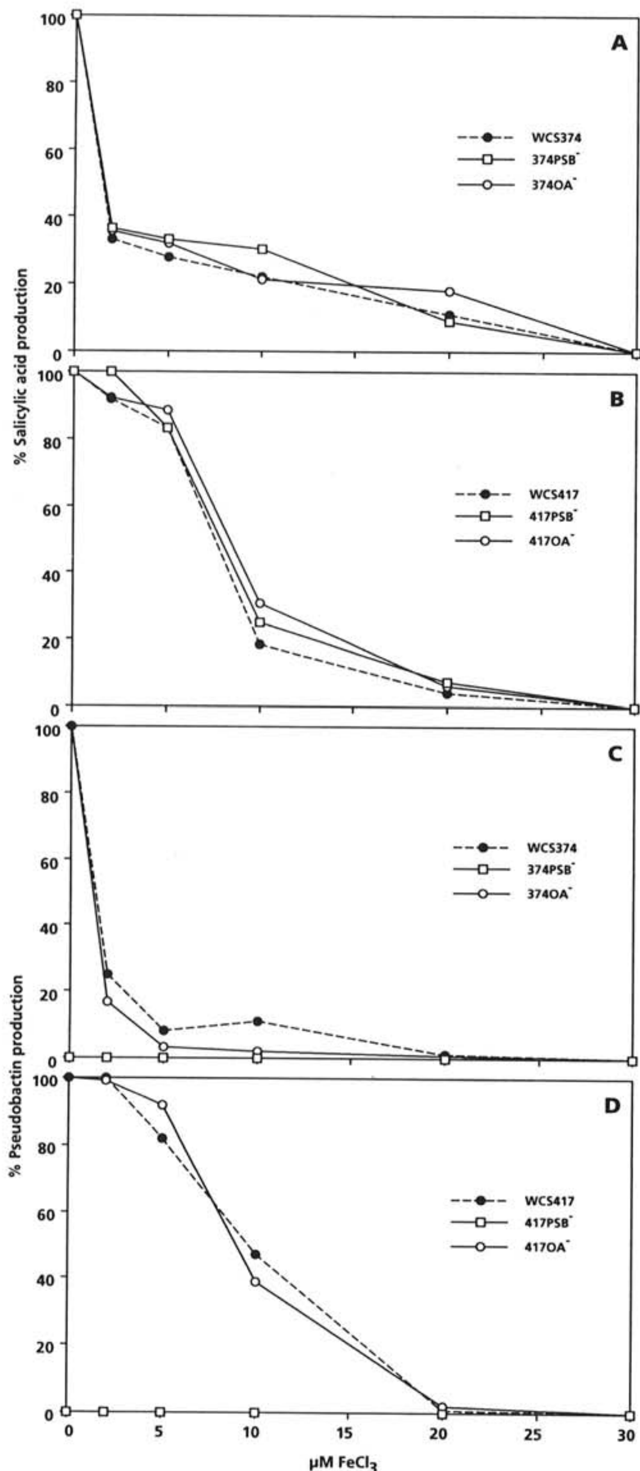

Fig. 2. Effect of increasing concentrations of $FeCl_3$ on production of salicylic acid **(A and B)** and pseudobactin **(C and D)** by *Pseudomonas fluorescens* strains WCS374 and WCS417 and their derivative pseudobactin-minus (PSB⁻) and O-antigen-minus (OA⁻) mutants as a percentage of the siderophore amounts produced in the absence of $FeCl_3$, as quantified by spectrophotometry. Strains were grown for 48 h in a standard succinate medium shake culture.

DISCUSSION

In this study, strains of *P. fluorescens* significantly suppressed Fusarium wilt of radish in the ISR rock wool bioassay, despite the fact that populations of the pathogen and the introduced bacteria were applied at separate locations on the plant root. Because a direct interaction between the two populations was impossible, induction of systemic resistance, as described in earlier studies (30,31), is the best explanation for these results.

The induction of systemic resistance at high iron availability by strains WCS374 and WCS417 of *P. fluorescens* and their pseudobactin-minus mutants, but not their O-antigen-minus mutants, agrees with the observation that purified LPSs of strains WCS374 and WCS417 induced systemic resistance against Fusarium wilt of radish (31). The inability of strain WCS358 and its pseudobactin-minus mutant to reduce disease in the ISR rock wool bio-

assay is also explained by the fact that their LPS is not able to induce resistance.

In the ISR rock wool bioassay, some of the *P. fluorescens* strains reduced disease more when the iron availability of the radish nutrient solution was low (Fig. 1). The determinant responsible for this effect was not the O-antigen of the LPS, because the O-antigen-minus mutants of WCS374 and WCS417 were also able to induce resistance at low iron availability. To explain the effect of iron availability on ISR, we studied pseudomonad metabolites regulated by iron availability. The production of both SA and pseudobactins by strains WCS374 and WCS417 and their O-antigen-minus mutants, and of SA by their pseudobactin-minus mutants, was demonstrated in vitro at low iron availability and decreased rapidly with the addition of iron to the medium. SA is considered to serve as a siderophore for some *Pseudomonas* spp. (43,56). SA may serve as a siderophore for strains WCS374 and WCS417 and their derivatives, since pyochelin is not produced (S. Buysens, *personal communication*).

