Comparison of Systemic Resistance Induced by Avirulent and Nonpathogenic *Pseudomonas* Species

Ellis Hoffland, Johanna Hakulinen, and Johan A. van Pelt

First and third authors: Department of Plant Ecology and Evolutionary Biology, Utrecht University, P.O. Box 80084, 3508 TB Utrecht, the Netherlands; and second author: Department of Biology, University of Joensuu, P.O. Box 111, SF 80101 Joensuu, Finland.
We thank M. Leeman, L. C. van Loon, C. Pieterse, I. van der Sluis, and P. Vogel for assistance and discussions on the manuscript.
Accepted for publication 15 April 1996.

ABSTRACT

Hoffland, E., Hakulinen, J., and van Pelt, J. A. 1996. Comparison of systemic resistance induced by avirulent and nonpathogenic *Pseudomonas* species. Phytopathology 86:757-762.

The plant growth-promoting rhizobacterium (PGPR) *Pseudomonas fluorescens* strain WCS417 has been shown to induce systemic resistance against *Fusarium oxysporum* in several plant species without inducing synthesis of pathogenesis-related proteins (PR). The aim of this study was to establish the spectrum of protection achieved by *P. fluorescens*-mediated induced resistance and to compare its effectiveness with pathogen-mediated systemic acquired resistance, which is associated with necrosis and induction of PR synthesis. We demonstrated that pretreatment with *P. fluorescens* protects radish (*Raphanus sativus*) through induction of systemic resistance not only against the fungal root pathogen *F. oxysporum* f. sp. *raphani*, but also against the avirulent bacterial leaf pathogen *P. syringae* pv. *tomato* and the fungal leaf pathogens *Alternaria brassicicola* and *F. oxysporum*. We thus demonstrated, for the first time, that one PGPR strain can induce resistance against multiple pathogens. The level of protection was at least as high as that achieved by the necrotizing, PR-inducing *P. syringae* pv. *tomato*, and the spectrum was even broader indicating that i) necrosis is not a prerequisite for effective, biological induction of resistance and ii) the absence of PR after induction by *P. fluorescens* does not lower the level of protection.

Once plants have been infected by a necrotizing pathogen, they commonly show enhanced resistance against further infection. Not only are the previously infected plant parts protected, but also parts distant from the site of the primary infection. This phenomenon was first described by Ross (27), who termed it systemic acquired resistance (SAR). SAR is also frequently referred to as systemic induced resistance (7,11). Later, it was demonstrated that, besides necrotizing pathogens, chemical agents such as salicylic acid (SA) (36), 2,6-dichloro-isonicotinic acid (INA) (21), and nonpathogenic microorganisms (i.e., specific strains of plant growth-promoting rhizobacteria [PGPR] [2,32,35]) can induce systemic resistance as well.

SAR is generally assumed to be effective against a broad spectrum of diseases including fungi, bacteria, and viruses. However, only a few reports of research are available in which this aspect of SAR has been studied systematically. Kuć (12) and Hammerschmidt and Yang-Cashman (8) reported that controlled infection with the necrotizing pathogen *Colletotrichum lagenarium* or tobacco necrosis virus protected cucumber plants against a wide range of pathogens. Also, the systemic resistance induced by INA has been shown to be a broad-spectrum resistance (21). Although SA induces resistance in many plant/pathogen combinations (18), in most cases this resistance cannot be considered systemic because disease reduction is demonstrated only in the SA-treated plant parts. Only in tobacco has SA been shown to induce systemic resistance (22,23,26). Its effectiveness indicates that this is probably also a broad-spectrum resistance. So far, nothing is known about the spectrum of protection achieved by PGPR-mediated induced systemic resistance. Liu et al. (17) claim to have demonstrated that PGPR-mediated induced resistance can control multiple pathogens but, in fact, different PGPR strains were used to demonstrate induction of resistance in cucumber against *C. orbiculare* (35) and *Pseudomonas syringae* pv. *tomato* (17).

All forms of SAR described so far are associated with accumulation of pathogenesis-related proteins (PR). Since some of these PR exhibit antifungal activity in vitro (19,25,28,33) and in vivo (1,3,30), it has often been suggested that PR accumulation and SAR are causally related. PR are, therefore, often used as markers for the expression of SAR (4,34).

Previously, we reported that PGPR strain *P. fluorescens* WCS417 induces systemic resistance in radish and Arabidopsis against *Fusarium oxysporum* Schlechtend.:Fr. f. sp. *raphani* J. B. Kendrick & W. C. Snyder (15,24), but does not induce accumulation of PR in either of the two plant species (9,24). This raised questions about putative, fundamental differences between *P. fluorescens*-mediated induced systemic resistance and pathogen-mediated SAR, and about the role of PR. Although we demonstrated that PR accumulation is not a prerequisite for the expression of induced systemic resistance, it could still be argued that PR contribute to disease reduction in the case of pathogen-induced resistance when they do accumulate (i.e., upon induction of SAR).

