European Journal of Plant Pathology **105:** 477–486, 1999.

Role of salicylic acid in systemic resistance induced by *Pseudomonas* spp. against *Pythium aphanidermatum* in cucumber roots

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Accepted 4 May 1999

Key words: cucumber root rot, high performance liquid chromatography, induced systemic resistance, plant growth-promoting rhizobacteria, *Pseudomonas aureofaciens*, *Pseudomonas corrugata*

Abstract

Pseudomonas corrugata strain 13 and *P. aureofaciens* strain 63-28, applied to roots, induced systemic resistance against *Pythium aphanidermatum* in cucumber roots. Salicylic acid (SA) from bacterial culture or plant tissues was quantified by high performance liquid chromatography. Both strains produced SA in King's B broth and also induced cucumber roots to accumulate endogenous SA one day after bacterial inoculation. Using a split root system, more SA accumulated in roots treated with bacteria than in distant roots on the opposite side of the root system in the first two days, but this difference disappeared after 3–4 days. SA levels were significantly higher in plants treated with bacteria compared to the split control, from one to five days after bacterization. SA did not inhibit mycelial growth of *Pythium aphanidermatum* at 100–200 µg ml^{-1} *in vitro*, but higher levels inhibited mycelial growth. Zoospore germination increased at concentrations of 10–500 µg ml^{-1}, but decreased at 1000 µg ml^{-1} compared to lower concentrations. Exogenously applied SA failed to induce local or systemic resistance against a challenge infection by the pathogen *in planta*. The results of this study show that exogenous applied SA does not induce systemic resistance to cucumber root rot caused by *P. aphanidermatum*, but endogenous SA accumulation in cucumber roots may be involved in induced systemic resistance.

Abbreviations: HPLC – high performance liquid chromatography; ISR – induced systemic resistance; PAL – phenylalanine ammonia-lyase; PGPR – plant growth-promoting rhizobacteria; SA – salicylic acid; SAG – SA-glucoside; SAR – systemic acquired resistance.

Introduction

Plant growth-promoting rhizobacteria (PGPR) can induce systemic resistance against a broad spectrum of diseases caused by viruses, bacteria (Liu et al., 1995), fungi (Wei et al., 1991) and even against plant insects (Zehnder et al., 1997). This phenomenon has been termed induced systemic resistance (ISR). The exact mechanism of ISR has not been elucidated. However, a signal, translocated from the induced site to the entire plant was proposed to be involved in ISR (Kloepper et al., 1992). ISR has many similarities to pathogen-inducible defense, called systemic acquired resistance (SAR) (reviewed by Sticher et al., 1997). Salicylic acid (SA) has been proposed as a systemic signal, inducer, or a required factor in SAR or ISR mechanisms in tobacco (Malamy et al., 1990), cucumber (Métraux et al., 1990), and beans (Dann et al., 1996; De Meyer and Höfte, 1997). SA could act as a systemic signal that triggers a local and systemic resistance response.

In the past decade, many studies indicated that SA accumulation was associated with plant physiological

responses to pathogen infection. SA levels increased as much as 20-fold after TMV infection on resistant cultivar tobacco leaves (Malamy et al., 1990). Similarly, the level of endogenous SA increased in tobacco leaves infected by TMV (Yalpani et al., 1991). In cucumber, SA appeared in the phloem exudate of plants infected by *Pseudomonas syringae* pv. *syringae* (Rasmussen et al., 1991). A similar increase of SA was measured in cucumber phloem exudates following infection with TNV or *Colletotrichum lagenarium* (syn. *C. orbiculare*), two necrotrophic pathogens that induce SAR in cucumber (Métraux et al., 1990). SA increase in cucumber leaves was maintained for up to five days after TNV or *Sphaerotheca fuliginea* inoculation (Conti et al., 1996). In 1979, White showed that exogenous application of SA induced resistance against TMV. Exogenous SA was found to induce PR-protein accumulation in tobacco, and this accumulation correlated with increased TMV resistance (Van Loon et al., 1982).