The amount of SA produced differed among the strains, but WCS374 and WCS417 produced at least 35 and five times more, respectively, than *P. fluorescens* CHA0 as reported by Meyer et al. (43). A pyoverdine-minus mutant of strain CHA0 produced nine to 14 times more SA than its wild type, which was explained by the assumption that the lack of pyoverdine is compensated for by increased production of SA, which serves as a siderophore. For strains WCS374 and WCS417, no increased SA production by the pseudobactin-minus mutants was observed, perhaps because the production of SA by these strains was already high compared with that by the CHA0 strain.

SA suppressed Fusarium wilt of radish in the ISR rock wool bioassay. The level of disease reduction by SA was independent of the applied concentration. Toxic effects of SA on a number of plant pathogens, including *F. oxysporum,* have been demonstrated (46,48). In the in vitro assay on the growth of hyphae of *F. oxysporum* f. sp. *raphani* in different SA concentrations, only high concentrations (> 1 mg/ml) reduced hyphal growth. Therefore, it is unlikely that the effects of SA in the bioassay were due to systemic transport and subsequent toxic effects at the pathogen infection site, because a concentration of 100 fg of SA per root (about 4×10^9 molecules of SA) was still effective in suppressing disease. It therefore is most likely that the observed disease suppression by SA is due to ISR.

Pseudobactin of strain WCS374, but not of strains WCS358 or WCS417, suppressed Fusarium wilt of radish in the ISR rock wool bioassay. The applied purified pseudobactin contained neither a detectable amount of LPS, as detected by the spectrophotometric method of Karkhanis et al. (24) (lower detection limit 500 ng per gram of talcum), nor SA (lower detection limit 3 ng per gram of talcum). However, the possibility cannot be excluded that concentrations of these compounds below the detection limits were active in inducing resistance. Although unlikely, it is also possible that the pseudobactin of WCS374, if taken up and translocated in the plant, inhibited the pathogen at the infection site. In vitro studies have demonstrated the inhibitory effects of the purified pseudobactins of WCS358 and WCS417 on the germination of conidia of *F. oxysporum* f. sp. *dianthi* (14).

Chou and Patrick (10) observed production of SA in the microbial decomposition of corn and rye residues. SA was detected in rhizosphere soil of mung bean and corn, whereas the levels of SA in the nonrhizosphere soil were either lower or not detectable, respectively (44). This suggested the involvement of rhizosphere microorganisms in the biosynthesis of SA. Therefore, the additional reduction of disease in the ISR rock wool bioassay at low iron availability compared with high iron availability by strain WCS374, its pseudobactin-minus and O-antigen-minus mutants, and the O-antigen-minus mutant of WCS417 is best explained by the assumption that SA was produced at low iron availability and increased disease control. For strains WCS374 and 374OA⁻, the possibility cannot be excluded that the pseudobactin was involved in the induction of resistance as well. However, because the pseudobactin-minus mutant 374PSB⁻ also increased disease control in the ISR rock wool bioassay at low iron availability, it is unlikely that the increased resistance induced by WCS374 and 374OA⁻ was due to the pseudobactin. On the other hand, the effect of SA, produced by both strains, may have masked the induction of resistance by the pseudobactin. Both pseudobactin and SA may be involved in the same induced resistance pathway(s) of the plant, so that induction of resistance by the pseudobactin and by SA is not additive. It is also possible that too little pseudobactin was produced in the rhizosphere to be effective in inducing resistance, or that too much was taken up or

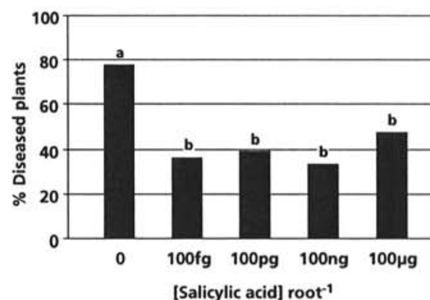

Fig. 3. Percentage of radish plants (cultivar Saxa×Nova) with Fusarium wilt in the ISR rock wool bioassay at low iron availability when the root tips were treated with increasing concentrations of salicylic acid (SA) in talcum 2 days before inoculation with the pathogen. The pathogen was inoculated on the root base in peat. The pathogen and SA were applied at separate locations on the roots. Bars with the same letter are not significantly different at $P \le 0.05$ according to Fisher's least significant difference test (unpooled data).