In this paper, we examined the range of pathogens against which the PGPR strain *P. fluorescens* WCS417 induces systemic resistance. To investigate whether this resistance is effective against pathogens other than the fungal root pathogen *F. oxysporum* f. sp. *raphani*, we tested the avirulent leaf pathogens *P. syringae* pv. *tomato*, *Alternaria brassicicola* (Schwein.) Wiltshire, a *F. oxysporum* isolate from *Arabidopsis thaliana* (L.) Heyhn., and the root pathogen *Rhizoctonia solani* Kühn. No virus was tested since out of five viruses examined, none produced symptoms on the radish cultivar used (H. Steijl, *personal communication*). We compared the effectiveness of resistance induced by nonpathogenic *P. fluorescens* strain WCS417 (without PR accumulation) with that induced by avirulent *P. syringae* pv. *tomato* (with PR accumulation). *P. syringae* pv. *tomato* triggers a hypersensitive response in radish, encompassing both necrosis and accumulation

Corresponding author: J. A. van Pelt; E-mail address: h.vanpelt@boev.biol.ruu.nl

Publication no. P-1996-0521-01R

of PR-1, PR-2, and PR-5 (9), and can thus be regarded as a "classic" inducer of pathogen-mediated SAR.

MATERIALS AND METHODS

Plant cultivation. Radish seeds (*Raphanus sativus* L. cv. Saxa 2 Nova; S & G Seeds B.V., Enkhuizen, the Netherlands) were sown in quartz sand, and 6-day-old seedlings were transferred to rock wool previously drenched in a nutrient solution (5 mM $Ca(NO_3)_2$, 5 mM KNO_3, 2 mM $MgSO_4$, 2 mM KH_2PO_4, 10 μM Fe-EDDHA, and trace elements).

The plants that were used for challenge inoculation with leaf pathogens were grown in containers (29 × 14 × 4.5 cm; 10 plants per container) filled with rock wool (24 × 12 × 4 cm). After laying the roots on the rock wool, roots were covered with small rock wool cubes to prevent dehydration.

Plants used for challenge inoculation with root pathogens were grown in a system described by Leeman et al. (15), allowing for spatial separation of induction treatment and challenge inoculation of the same root system. Seedlings were placed horizontally on two rock wool cubes that were individually enclosed in plastic bags. Roots were positioned through an incision in the bags so that the lower part contacted one cube and the upper part the other. Both parts were covered with small cubes to prevent dehydration. Three plants were positioned together on two rock wool cubes (8 × 8 × 4 cm each).

Plants were grown during the summer months in a temperature-controlled glasshouse at 22°C at night and 24°C during the day, 70% relative humidity, and 16 h of light supplemented with Son-T (high-pressure sodium lamp with photosynthetic photon flux density approximately 200 μmol m^{-2} s^{-1} at plant height; Philips B.V., Twinhout, Belgium). Plants were watered twice a week. Nutrient solution was added once a week.

Microorganisms: Growth and inoculum preparation. Rifampicin-resistant *P. fluorescens* strain WCS417r (14) and *P. syringae* pv. *tomato* DC3000 (obtained from A. J. Slusarenko, Institut für Pflanzenbiologie, Zürich) were grown for 24 to 48 h at 27°C on King's medium B agar plates (10). The bacterial cells were collected and suspended in 0.01 M $MgSO_4$. The concentration was adjusted to 10^9 cells per ml for induction treatment. *P. syringae* pv. *tomato* was also used as a challenging avirulent pathogen. For challenge inoculation, the concentration was adjusted to 5×10^7 cells per ml.

A. brassicicola (supplied by B. Mauch-Mani, Institut für Pflanzenbiologie) was maintained on potato-carrot agar, a *F. oxysporum* isolate from the roots of *Arabidopsis thaliana* accession Landsberg erecta (referred to here as *F. oxysporum*; 20). *F. oxysporum* f. sp. *raphani* strain WCS600 (referred to here as *F. oxysporum* f. sp. *raphani*) and *R. solani* were maintained on potato-dextrose agar.

To prepare inoculum of *F. oxysporum* and *A. brassicicola*, conidia were collected from the plates, suspended in 0.01 M $MgSO_4$, centrifuged (15 min at 4500 × *g*), and adjusted to a concentration of 2.5×10^5 conidia per ml in 0.01 M $MgSO_4$.

A conidial suspension (±10^6 conidia per ml of 0.01 M $MgSO_4$) of *F. oxysporum* f. sp. *raphani* was prepared after growth of the fungus on liquid malt (2%) medium for 2 weeks. The suspension was added to sterilized peat (0.1 ml g^{-1}), and the fungus was allowed to germinate and colonize the peat for 2 days at 22°C. The number of CFU reached was determined by plating a peat suspension diluted in 0.01 M $MgSO_4$ on potato-dextrose agar. The peat was mixed with quartz sand to obtain an inoculum density of 10^5 CFU of *F. oxysporum* per g.

A potato-dextrose agar plate (9 cm in diameter) completely covered with *R. solani* was cut into pieces and mixed with 50 g of sterilized peat. The mixture was incubated for 2 days at 22°C and mixed with quartz sand (1:10, wt/wt) before use as challenge inoculum.

Induction treatments. Induction treatments were applied to either leaves or roots of radish plants 12 days after transfer of the plants to the rock wool. For induction on the leaf, the plants were transferred to humid glass chambers about 18 h before induction. They remained there until about 20 h after induction. The lower side of one of the first pairs of true leaves was wetted with 0.01 M $MgSO_4$ (control) or a bacterial suspension of either avirulent *P. syringae* pv. *tomato* or nonpathogenic *P. fluorescens* (both 10^9 cells per ml) using a paintbrush. About 1 ml of inoculum was applied to each leaf. For induction on the root, the roots of each plant were covered with ±0.5 g of a talcum suspension consisting of a mixture (1:1; wt/vol) of talcum (E. Merck, Darmstadt, Germany) and 0.01 M $MgSO_4$ (control) or a bacterial suspension of either *P. syringae* pv. *tomato* or *P. fluorescens* (both 10^9 cells per ml). In case the plants were to be challenge inoculated with a root pathogen, treatment was restricted to the lower part of the roots.