On the other hand, some studies indicated that SA may not be a translocated primary signal for SAR, and SA may only play a regulatory role in the expression of SAR genes (Vernooij et al., 1994). Pieterse et al. (1996) proved that SA was not involved in ISR as a signal with transgenic Arabidopsis NahG plants, which express a bacterial SA-degrading enzyme. Further reports supported the suggestion that SA does not play a role in SAR and that it may not be a cause or inducer for SAR (Silverman et al., 1995; Penninckx et al., 1996; Seah et al., 1996). Chen et al. (1996) proved that SA could induce pathogenesis-related protein (PR-1a) expression in tobacco, but *Bacillus cereus* UW85, which suppresses tobacco seedling damping-off disease caused by *Pythium* spp. did not induce the expression of PR-1a. Press et al. (1997) showed that an ISR^- mutant of PGPR produced the same amount of SA as its parent, while SA^- mutant strains still had as much ISR activity as the wild strain. Moreover, Seah et al. (1996) failed to induce resistance against take-all in wheat with exogenous SA applications. Obviously, SA may not be involved in all cases of systemic resistance and may not act as an exogenous inducer against all plant pathogens.

In general, SA may play a more important role for SAR than for ISR. Based on our knowledge, SA still is an uncertain factor as a signal for SAR or ISR. SA could be produced in infected plants or by PGPR strains, and could induce resistance against *Pythium* root disease on tobacco (Chen et al., 1996). But it is not clear what role SA plays in resistance to cucumber root disease. In this current study, we tested whether exogenous SA could directly suppress mycelial growth or zoospore germination of *P. aphanidermatum*. We also determined whether SA could be produced *in vitro* by the selected strains and whether PGPR bacterization could induce higher levels of endogenous SA in cucumber roots. In addition, we tested whether exogenously applied SA could induce systemic resistance against cucumber root disease caused by *P. aphanidermatum*.

Material and methods

Bacterial strains

Two PGPR strains were selected for testing, including *Pseudomonas aureofaciens* strain 63-28 (from Agrium Inc. Saskatoon, SK, Canada) and *Pseudomonas corrugata* strain 13, one of the best strains from the work of Paulitz et al. (1992). Cell suspensions of each strain were prepared by streaking PGPR strains from nutrient broth + 10% glycerol stored at −80 °C onto tryptic soy agar (TSA) plates and incubating at 25 °C for 36 h. A tube containing 20 ml of tryptic soy broth (TSB) was inoculated with a single colony of each bacterial strain. The TSB tube was incubated on a shaker at 120 rpm at room temperature (22–24 °C) for 48 h. Bacterial cells were centrifuged at 3000*g* for 10 min, the pellets were washed and re-suspended in 0.1 M $MgSO_4$ to a concentration of 10^8 cells ml^{-1} for inoculation.

A bioluminescent reporter strain *Pseudomonas fluorescens* HK44 (pUTK21, from Dr. G. Sayler, University of Tennessee, Knoxville, TN, USA) was employed to monitor salicylic acid production by PGPR strains. The plasmid pUTK21 contains a transcriptional *nahG-luxCDABE* gene fusion between a *luxCDABE* gene cassette from *Vibrio fischeri* and the *nahG* gene of the salicylate operon (Heitzer et al., 1992). This strain can degrade both salicylate and naphthalene and allows naphthalene and salicylate catabolism to be detected by emission of bioluminescent light.

Root pathogen

P. aphanidermatum isolate 186 (provided by Dr. W. Jarvis, Agriculture and Agri-Food Canada, Harrow, Ontario, Canada), which causes cucumber root disease, was used in this study. *P. aphanidermatum* 186

was maintained on sterilized soil amended with 1% rolled oats. Soil was allowed to dry after the fungus had colonized the soil. The stored isolate was plated on *Pythium* selective medium (Mircetich and Kraft, 1973), and after 2 days of incubation at 28 °C it was transferred to a V8 juice agar plate. The pathogen isolate was used for producing zoospores as challenge inoculum on cucumber roots. Inoculum of *P. aphanidermatum* zoospores was prepared as previously reported by Paulitz et al. (1992).

Induced systemic resistance by selected PGPR strains

A split root technique was utilized to prove induced systemic resistance by PGPR with a gnotobiotic system in a greenhouse. The roots of ten-day-old plants (*Cucumis sativus* L. cv. Corona) were split with a razor and transplanted into inverse V-shaped PVC tubes. The tubes were filled with vermiculite (medium grade, Vil Vermiculite Inc., Montreal, Canada) and the ends were immersed in pots containing vermiculite. Cucumber roots grew down into two plastic pots with half in each pot. Two weeks later, one side of the root system was bacterized with either *Pseudomonas corrugata* 13 or *P. aureofaciens* 63-28 or treated with 0.1 M $MgSO_4$ buffer. Fifty milliliters of bacterial suspension (10^8 cells ml^{-1}) were added to the bacterized side. Three days later, the other side of the root system was challenged with zoospores of *P. aphanidermatum* (Edson) Fitzpatrick by pot drenching (approx. 10^5 zoospores per pot). After treatment, each bacterized pot was covered with a plastic bag to prevent pathogen contamination from other pots. The seedlings were fertilized every other day with Peter's 10-52-10 fertilizer (2 g l^{-1}, W.R. Grace, Co. Fogelsville, PA). The humidity in the greenhouse was maintained at around RH 70%; and temperature was adjusted to 25 °C/20 °C day/night.