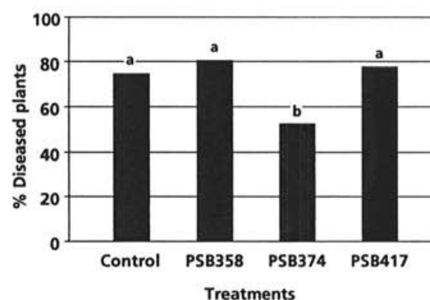

Fig. 4. Percentage of radish plants (cultivar Saxa×Nova) with Fusarium wilt in the ISR rock wool bioassay at low iron availability when the root tips were treated with 70 µg per root system of purified pseudobactins of *Pseudomonas putida* WCS358 or *P. fluorescens* WCS374 or WCS417 in talcum 2 days before inoculation with the pathogen. The pathogen was inoculated on the root base in peat. The pathogen and pseudobactin were applied at separate locations on the roots. Bars with the same letter are not significantly different at $P \le 0.05$ according to Fisher's least significant difference test (unpooled data).

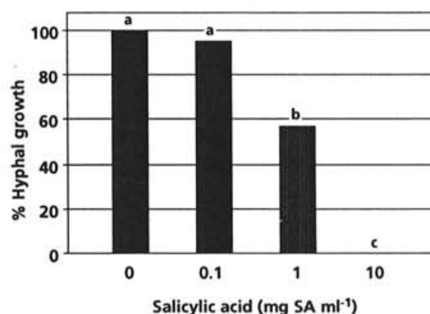

Fig. 5. In vitro growth of hyphae of *Fusarium oxysporum* f. sp. *raphani* from germinated microconidia on potato-dextrose agar in increasing concentrations of salicylic acid. Bars with the same letter are not significantly different at $P \le 0.05$ according to Fisher's least significant difference test (unpooled data).

degraded by other microorganisms. Maurhofer et al. (40) reported that the pyoverdine-minus mutant of *P. fluorescens* CHA0, which produces SA, induced significantly less resistance in tobacco against tobacco necrosis virus than the wild-type strain.

The fact that neither strains WCS358 and WCS417 nor their pseudobactin-minus mutants 358PSB⁻ and 417PSB⁻ increased control of disease in the ISR rock wool bioassay at low iron compared with high iron availability may be explained by the fact that these strains do not produce a pseudobactin that induces resistance and did not produce SA (WCS358) or produced substantially less SA (WCS417) than WCS374. However, induction of resistance by SA occurs in the femtomolar range, and the O-antigen-minus mutant 417OA⁻ is able to produce the same amount of SA as its wild type, yet it induced resistance at the low iron availability. Strain WCS417 and its mutants suppressed disease less (not significant) than strain WCS374 and its mutants. Thus, the situation is complex, and additional iron-regulated metabolites (siderophores) may play a role in WCS374.

In the classical SAR in plants, resistance to fungal, bacterial, and viral pathogens can be induced by exogenously applied SA or acetylsalicylic acid (16,38,61) or by preinoculation with necrosis-causing virulent strains or avirulent or incompatible strains of disease-causing organisms (13). SAR enhances the plant's resistance to subsequent challenge infections of a variety of viral, bacterial, and fungal pathogens (27). Development of SAR correlates with the expression of a set of genes including those which encode for pathogenesis-related proteins (55). The exact function of SA is still not clear. SA may serve as an endogenous signal molecule required for SAR expression (41,55,61). SA may also specifically inhibit the catalase activity of certain SA-binding proteins, resulting in an increase of H_2O_2, which in turn induces the expression of genes associated with SAR (9). How SA produced by PGP *Pseudomonas* spp. induces resistance is not yet clear. When taken up by plant roots, it may act as a systemic or local signal and add to or modulate other signals triggered by other bacterial traits. Whether the observed ISR in the Fusarium wilt of radish model differs from SAR is also not clear. Hoffland et al. (22) demonstrated that ISR in this model is not associated with accumulation of pathogenesis-related proteins, which distinguishes ISR from SAR.

In conclusion, the results described are evidence for the induction of systemic resistance against Fusarium wilt of radish by determinants of *P. fluorescens* strains WCS374 and WCS417 that are regulated by iron availability. Strain WCS374 apparently possesses several determinants involved in ISR: O-antigen, SA, and possibly pseudobactin. For strain WCS417, the O-antigen and most likely the production of SA are involved in ISR. Strain WCS358 neither produces SA nor possesses a suitable pseudobactin or O-antigen to induce systemic resistance in radish. Most of the studies that have suggested a role for siderophore-mediated competition for iron at low iron availability as a biological control mechanism (reviewed in 5,34,36) have not considered the possible role of these siderophores and iron availability in induction of disease resistance. Siderophore-mediated competition for iron may be of no or minor importance as a mechanism of biological control in some if not most of the cases studied. Therefore, it seems appropriate to reevaluate the role of competition for iron and iron availability in suppression of disease. Further research is necessary to elucidate the precise nature and function of iron availability-regulated determinants in PGP *Pseudomonas*-mediated ISR.

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