Challenge inoculation. Challenge inoculation took place 3 days after induction treatment. For challenge inoculation with avirulent leaf pathogens (*A. brassicicola*, *F. oxysporum*, and *P. syringae* pv. *tomato*), plants were put into humid glass chambers as described above. The lower side of one of the first pairs of true leaves (the one not previously induced) was wetted with a conidial or bacterial suspension (2.5×10^5 conidia per ml for *A. brassicicola* and *F. oxysporum*; 5×10^7 cells per ml for *P. syringae* pv. *tomato*) using a paintbrush. About 1 ml of inoculum was applied to each leaf. For inoculation with root pathogens (*F. oxysporum* f. sp. *raphani* and *R. solani*), about 0.2 g of the peat/sand mixture containing the pathogen was put on the upper part of the roots.

Evaluation of disease severity, experimental set-up, and data analysis. The disease severity caused by the avirulent leaf pathogens was evaluated by counting the number of lesions on the challenge-inoculated leaf 3 (*P. syringae* pv. *tomato*) or 10 days (*F. oxysporum* and *A. brassicicola*) after challenge inoculation. Fifty plants were used per treatment. If the condition of homogeneity of variance was not met, data were transformed before a nested analysis of variance was carried out (with the "container effect" nested within induction treatments), followed by Tukey's studentized range (honestly significant difference) test, using the SAS statistical package (SAS Institute Inc., Cary, NC).

The percentage of plants diseased after challenge inoculation with *F. oxysporum* f. sp. *raphani* was determined 15 days after challenge inoculation. *R. solani*-challenge-inoculated plants were evaluated 4, 6, 8, and 10 days after treatment. For both root pathogens, 16 replicates of three plants were used. The plants inoculated with *F. oxysporum* f. sp. *raphani* were inspected for external (wilting) and internal (browning of the vascular tissue) symptoms, based on observation of cross sections of the tap root. The plants inoculated with *R. solani* were inspected for rot of the radish tap root, cotyledons, or leaves. All plants showing symptoms, either external or internal, were scored as diseased. Treatment effects were determined after analysis of variance and a subsequent Tukey test. To evaluate differences in disease severity among the plants inoculated with either of the two root pathogens, the nonparametric Kruskal-Wallis test was used after dividing the diseased plants over different classes, depending on the plant part to which the symptoms extended. All experiments were repeated at least once.

Plant colonization by inducing bacteria. Freshly harvested plant material (0.3 g of roots and 0.5 g of leaves originating from three plants) was collected, ground using mortar and pestle, and diluted in 0.01 M $MgSO_4$. Suspensions from control plants and from plants induced with *P. syringae* pv. *tomato* were plated onto King's medium B agar supplemented with rifampicin (50 μg/ml), cycloheximide (100 μg/ml), ampicillin (40 μg/ml), and chloramphenicol (13 μg/ml). Suspensions from plants induced with *P. fluorescens* were plated onto the same medium containing 150, instead of 50, μg/ml of rifampicin. After 2 days of incubation at 28°C, the number of colonies was determined.

Protein extraction and Western blot analysis. Intercellular fluid of leaves was collected by vacuum infiltration with a phos-

phate buffer (20 mM, pH 7.0) followed by centrifugation (10 min at 1,000 × *g*). Proteins were precipitated overnight in 80% acetone at –20°C. Protein concentrations were determined using the bicinchoninic acid (BCA)-method (29). Amounts of proteins equivalent to a 0.2-g fresh weight sample were loaded on a sodium dodecyl sulfate (SDS)-polyacrylamide gel. Electrophoresis was carried out according to Laemmli (13) in a Mini-Protean II System (Bio-Rad Laboratories, Richmond, CA) using 15% separating gels.

For immunodetection, the proteins were electroblotted onto a nitrocellulose membrane (0.2 μm) in a 25 mM Tris, 192 mM glycine, and 20% (vol/vol) methanol transfer buffer for 60 min at 100 V. The blots were blocked in Tris-buffered saline (TBS)/Tween (10 mM Tris, 20 mM NaCl, and 0.5% Tween 20, pH 8.0) containing 5% defatted milk powder and incubated with primary antibodies diluted in TBS/Tween containing 1% bovine serum albumin. A specific antiserum against tobacco PR-1 (31) was used as heterologous antiserum for detection of PR-1 homologs in radish. Antigens were visualized upon incubation with goat anti-rabbit IgG conjugated to alkaline phosphatase (Bio-Rad Laboratories) using 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium as substrate.