Disease severity was examined two weeks after *P. aphanidermatum* challenge. Disease index was calculated as follows:

$$\text{Disease index (DI)} = \left[\sum (S_i * N_i)/5T\right] \times 100, \quad (i = 0, 1, 2, 3, 4, 5),$$

where S is the appropriate disease class from 0 to 5, N = number of the diseased plants in the same class, and T = total number of plants rated (Zhou and Paulitz, 1994).

Influence of SA on zoospore behaviour in vitro

Zoospores were generated according to the methods described in an earlier report (Paulitz et al., 1992). A zoospore suspension *P. aphanidermatum* (0.5 ml) was mixed with 0.5 ml SA solution (adjusted to pH 6.5 with 0.1 N NaOH) in a microtube. The final SA concentrations ranged from 0.01 to 1000 µg ml^{-1}. The mixture containing approximately 10^5 spores per ml was incubated at room temperature (22 °C) for five hours. Zoospores were killed with 95% ethanol and stained by adding 50 µl of 0.05% trypan blue in lactophenol to each microtube. Zoospores, cysts, and germinated cysts were observed and counted under a microscope at a magnification of 200×. The experiment was conducted twice with three replications for each treatment.

Influence of SA on mycelial growth

A Petri dish trial was conducted to test the direct influence of SA on the growth of *P. aphanidermatum in vitro*. The *P. aphanidermatum* isolate was grown on V8 juice agar plates for 48 h at 25 °C. Five-mm discs of the culture were removed. One disc was placed on the center of each SA-amended V8 juice agar plate which was adjusted to pH 6.5–7.0 with 0.1 N NaOH. The SA concentrations in the media were from 0 to 3000 µg ml^{-1}. The diameter (mm) of colony on the SA plate was measured from the inoculum disc edge to the growing colony edge 24 h after inoculation. Six replications for each concentration were set up in two separate experiments at concentrations from 0 to 1000 µg ml^{-1} and from 0 to 3000 µg ml^{-1}, respectively.

Influence of exogenous SA on root disease and growth promotion

To detect an influence of SA on cucumber root disease, seven-day-old cucumber seedlings were placed in glass tubes (25 × 90 mm) containing 25 ml plant nutrient solution that consisted of 0.97 g l^{-1} of Peter's Hydro-sol fertilizer (5-11-25) and 0.64 g l^{-1} of $Ca(NO_3)_2$ (Zhou and Paulitz, 1993). Three days later, each plant was injected with a sterile syringe at the base of the stem with 10 µl of SA solution (adjusted to pH 6.5–7.0 with 0.1 N NaOH) at various concentrations of 0, 100, 200, 500, 1000 and 2000 µg ml^{-1}. In another experiment, plants were treated with SA by adding 1 ml of each solution of the above concentrations to the tubes containing the nutrient solution so that the SA

concentrations in the tubes were 0, 4, 8, 20, 40, and 80 µg ml^{-1}. Four days after SA treatment, plants were inoculated with a zoospore suspension of *P. aphanidermatum* (10^4 spores per ml) for both experiments by injecting one ml of the zoospore suspension to the bottom of the tube with a syringe. Disease severity was examined for the next ten days and rated according to the method of Zhou and Paulitz (1994).

To determine if cucumber growth was promoted by exogenous SA, a similar experiment was set up with a hydroponic system. *Pythium* challenge was replaced by a second SA boosting treatment four days after the first one. Plants were harvested two weeks after the first treatment and the data on plant height and fresh root weight were collected. Both experiments were set up on an orbital shaker at 80 rpm to aerate the culture tubes on a growth bench with eight replications for each treatment. The temperature on the bench was 25 °C during the 16 h day, and 20 °C for the 8 h night. Nutrient solution was refilled twice a day. The experiment was repeated.