RESULTS

Plant colonization by inducing bacteria. To investigate local and systemic plant colonization by inducing bacteria, plant material was ground, diluted, and plated onto media selective for the two inducing species. Two days after leaf induction, *P. syringae* pv. *tomato* and *P. fluorescens* were found to be equally effective in surviving on the induced leaf (Table 1). In the leaves of control plants treated with $MgSO_4$, no rifampicin-resistant bacteria were detected. Neither were any detected on the noninduced leaves that were to be challenge inoculated one day later. In root samples of leaf-induced plants, some colonies were detected on plates containing 50 μg/ml of rifampicin that were used for the control and *P. syringae* pv. *tomato*-induced plants. Considering their morphology, these were most likely bacteria other than *P. syringae* pv. *tomato*. Apparently, a concentration of 50 μg/ml of rifampicin allows growth of some rhizosphere bacteria. On the plates used for *P. fluorescens*-treated plants (containing 150 μg/ml of rifampicin), no bacteria were detected on the roots of leaf-induced plants.

On the root-induced plants, similar numbers of CFU from *P. syringae* pv. *tomato* and *P. fluorescens* were detected on the treated root parts. No introduced bacteria were detected on the leaves of those plants. Again, some bacteria, probably not *P. syringae* pv. *tomato*, were detected on plates (containing 50 μg/ml of rifampicin) from roots of control plants.

Twelve days after induction treatments, the results were similar. Still, no introduced bacteria were detected on the noninduced leaves that had been challenge inoculated.

Disease suppression. One day after induction, the plants induced with *P. syringae* pv. *tomato* showed a large number (>100 per leaf) of small, brownish/black, necrotic lesions (±1 mm in diameter) on the induced leaf only. The other leaf-induced plants and all root-induced plants remained symptomless throughout the experiments.

Three days after induction treatments, the plants were challenge inoculated. All leaf pathogens caused limited necrotic lesions (±1 mm in diameter). The effect of induction treatments could be expressed as a reduction in the number of lesions, but not in the size of the lesions. These numbers are given in Figure 1A, B, and C.

Against *A. brassicicola*, both *P. syringae* pv. *tomato* and *P. fluorescens* significantly ($P \leq 0.05$) induced resistance when applied to the leaf (Fig. 1A). When induction treatment was applied to the root, only *P. fluorescens* significantly reduced the number of lesions per leaf. Surprisingly, both control treatments (0.01 M $MgSO_4$ on either leaf or root) reduced the number of lesions per leaf compared with the nontreated plants. This phenomenon was only observed when *A. brassicicola* was used as a challenging pathogen. Apparently, the application procedure triggers some reactions that render the plant more resistant to *A. brassicicola*.

When a *F. oxysporum* isolate from Arabidopsis was used as a challenge pathogen on the leaf, results were similar. Again, both inducing species were effective when applied to the leaf but, on the root, only *P. fluorescens* induced resistance (Fig. 1B).

P. syringae pv. *tomato* was not only used as an inducing agent, but also for challenge inoculation (Fig. 1C). The effects of induction treatments were qualitatively the same as those obtained for the two avirulent fungal leaf pathogens. Resistance against *P. syringae* pv. *tomato* was significantly induced by pretreatment with itself at the leaf only, and by pretreatment with *P. fluorescens* at both leaf and root.

Since *P. syringae* pv. *tomato* did not induce resistance against avirulent leaf pathogens when applied to the roots, its resistance-inducing action against root pathogens was only tested after application to the leaf. *P. fluorescens* was only applied to the root.

F. oxysporum f. sp. *raphani* caused clear wilting symptoms: yellowing of the leaves starting from the veins, resulting in death in some plants. A large part of the plants showed only internal symptoms (browning of the vascular tissue of the main root or tap root). To *F. oxysporum* f. sp. *raphani* (Fig. 1D), only *P. fluorescens* induced resistance; *P. syringae* pv. *tomato* was ineffective.

R. solani caused rot of the outside of the tap root, followed by wilting of the cotyledons, leaves, and entire shoot. In Figure 1E, the percentages of diseased plants 6 days after challenge inoculation are

TABLE 1. Local and systemic plant colonization[a] by the bacterial species used to induce resistance 2 and 12 days after induction treatment[b]

Days after induction	Induction site	Sample	Induction treatment		
			Control[c]	*P. syringae* pv. *tomato*[c]	*P. fluorescens*[d]
2	Leaf	Induced leaf	n.d.[e]	$(4.9 \pm 0.5) \times 10^7$	$(4.7 \pm 0.5) \times 10^7$
		Noninduced leaf	n.d.	n.d.	n.d.
		Root	$(3.3 \pm 4.4) \times 10^2$	$(3.3 \pm 4.7) \times 10^2$	n.d.
	Root	Induced root part	$(9.3 \pm 12.5) \times 10^1$	$(4.3 \pm 6.1) \times 10^4$	$(5.4 \pm 2.0) \times 10^5$
		Leaf	n.d.	n.d.	n.d.
12	Leaf	Induced leaf	n.d.	$(7.4 \pm 2.6) \times 10^5$	$(6.4 \pm 4.7) \times 10^5$
		Noninduced leaf	n.d.	n.d.	n.d.
		Root	$(8.7 \pm 3.1) \times 10^2$	$(2.7 \pm 1.1) \times 10^3$	n.d.
	Root	Induced root part	$(6.6 \pm 4.7) \times 10^3$	$(4.6 \pm 2.3) \times 10^5$	$(6.0 \pm 2.6) \times 10^4$
		Leaf	n.d.	n.d.	n.d.