Detection of SA produced by bacterial strains

A. Bioassay with an indicator strain

An SA-indicating bacterium was employed to rapidly identify whether the test strains produced SA. Bacteria were spotted on tryptone-yeast agar (TYA, Difco, Detroit, Michigan, USA, one litre containing Bacto tryptone, 10.0 g, Bacto yeast, 5.0 g, NaCl, 10.0 g, and agar, 20.0 g, adjusted to pH 7.5 with NaOH) and grown for 48 h at 28 °C. *P. fluorescens* strain HK44(pUTK21), an SA-indicating bacterial strain, was grown for 18 h in Minimal Medium Davis (MMD, Difco), amended with 10 g l^{-1} sucrose. To examine SA production, HK44(pUTK21) cultures were diluted 100-fold in 20 mM phosphate buffer to 10^8 cells ml^{-1} and over sprayed onto TYA plates with colonies of PGPR bacterial strains. Plates were incubated for 24 h at 25 °C. To visualize SA on the plates, 10 µl of n-decyl aldehyde (Sigma, St. Louis, MO, USA) was spotted on the lid of each plate, and bioluminescence was observed in the dark. SA-producing strains were identified by a bright halo surrounding the colony (Press et al., 1997).

B. Detection by HPLC

Each bacterial strain was seeded into a plastic tube containing 20 ml King's B (KB) broth, from a single colony on a TSA plate where bacteria were grown for 24 h at 28 °C. SA was extracted after 48 h from the stationary-phase culture. At that time, bacterial cultures contained 10^{10}–10^{11} cells ml^{-1}. The liquid culture was centrifuged at 2800*g* for 20 min at 4 °C. The supernatant was acidified to pH 2 with 1 N HCl. The solution was filtered through 0.20 µm nylon membrane under a vacuum, partitioned twice with 2 ml $CHCl_3$, and finally dried under a nitrogen stream at 40 °C. Each sample was re-suspended in 1 ml of 23% methanol in 20 mM sodium acetate buffer (pH 5.0) before it was analyzed with HPLC (Meyer et al., 1992; Press et al., 1997).

SA extraction from PGPR-bacterized or induced cucumber root tissue

One gram of fresh roots was homogenized in a 10 × 130 mm culture tube with liquid nitrogen. The ground frozen tissue was rinsed with 2.5 ml of 90% methanol, sonicated for 20 min and centrifuged at 2800*g*. The pellet was extracted again with 2 ml of 100% methanol. The supernatants from the double extraction were dried using a stream of nitrogen. The residue was re-suspended in 2.0 ml 5% trichloroacetic acid (TCA) and centrifuged at 2800*g* for 15 min. The supernatant was partitioned twice with an extraction medium (ethyl acetate : cyclopentane : isopropanol at 100 : 99 : 1). The top phase was combined and dried under a nitrogen stream (Raskin et al., 1989). Residues were re-suspended in 1 ml of 23% methanol in 20 mM sodium acetate buffer, and the solution was passed through 0.20 µm nylon membrane via a vacuum at 250 mm Hg. The sample was stored at −80 °C and prepared for high performance liquid chromatography (HPLC) analysis.

Extracted SA from bacterial culture or root tissues was scanned with a spectrophotometer (Beckman 664) and the spectrum was compared with the commercial standard (Sigma).

HPLC analysis of SA production in split roots

For HPLC analysis of SA extracts from both root tissue and bacterial cultures, a Waters 441 model (Millipore Corporation, Milliford, MA, USA) with UV/VIS detector was used. Separation of the compounds, detected at 280 nm (Manuch-Mani and Slusarenko, 1996; Manihe et al., 1992), was performed with a Bondapak C18 column (125 Å, 10 V m, 3.9 × 300 mm). Column temperature was kept at 40 °C (Meyer et al., 1992) with a mobile phase flow rate of 1 ml min^{-1}. SA was separated isocratically with 23% methanol (v/v)

in 20 mM sodium acetate buffer (pH 5.0) (Yalpani et al., 1991). Fifty µl of each sample was injected into the column, which had a pressure of 2000 mm Hg (2.8 kg cm^{-2}). With this technique and procedure, 40% of the extractable SA was recovered, which was determined by spiking root tissue with standard SA (Sigma). The SA peak was well separated from the others, and the detection limit was equivalent to 14 ng g^{-1} of fresh root weight. Ultra-pure water was used for the dilution and the preparation of the solutions. The experiment was conducted twice with a randomized complete block design and three replicates per treatment.

Results

Pseudomonas strains 13 and 63-28 systemically induced resistance against cucumber root rot caused by *P. aphanidermatum*, confirming previous work (Zhou and Paulitz, 1994; Chen et al., 1998). The disease index on the induced root was significantly lower than that of the split or intact controls (Table 1).

Salicylic acid *in vitro* did not significantly inhibit mycelial growth of *P. aphanidermatum* at concentrations of 100 µg ml^{-1} or less. Growth was inhibited by concentrations of 300–3000 µg ml^{-1} in V8 juice agar plates. At a concentration of 3000 µg ml^{-1}, mycelial growth was completely inhibited (Figure 1).