[a] CFU per g of fresh weight. Means ± SE, $n = 3$.
[b] *Pseudomonas syringae* pv. *tomato* and *P. fluorescens* were detected on selective media. For the control treatment, the medium selective for *P. syringae* pv. *tomato* was used.
[c] Plates containing 50 μg/ml of rifampicin were used.
[d] Plates containing 150 μg/ml of rifampicin were used.
[e] Not detected. Detection limit: 100 CFU per g of fresh weight.

presented. The number of diseased plants did not increase after this time. Two days earlier, the percentages were lower, but the effect of induction treatments was comparable. None of the two induction treatments resulted in significant suppression of disease caused by this pathogen. On the contrary, induced plants tended to be more diseased than did the noninduced controls.

All plants showing any symptoms of *F. oxysporum* f. sp. *raphani* or *R. solani* were scored as diseased, and it might have been that resistance induction lead to reduction in disease severity. However, this was not the case. When only the diseased plants were considered, there was no difference ($P \leq 0.05$) in the severity of the disease caused by either of the root pathogens among the different induction treatments (data not shown).

In order to compare the effectiveness of the two inducing species, the disease reduction obtained by each was compared with their respective controls (0.01 M $MgSO_4$ at the root or leaf; Fig. 2). The number of lesions per leaf (for the leaf pathogens) or the percentage of diseased plants (for the root pathogens) in each control treatment was taken as 100%. When only the leaf pathogens were considered (Fig. 2A, B, and C), leaf induction by *P. fluorescens* was generally as effective as leaf induction by *P. syringae* pv. *tomato*. In contrast, when applied to the root, *P. syringae* pv. *tomato* did not induce resistance against either of the three leaf pathogens used for challenge inoculation. The site of application of *P. fluorescens* did not affect the extent of resistance attained.

Against the root pathogens, induction by *P. syringae* pv. *tomato* at the leaf was ineffective in both cases (Fig. 2D and E). Induction by *P. fluorescens* at the root was highly effective against *F. oxysporum* f. sp. *raphani* (Fig. 2D), but ineffective against *R. solani* (Fig. 2E). All above described experiments were repeated at least once, yielding similar results.

Accumulation of PR-1. PR-1, PR-2, and PR-5 are coordinately, locally induced in radish when *P. syringae* pv. *tomato* is applied to the leaf (9). To ascertain induction of PR as a result of the different inducer treatments, Western blot analysis was applied to the intercellular fluid of the leaves using PR-1 as a marker. Only nonchallenge-inoculated plants were used. For plants that were induced at the leaf, both the induced leaf and the noninduced leaf were analyzed to check for local and systemic induction of PR accumulation, respectively. In the plants that were induced at the root, only systemic induction in the first pair of true leaves was investigated.

Induction treatment with *P. syringae* pv. *tomato* at the leaf caused both local and systemic accumulation of PR-1 within 2 days (Fig. 3). Neither the control treatment nor induction with *P. fluorescens* at the leaf induced PR-1 accumulation. No PR-1 homologs could be detected in leaves of plants that were induced by either of the two species at the root (data not shown). Twelve days after induction treatments, the results were similar.

DISCUSSION

The results of the plant colonization study demonstrated that the two bacterial species used to induce resistance did not colonize the leaves used for challenge inoculation (Table 1). It was shown previously that the bioassay used for the root pathogens guarantees spatial separation of inducing and challenging microorganisms (9,15). Consequently, effects on disease development must have been plant-mediated. We also demonstrated that *P. syringae* pv. *tomato* and *P. fluorescens* survived on the induced plant parts to similar extents. Differences between the species, therefore, cannot be because of differences in bacterial numbers.

P. syringae pv. *tomato* proved to possess all the characteristics commonly associated with the induction of SAR: i) it caused rapidly appearing necrotic lesions when applied to the leaf, typical of a hypersensitive response; ii) it induced local and systemic accumulation of PR-1, PR-2, and PR-5 (Fig. 3; [9]); and iii) the resistance induced was effective against multiple pathogens, i.e., itself

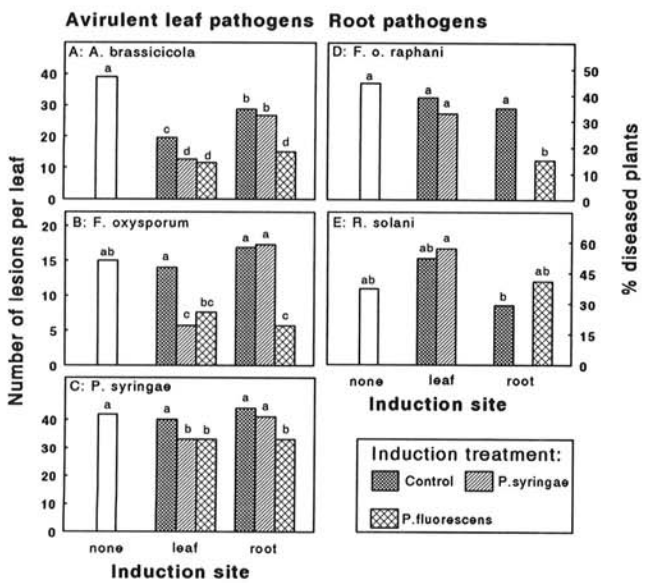

Fig. 1. Disease incidence among plants challenge inoculated with **A,** *Alternaria brassicicola*; **B,** *Fusarium oxysporum*; **C,** *Pseudomonas syringae* pv. *tomato*; **D,** *F. oxysporum* f. sp. *raphani*; and **E,** *Rhizoctonia solani*. Nontreated plants (left, white bars) were not treated at all before challenge inoculation. The control plants of the leaf- or root-induced plants were treated with 0.01 M $MgSO_4$. Treatments with the same letter are not significantly different (Tukey's studentized range test).

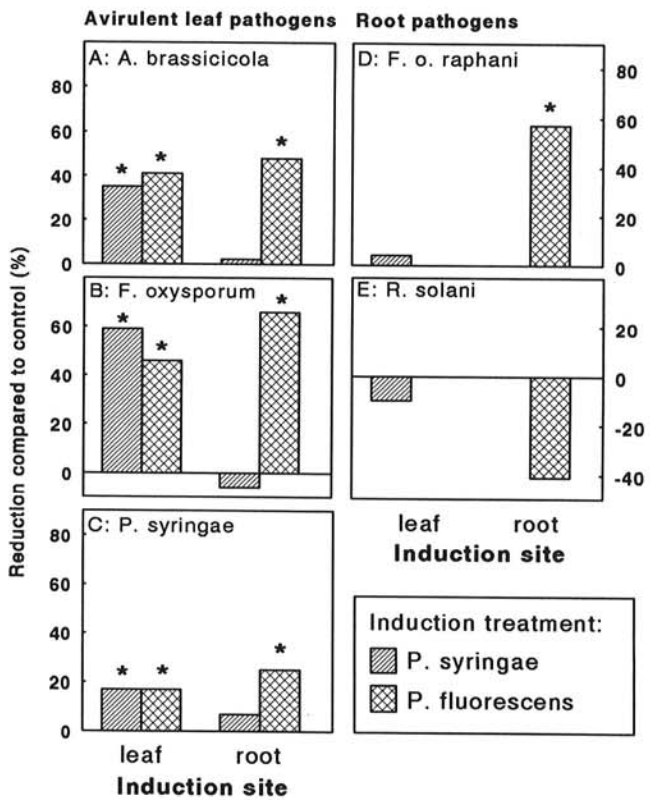

Fig. 2. The reduction of disease incidence among plants challenge inoculated with **A,** *Alternaria brassicicola*; **B,** *Fusarium oxysporum*; **C,** *Pseudomonas syringae* pv. *tomato*; **D,** *F. oxysporum* f. sp. *raphani*; and **E,** *Rhizoctonia solani* induced by *P. syringae* pv. *tomato* or *P. fluorescens*. The percentage of diseased plants in the respective control plants (treated with 0.01 M $MgSO_4$ at either leaf or root) was taken as 100%. An * means that induction treatment resulted in a significant disease reduction compared with the control treatment (Tukey's studentized range test). A negative reduction (**B** and **E**) indicates that the respective induction treatments increased disease incidence.

(an avirulent bacterial pathogen on radish; Fig. 1C) and two avirulent fungal pathogens (*A. brassicicola* and *F. oxysporum*; Fig. 1A and B, respectively). *P. syringae* pv. *tomato* is, therefore, suitable as a reference to evaluate the characteristics of *P. fluorescens*-mediated induced disease resistance in comparison with classic SAR.

P. fluorescens induced resistance against the same three avirulent leaf pathogens and, in contrast to *P. syringae* pv. *tomato*, also against the fungal root pathogen *F. oxysporum* f. sp. *raphani*. This demonstrated, for the first time, that one and the same PGPR strain can protect a plant against multiple pathogens. The spectrum of resistance can even be broader than that of classic SAR. In accordance with Liu et al. (17), we demonstrated that leaf application of a PGPR strain is effectively inducing resistance against leaf pathogens. The results of Leeman et al. (16) strongly suggest that leaf application might also be effective against root pathogens, indicating great possibilities for foliar application of *P. fluorescens* in practical agriculture.

It is not clear why neither *P. fluorescens* nor *P. syringae* pv. *tomato* could induce resistance against *R. solani*. No other reports on SAR against *R. solani* are known. In radish, no resistance against *R. solani* has ever been described. We suggest that induction of systemic resistance is only possible when a basic level of genetic resistance is already present. This is probably lacking in radish.

In general, it can be concluded that, when applied to the leaf, *P. fluorescens* is as effective an inducer as *P. syringae* pv. *tomato* (Fig. 2A, B, and C). This demonstrates that hypersensitive-type necrosis is not required for biological induction of resistance. When applied to the root, *P. fluorescens* was much more effective as an inducer than *P. syringae* pv. *tomato*. Since nonchallenged plants, root-induced with *P. syringae* pv. *tomato*, showed neither disease symptoms nor growth reduction nor visible necrosis, it is unlikely that *P. syringae* pv. *tomato* acted as a pathogen on radish roots. It seems, therefore, that *P. syringae* pv. *tomato*-mediated SAR is related to necrosis, whereas *P. fluorescens*-mediated induced systemic resistance is not. Although the occurrence of a hypersensitive response seems to be unconditionally related to induced resistance in Arabidopsis (5,6), biologically induced resistance in radish is clearly not depending on the occurrence of a hypersensitive response.