P. aphanidermatum zoospores were more sensitive to SA than mycelium at low concentrations. Ten to 500 µg ml^{-1} of SA stimulated zoospore germination (Figure 2). At 50 µg ml^{-1}, germination was increased

Table 1. Induced systemic resistance against *Pythium aphanidermatum* by PGPR on split cucumber roots

Treatment[1]	Disease index[2]
Strain 13	30b[3]
Strain 63-28	33b
Split control	53a
Intact control	50a

[1]The roots were split ten days after planting. Two weeks later, one side root was bacterized by PGPR, and three days later, the distant side root was challenged by *P. aphanidermatum*. Disease severity was examined on the challenged half roots two weeks later.

[2]Disease index $= \left[\sum(S_i * N_i)/5T\right] \times 100$, $(i = 0, 1, 2, 3, 4, 5)$, where S is the disease class from 0 to 5, $N =$ number of the diseased plants in the same class, and $T =$ total number of plants rated (Zhou and Paulitz, 1994).

[3]Value shown is a mean of six replicates. Means followed by the same letter indicates no significant difference, based on Fischer's protected LSD test ($P < 0.05$).

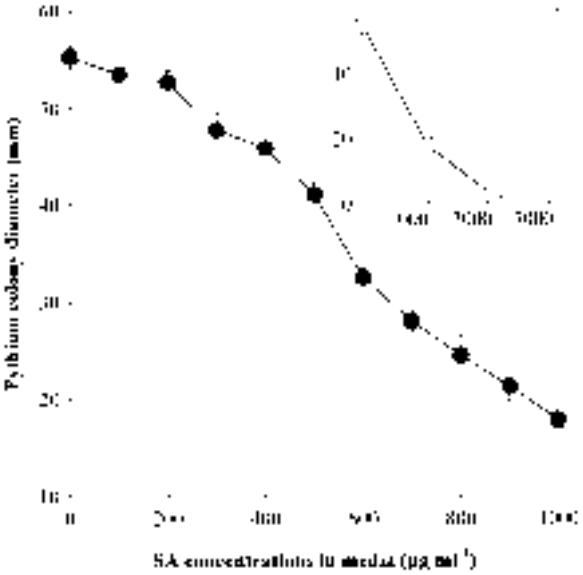

Figure 1. Inhibition of *P. aphanidermatum* mycelial growth by salicylic acid (SA) in a petri dish assay. V8 agar medium containing SA was adjusted to pH 6.5–7.0 with 0.1 N NaOH. Cultured plates were incubated at 25 °C, and colony diameter was measured after 24 h.

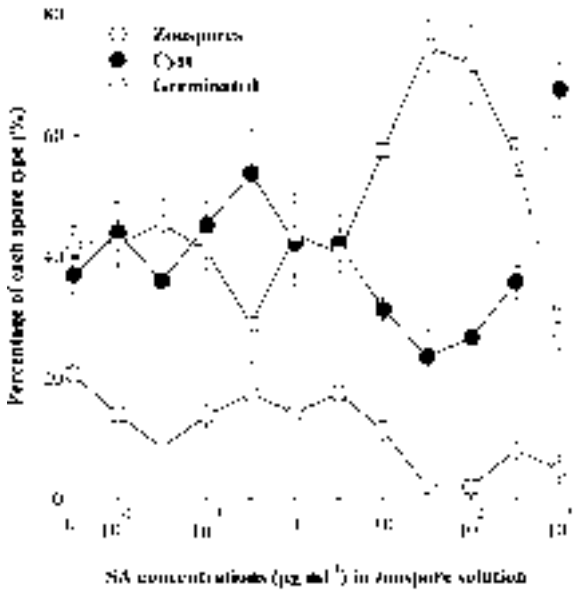

Figure 2. The effect of salicylic acid (SA) on *Pythium* zoospore behavior *in vitro*. Solutions were adjusted to pH 6.5–7.0 with 0.1 N NaOH. Swimming zoospores were incubated in SA solutions at room temperature (22 °C) for 5 h, then the zoospores were killed with 95% ethanol, stained with 0.05% trypan blue, and zoospores were examined under a microscope.

by 70% or more, compared to the water control. Higher concentrations (1000 µg ml^{-1}) reduced zoospore germination. Zoospores were not affected at low SA concentrations (<5 µg ml^{-1}). The results indicated that SA at 5 µg ml^{-1} or less could not directly influence zoospore germination of *P. aphanidermatum in vitro*.