In plants treated with *P. syringae* pv. *tomato* on the leaf, induction of resistance was associated with systemic induction of PR, whereas in plants induced by *P. fluorescens*, at either leaf or root, it was not (Fig. 3; [9]). Since cell walls of all four fungal pathogens used contain glucans, a putative effect of PR-2-type glucanases could be expected. Consequently, if PR would play a role in disease suppression, one would expect resistance induced by *P. syringae* pv. *tomato* to be more effective than that induced by *P. fluorescens*. We did not find such a difference (Fig. 2A, B, and C). This indicates that induction of PR associated with SAR does not necessarily lead to a more effective disease suppression compared with biological induction of resistance without induction of PR.

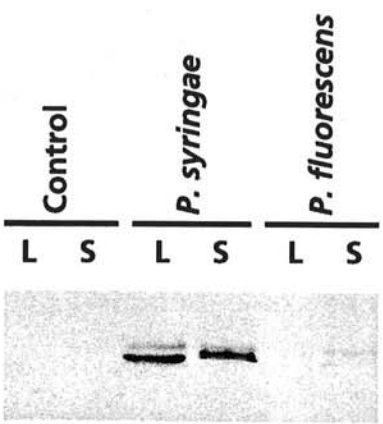

Fig. 3. Local (L) and systemic (S) induction of a PR-1 homolog in the intercellular fluid of radish leaves upon treatment of the leaf with 0.01 M $MgSO_4$ (Control), *Pseudomonas syringae* pv. *tomato*, or *P. fluorescens*. Western blots of samples collected 2 days after induction treatment are shown. To establish systemic induction, the leaves opposite the induced ones were used. In other plants, this leaf was used for challenge inoculation.

In conclusion, we demonstrated that *P. fluorescens*-mediated induced systemic resistance resembles classic SAR with respect to the broadness and effectivity of protection. The absence of necrosis and PR synthesis upon induction by this PGPR strain suggests that, although the protection is the same as with classic SAR, it is achieved via a (partly) different pathway. The pathway leading to induction of PR may be branching off the pathway leading to induction of resistance. This is in accordance with results obtained with Arabidopsis, which also demonstrates that *P. fluorescens* induces resistance along a pathway different from that controlling classic SAR (24). Induction of PR in the case of SAR does not seem to exert any additional effect on disease reduction.

LITERATURE CITED

1. Alexander, D., Goodman, R. M., Gut-Rella, M., Glascock, C., Weymann, K., Friedrich, L., Maddox, D., Ahl-Goy, P., Luntz, T., Ward, E., and Ryals, J. 1993. Increased tolerance to two oomycete pathogens in transgenic tobacco expressing pathogenesis-related protein 1a. Proc. Natl. Acad. Sci. U.S.A. 90:7327-7331.
2. Alström, S. 1991. Induction of disease resistance in common bean susceptible to halo blight bacterial pathogen after seed bacterization with rhizosphere pseudomonads. J. Gen. Appl. Microbiol. 37:495-501.
3. Broglie, K., Chet, I., Holliday, M., Cressman, R., Biddle, P., Knowlton, S., Muavals, C. J., and Broglie, R. 1991. Transgenic plants with enhanced resistance to the fungal pathogen *Rhizoctonia solani*. Science 254:1194-1197.
4. Chen, Z., Silva, H., and Klessig, D. F. 1993. Active oxygen species in the induction of plant systemic acquired resistance by salicylic acid. Science 262:1883-1886.
5. Dietrich, R. A., Delaney, T. P., Uknes, S. J., Ward, E. J., Ryals, J. A., and Dangl, J. L. 1994. Arabidopsis mutants simulating disease resistance response. Cell 77:565-577.
6. Greenberg, J. T., Guo, A., Klessig, D. F., and Ausubel, F. M. 1994. Programmed cell death in plants: A pathogen-triggered response activated coordinately with multiple defense reactions. Cell 77:551-563.
7. Hammerschmidt, R., and Kuć, J. 1995. Induced Resistance to Disease in Plants. Developments in Plant Pathology 4. Kluwer Academic Publishers, Dordrecht, the Netherlands.
8. Hammerschmidt, R., and Yang-Cashman, P. 1995. Induced resistance in cucurbits. Pages 63-85 in: Induced Resistance to Disease in Plants. Developments in Plant Pathology 4. R. Hammerschmidt and J. Kuć, eds. Kluwer Academic Publishers, Dordrecht, the Netherlands.
9. Hoffland, E., Pieterse, C. M. J., Bik, L., and van Pelt, J. A. 1995. Induced systemic resistance in radish is not associated with accumulation of pathogenesis-related proteins. Physiol. Mol. Plant Pathol. 46:309-320.
10. King, E. O., Ward, M. K., and Raney, D. E. 1954. Two simple media for demonstration of pyocyanin and fluorescin. J. Lab. Clin. Med. 44:301-307.
11. Kloepper, J. W., Tuzun, S., and Kuć, J. A. 1992. Proposed definitions related to induced disease resistance. Biocontrol Sci. Technol. 2:349-351.
12. Kuć, J. 1982. Induced immunity to plant disease. BioScience 32:854-860.
13. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.
14. Lamers, J. G., Schippers, B., and Geels, F. P. 1988. Soil-borne disease of wheat in the Netherlands and results of seed bacterization with pseudomonads against *Gaeumannomyces graminis* var. *tritici*. Pages 134-139 in: Cereal Breeding Related to Integrated Cereal Production. M. L. Jorna and L. A. Slootmaker, eds. Pudoc, Wageningen, the Netherlands.
15. Leeman, M., van Pelt, J. A., den Ouden, F. M., Heinsbroek, M., Bakker, P. A. H. M., and Schippers, B. 1995. Induction of systemic resistance by *Pseudomonas fluorescens* in radish cultivars differing in susceptibility to Fusarium wilt, using a novel bioassay. Eur. J. Plant Pathol. 101:655-664.
16. Leeman, M., van Pelt, J. A., den Ouden, F. M., Heinsbroek, M., Bakker, P. A. H. M., and Schippers, B. 1995. Induction of systemic resistance against Fusarium wilt of radish by lipopolysaccharides of *Pseudomonas*