Exogenous SA did not stimulate systemic resistance against *Pythium* cucumber root rot by SA stem-injection (Table 2) or root application (Table 3). Exogenously applied SA could not replace PGPR as an inducer to protect cucumber against root disease. Similarly, SA did not promote cucumber growth in either plant height and root weight (Table 2) as did both PGPR strains in previous studies (Chen, 1999).

Both PGPR strains could produce SA in culture and the indicator bacterial assay and HPLC analyses indicated that strain 13 generated more SA in culture filtrate than strain 63-28 (Table 4). However, both strains equally induced systemic resistance against *P. aphanidermatum in vivo* (Table 1). The indicator bacterial strain HK44(pUTK21) could rapidly distinguish bacteria which produced SA at a high level, but HPLC was more precise in detecting SA production in both bacterial culture and plant extract. Strain HK44(pUTK21) could not be used to quantify SA produced in plant tissue or bacterial cultures. SA extracted from root tissues or bacterial cultures had a similar UV/VIS spectrum to the SA standard (data not shown).

SA levels were significantly greater in root tissues one day after they were bacterized with either of the two PGPR strains than in the untreated controls. SA levels detected in roots bacterized with strain 13 were significantly greater than in roots treated with strain 63-28. SA levels were also significantly greater in distant roots induced with strain 13 than in roots induced with strain 63-28 after one day. This difference lasted until 3 days after bacterization. In general, the SA levels were initially greater in the bacterized side than the distant side. The difference in SA levels between the bacterized side and the distant induced side decreased 2–3 days later, and subsided after 4 days (Figure 3). The repeat experiment showed a similar trend.

Table 2. Influence of exogenous salicylic acid (SA) on root disease severity and growth promotion of cucumber grown in a hydroponic system[1]

SA (µg ml^{-1})	Disease index[2]	Plant height[3] (cm)	Fresh root weight (g)
0	38	11.9	9.26
100	48	12.2	9.68
200	45	13.0	9.09
500	35	13.1	9.38
1000	35	11.7	9.05
2000	33	11.6	8.75
$LSD_{0.05}$	25	1.8	1.61

[1]Ten µl of SA was injected into the stem of each plant. *P. aphanidermatum* zoospores were inoculated onto cucumber root at 10^4 spores per plant four days after SA application. Disease was examined ten days after zoospore inoculation. In the growth promotion experiment, plants were not challenged by *Pythium*. There were eight replications for each concentration.
[2]Disease index was calculated as in Table 1.
[3]Plant height was measured from the crown to the terminal bud.

Table 3. The effect of exogenous SA on root disease severity and growth promotion of cucumber[1]

SA (µg ml^{-1})	Disease index[2]	Plant height[3] (cm)	Fresh root weight (g)
0	40	11.8	8.62
4	35	11.7	9.06
8	45	11.9	9.04
20	35	12.4	9.63
40	48	11.7	9.49
80	43	11.5	9.19
$LSD_{0.05}$	29	1.9	1.60

[1]SA was applied in the nutrient solution. *P. aphanidermatum* zoospores were inoculated to cucumber root at 10^4 spores per plant four days after SA application. Disease was examined ten days after zoospore inoculation. No *Pythium* was inoculated on plants in the growth promotion experiment and there were eight replications for each SA concentration.
[2]Disease index was calculated as in Table 1.
[3]Plant height was measured from the crown to the terminal bud.

Table 4. Salicylic acid (SA) produced by PGPR detected with indicator bacterial strain HK44(pUTK21) and HPLC

PGPR strains	SA production	
	Indicator strain[1]	HPLC (µg ml^{-1})
Strain 13	+	11.3a[2]
Strain 63-28	–	3.0b
Control	–	ND[3]

[1]Positive (+) or negative (–) indicates SA production detected by indicator testing strains.
[2]SA concentration in each bacterial supernatant, means followed by the same letter indicates no significant difference at 5% level.
[3] ND – Not detected.

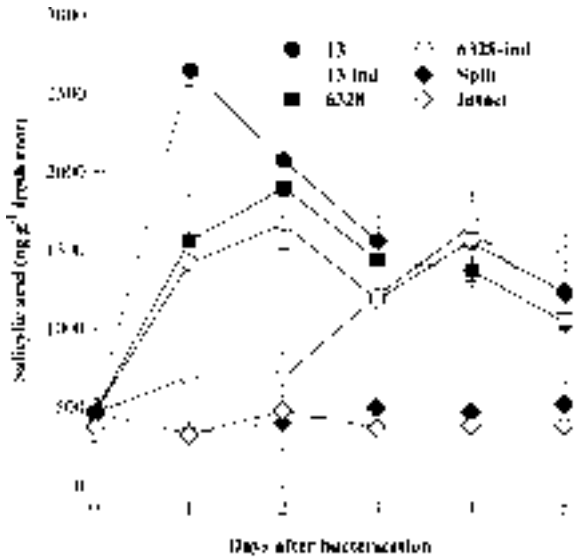

Figure 3. Endogenous levels of salicylic acid (SA) in bacterized and distant induced cucumber roots.