fluorescens. Phytopathology 85:1021-1027.
17. Liu, L., Kloepper, J. W., and Tuzun, S. 1995. Induction of systemic resistance in cucumber against bacterial angular leaf spot by plant growth-promoting rhizobacteria. Phytopathology 85:843-847.
18. Malamy, J., and Klessig, D. J. 1992. Salicylic acid and plant disease resistance. Plant J. 2:643-654.
19. Mauch, F., Mauch-Mani, B., and Boller, T. 1988. Antifungal hydrolases in pea tissue. II. Inhibition of fungal growth by combinations of chitinase and β-1,3-glucanase. Plant Physiol. 88:936-942.
20. Mauch-Mani, B., and Slusarenko, A. J. 1994. Systemic acquired resistance in *Arabidopsis thaliana* induced by a predisposing infection with a pathogenic isolate of *Fusarium oxysporum*. Mol. Plant-Microbe Interact. 7:378-383.
21. Métraux, J. P., Ahl-Goy, P., Staub, T., Speich, J., Steinemann, A., Ryals, J., and Ward, E. 1991. Induced systemic resistance in cucumber in response to 2,6-dichloro-isonicotinic acid and pathogens. Pages 432-439 in: Advances in Molecular Genetics of Plant-Microbe Interactions. Vol. 1. H. Hennecke and D. P. S. Verma, eds. Kluwer Academic Publishers, Dordrecht, the Netherlands.
22. Palva, T. K., Hurtig, M., Saindrenan, P., and Palva, E. T. 1994. Salicylic acid induced resistance to *Erwinia carotovora* subsp. *carotovora* in tobacco. Mol. Plant-Microbe Interact. 7:356-363.
23. Pennazio, S., Roggero, P., and Abbatista Gentile, I. 1985. Effects of salicylate on virus-infected tobacco plants. Phytopathol. Z. 114:203-213.
24. Pieterse, C. M. J., van Wees, S. C. M., Hoffland, E., van Pelt, J. A., and van Loon, L. C. Systemic resistance in Arabidopsis induced by biocontrol bacteria is independent of salicylic acid accumulation and pathogenesis-related gene expression. Plant Cell. In press.
25. Ponstein, A. S., Bres-Vloermans, S. A., Sela-Buurlage, M. B., van den Elzen, P. J. M., Melchers, L. S., and Cornelissen, B. J. C. 1994. A novel pathogen- and wound-inducible tobacco (*Nicotiana tabacum*) protein with antifungal activity. Plant Physiol. 104:109-118.
26. Roggero, P., and Pennazio, S. 1988. Effects of salicylate on systemic invasion of tobacco plants by various viruses. J. Phytopathol. 123:207-216.
27. Ross, A. F. 1961. Systemic acquired resistance induced by localized virus infections in plants. Virology 14:340-358.
28. Schlumbaum, A., Mauch, F., Vögeli, U., and Boller, T. 1986. Plant chitinases are potent inhibitors of fungal growth. Nature 324:365-367.
29. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. 1985. Measurement of protein using bicinchoninic acid. Anal. Biochem. 150:76-85.
30. van den Elzen, P. J. M., Jongedijk, E., Melchers, L. S., and Cornelissen, B. J. C. 1993. Virus and fungal resistance: From laboratory to field. Philos. Trans. R. Soc. Lond. B Biol. Sci. 342:271-278.
31. van Loon, L. C., Gerritsen, Y. A. M., and Ritter, C. E. 1987. Identification, purification, and characterization of pathogenesis-related proteins from virus-infected Samsun NN tobacco leaves. Plant Mol. Biol. 9:593-609.
32. van Peer, R., Niemann, G. J., and Schippers, B. 1991. Induced resistance and phytoalexin accumulation in biological control of Fusarium wilt of carnation by *Pseudomonas* sp. strain WCS417r. Phytopathology 81:728-734.
33. Vigers, A. J., Wiedemann, S., Roberts, W K., Legrand, M., Selitrennikoff, C. P., and Fritig, B. 1992. Thaumatin-like pathogenesis-related proteins are antifungal. Plant Sci. 83:155-161.
34. Ward, E. R., Uknes, S. J., Williams, S. C., Dincher, S. C., Wiederhold, D. L., Alexander, D. C., Ahl-Goy, P., Métraux, J.-P., and Ryals, J. 1991. Coordinate gene activity in response to agents that induce systemic acquired resistance. Plant Cell 3:1085-1094.
35. Wei, G., Kloepper, J. W., and Tuzun, S. 1991. Induction of systemic resistance of cucumber to *Colletotrichum orbiculare* by select strains of plant growth-promoting rhizobacteria. Phytopathology 81:1508-1512.
36. White, R. F. 1979. Acetylsalicylic acid induces resistance to tobacco mosaic virus in tobacco. Virology 99:410-412.