Discussion

Various biotic or abiotic factors can systemically induce resistance in plants. This cascade of events could be triggered by an elicitor which activates a systemic signal. Many organic compounds were reported to be putative triggers or signals in an interaction between plants and inducers. SA has been shown to play a critical role as a systemic signal translocated in the induced tobacco and cucumber plants (Yalpani et al., 1991; Malamy et al., 1990; Métraux et al., 1990). Most previous studies on SA were focused on the interactions between plants and virulent or avirulent pathogens on leaves (Conti et al., 1996; Gaffiney et al., 1993; Yalpani et al., 1991). However, little work has been done on detecting SA from plant root tissues, but it was detected from cucumber and tobacco phloem sap (Rasmussen et al., 1991; Métraux et al., 1990; Yalpani et al., 1991). Recent work with transgenic Arabidopsis plants has indicated that jasmonic acid and ethylene may be important signals in ISR, unlike SAR (Pieterse et al., 1998).

The ability of PGPR to produce SA may not be correlated with its ISR activity. Although the two selected PGPR strains varied in SA production in culture, they induced the same resistance against *Pythium* root rot of cucumber. A recent study showed that SA^- mutant PGPR strains retained the same ISR activity as their parent strain *Serratia marcescens* 90-166 in cucumber against the pathogen, *Colletotrichum orbiculare* (Press et al., 1997).

Our results indicate that the PGPR could stimulate cucumber plants to accumulate SA in their roots. The SA detected in bacterized cucumber root is probably produced by the plant itself, and not of bacterial origin, even though the bacteria could produce it in culture. If SA production in culture (10^{11} cells ml^{-1}) is extrapolated to the root system only about 0.1 ng of SA would be produced per gram of root (10^6 cells g^{-1} root tissue, unpublished data), which is much lower than the levels actually detected in the root. In PGPR-induced roots it is unclear whether the increased amount of SA is locally generated or systemically transferred from bacterized or other tissues, since free SA is translocated in plant phloem (Métraux et al., 1990; Rasmussen et al., 1991). Comparing SA levels in both the bacterized side and distant induced side, SA is initially more concentrated in the bacterized side, but the difference of SA levels in both sides decreased after 2–3 days. This suggests that the PGPR initially stimulated local SA accumulation in bacterized cucumber roots. SA may be translocated from bacterized roots to induced roots later since SA accumulation was detected first in the bacterized side and then in the distant induced side. Similarly, Meuwly and Métraux (1993) demonstrated that the free SA increased 33-fold locally in the infected cucumber leaf, and 4.2-fold systemically in the second leaf compared to controls 5 days after infection by *Pseudomonas lachrymans*. Therefore, we speculated that some SA could be transferred to the induced side from the bacterized side. This transfer may differ from the signal transduction in SAR, because a signal transduction should occur in a shorter time. In a detaching trial, a chemical signal was generated and translocated in induced tissues within 4 h after induction by the hypersensitive response (HR)-inducing bacterium, *Pseudomonas syringae* pv *syringae* in cucumber leaves (Rasmussen et al., 1991).

SA inhibited *Pythium* mycelial growth and zoospore germination only at high concentrations. Usually cucumber phloem contains about 0–0.7 µM of SA (Métraux et al., 1990), even in induced cucumber plants SA was only present at 7 µM (approx. 1 µg ml^{-1}). Therefore, SA produced by the roots or bacteria would not reach a concentration sufficient enough to affect *P. aphanidermatum* mycelial growth or zoospore germination in induced cucumber roots. Although the

exogenous SA injection studies by White (1979) and Rasmussen et al. (1991) showed resistance against further infection by tobacco mosaic virus (TMV) and *Colletotrichum lagenarium* on tobacco and cucumber leaves, respectively, this resistance occurred on the SA-injected leaves. Their studies support the concept that the exogenously applied SA induced a local but not systemic resistance.

A number of conjugated forms of SA have been identified in plants, mainly in the form of β-glucosyl conjugate. In bean leaves, more than 90% of total SA is bound with sugar (Dann et al., 1996). SA is conjugated to form SA-glucoside (SAG) by an SA-inducible glucosyltranferase (SA-GTase). Six hours after SA feeding, the levels of SA-GTase were increased approx. 10-fold in rice seedlings (Silverman et al., 1995). Henning et al. (1993) found that most of the injected SA was bound to form SAG in 2–8 h in tobacco leaves. Since SA-glucoside is inactive as an SAR inducer, it could be hydrolyzed to release free SA to stimulate a plant resistance response. The deconjugation of SAG may play an important role if SA were to be associated with SAR or ISR. Moreover, a metabolite of SA, methyl salicylate, has been found in many plants, but it is not clear if methyl salicylate serves as signal or inducer in SAR or ISR.

The endogenous SA levels increased in bacterized or distant induced cucumber roots. However, to conclusively show that SA is involved in this system, experiments would have to be done with plants engineered with salicylate-degrading bacterial enzymes, such as done by Pieterse et al. (1996) with Arabidopsis. SA may serve as a SAR signal in some plant–pathogen interactions, but not in others. Silverman et al. (1995) showed that SA levels did not increase in rice seedlings after inoculation either with the avirulent pathogen *Pseudomonas syringae* D20 or with the rice pathogens *Magnaporthe grisea* and *Rhizoctonia solani*. They speculated that in rice, unlike tobacco, SA may not play a signaling role in disease resistance. In wheat, exogenous SA applied by foliar dip, foliar wipe, root drench or pre-germination soak failed to induce a resistance response against take-all, a wheat root disease caused by *Gaeumannomyces graminis* var *tritici* (Seah et al., 1996). Similarly, SA may not be a primary signal but it certainly accumulated to a greater amount when roots were bacterized or induced by PGPR. Therefore, SA accumulation may play a role in ISR against cucumber *Pythium* root by PGPR, or it could be coincidental.

SA is a phenolic compound. In a putative SA biosynthetic pathway, phenylalanine is converted to cinnamic acid, then to benzoic acid or coumaric acid, and finally to SA (Lee et al., 1995). The pathway has been proven by labeled ^{14}C-benzoic or ^{14}C-cinnamic acid in higher plants (Ellis and Amrhein, 1971). SA accumulation may be involved in phenylalanine ammonia-lyase (PAL) activity since phenylalanine is a precursor of SA in the pathway. However, biosynthesis of lignin and other phenolic compounds are also associated with phenylalanine production in the upstream part of the pathway (Ward et al., 1991). Previous reports demonstrated that PAL and lignin were involved in plant defense response in SAR (Bruce et al., 1989; Pellegrini et al., 1994; Siegrist et al., 1994; Mauch-Mani and Slusanenko, 1996). Our previous study also indicated that the PAL activity increased in cucumber roots induced by strains 13 and 63-28 (Chen et al., 1997). The increasing PAL activity could result in SA accumulation; coincidentally, this increase could also stimulate lignin biosynthesis and phytoalexin formation in another biosynthetic pathway (Ongena et al., 1999). The two pathways come from the same precursor phenylalanine which is a substrate of PAL. Conti et al. (1996) indicated that cell walls became more lignified in SA pre-treated cucumber leaves infected with the fungal pathogen *Sphaerotheca fuliginea*. The percentage of cells having lignified walls significantly increased from 2–10 days after infection compared to the pre-treated control without SA.

In conclusion, we speculate that PGPR may activate PAL, which leads to increased biosynthesis of SA and lignin or phytoalexins. SA may be indirectly involved in ISR induced by PGPR against cucumber root diseases. SA might be a symptom rather than a cause of the induction of defense reactions. The accumulation of lignin or/and phytoalexins may play a more important role than SA in cucumber systemic resistance against *P. aphanidermatum*. On the other hand, SA may be an important translocated signal involved in ISR, and accumulates in bacterial treated roots. Future research is needed to elucidate the exact role of SA in ISR.

In this study, we demonstrated that although PGPR induce resistance, exogenous SA does not induce resistance to cucumber root disease caused by *P. aphanidermatum*. Exogenously applied SA may be quickly conjugated into a β-glucosyl form, which is not active as an inducer.

Acknowledgements

We thank Mr. Stewart Leibovitch and Mr. Bo Pan for technical advice on HPLC. This work was financed by an operating grant from the Natural Sciences and Engineering Research Council of Canada, the Fonds pour la Formation de Chercheurs et l'Aide à Recherche du Québec, and a grant from the Synergie program of the fonds de Développement Technologique, Ministère de l'Enseignement Supérieur et de la Science du Québec.

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