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Systemic Induction of Salicylic Acid Accumulation in Cucumber after Inoculation with *Pseudomonas syringae* pv *syringae*[1]

Jack B. Rasmussen, Raymond Hammerschmidt*, and Michael N. Zook

Department of Botany and Plant Pathology, Michigan State University, East Lansing, Michigan 48824–1312

ABSTRACT

Inoculation of one true leaf of cucumber (*Cucumis sativus* L.) plants with *Pseudomonas syringae* pathovar *syringae* results in the systemic appearance of salicylic acid in the phloem exudates from petioles above, below, and at the site of inoculation. Analysis of phloem exudates from the petioles of leaves 1 and 2 demonstrated that the earliest increases in salicylic acid occurred 8 hours after inoculation of leaf 1 in leaf 1 and 12 hours after inoculation of leaf 1 in leaf 2. Detaching leaf 1 at intervals after inoculation demonstrated that leaf 1 must remain attached for only 4 hours after inoculation to result in the systemic accumulation of salicylic acid. Because the levels of salicylic acid in phloem exudates from leaf 1 did not increase to detectable levels until at least 8 hours after inoculation with *P. s.* pathovar *syringae*, the induction of increased levels of salicylic acid throughout the plant are presumably the result of another chemical signal generated from leaf 1 within 4 hours after inoculation. Injection of salicylic acid into tissues at concentrations found in the exudates induced resistance to disease and increased peroxidase activity. Our results support a role for salicylic acid as an endogenous inducer of resistance, but our data also suggest that salicylic acid is not the primary systemic signal of induced resistance in cucumber.

Inoculation of one leaf of cucumber plants and other cucurbits with necrotic lesion-inducing pathogens (7–9) or necrosis/chlorosis-inducing chemicals (3, 4) results in the expression of systemic resistance against disease caused by a number of pathogens. The onset of resistance has been correlated with the initial appearance of necrotic lesions and generally begins to develop 3 to 4 d after the resistance-inducing inoculation (7–9).

We have recently demonstrated that systemic resistance can be induced in cucumber within 24 h by inoculating leaf 1 with the HR[2]-inducing bacterium *Pseudomonas syringae* pv *syringae* (17). This work further demonstrated the correlation between the onset of resistance and the systemic appearance of acidic, apoplastic peroxidase isoforms. Detaching the first true leaf at intervals after inoculation with *P. s.* pv *syringae* demonstrated that this leaf must remain attached for only 6 h to result in the systemic expression of enhanced peroxidase activity and a small, but detectable, increase in the level of systemic disease resistance. Allowing the first leaf to remain on the plant for up to 12 h after inoculation with *P. s.* pv *syringae* resulted in a further increase in the level of systemic resistance as compared with plants that had the inoculated first leaf detached 6 h after inoculation. Tn*5* mutants of *P. s.* pv *syringae* that had lost the ability to induce the HR were also unable to induce systemic resistance and peroxidase activity.

Dean and Kuć (1, 2) have provided strong evidence that the systemic signal(s) for induced resistance was generated in and mobilized out of the leaves that were initially inoculated ("source" leaves) with resistance-inducing pathogens. Metraux *et al.* (12) recently reported that cucumber plants inoculated with either *Colletotrichum lagenarium* or tobacco necrosis virus on one leaf had higher levels of salicylic acid (an exogenous inducer of resistance in cucumber [13] and tobacco [18, 19]) in phloem exudates just prior to the expression of induced resistance. We further examined the possible involvement of salicylic acid in the induction of systemic resistance in cucumber with *P. s.* pv *syringae* as the inducing agent. Because inoculation with *P. s.* pv *syringae* induces resistance in cucumber more rapidly than *C. lagenarium* or tobacco necrosis virus, it is possible that the time course and production of systemic signals can be monitored more precisely after inoculation with this bacterium.

MATERIALS AND METHODS

Growth of Plants and Pathogens

Established protocols were used to grow cucumber (*Cucumis sativus* L.) cv SMR 58 (9), *Pseudomonas syringae* pv *syringae* D20 (wild type) (17), *C. lagenarium* (9), and *P. s.* pv *syringae* D21, a Tn*5* mutant of strain D20 unable to induce systemic resistance (17).

Inoculations

Unless specified otherwise, cucumber plants with two true leaves were inoculated by injection into one leaf with cells of *P. s.* pv *syringae* strain D20 at 15 sites per leaf as described (17). Controls were either left untreated or injected with water or buffer. The level of inoculum used was 1×10^8 cfu/mL unless stated otherwise.

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[2] Abbreviations: HR, hypersensitive response; pv, pathovar; TMS, trimethylsilyl; cfu, colony-forming unit.

Extraction and Assay of Peroxidase

Total soluble and apoplastic peroxidase activity and in-gel peroxidase activity after native PAGE were determined as previously described (16). The increase in peroxidase was used as a marker for the initial phase of systemic resistance induction (17).

Collection of Phloem Exudates

Phloem exudates were collected from the cut ends of petioles (14) with 50 μL capillary pipettes. Known volumes of exudate were placed into three volumes of ethanol to precipitate the proteins and other high mol wt materials. After removal of the insoluble materials by centrifugation (10,000*g*, 5 min), the pellets were reextracted with ethanol. The combined ethanol extracts were concentrated to dryness under vacuum and redissolved in 50% ethanol to a volume equal to that of the collected exudate.

Analysis of Salicylic Acid

The ethanol extracts were spotted onto silica gel 60 A chromatography plates (Whatman) and developed in toluene:dioxane:acetic acid (90:25:4, v/v) for routine analysis. Typically, a 50-μL equivalent of phloem exudate was analyzed. Salicylic acid was visualized on the plate by viewing under UV light (302 nm). The fluorescent band corresponding to salicylic acid was eluted from the silica gel with 1 mL of 95% ethanol and used for quantitation or other analyses. Quantitative analysis was performed with an SLM 4800 fluorometer (SLM Instruments, Inc.) upgraded with Olis (Jefferson, GA) electronics and software (excitation wavelength = 310 nm, emission wavelength = 400 nm). Each data point reported is the average of three replicate samples (three plants/sample) from one representative experiment. Each experiment was performed at least three times. The value of each replicate is the average of 20 fluorescent readings taken over 20 s. The limit of detection of salicylic acid in a final volume of 1 mL was 0.2 nmol.

To determine if the exudates contained conjugates of salicylic acid, 200 μL of the ethanol-soluble material from phloem exudates was adjusted to approximately pH 3 and extracted with diethyl ether to remove free salicylic acid. The remaining aqueous phase was then adjusted to 0.1 M HCl and then heated at 60°C in a sealed tube for 1 h. After cooling, the hydrolysate was extracted with diethyl ether (v/v, three times). The ether extracts were pooled, the ether was evaporated under a stream of N_2, and the residue was dissolved in ethanol prior to quantitative analysis as described above.

UV spectra were recorded with a Zeiss PM6 Spektralphotometer. For gas chromatography/mass spectral analysis, the unknown and authentic salicylic acid were derivatized with Regisil (Regis) to form the TMS derivative. GC-MS was carried out with a Shimidzu GC-9A gas chromatograph connected to an LKB 2091 single focusing mass spectrometer. Gas chromatographic separation was carried out on an HP ULTRA II capillary (0.57 mm internal diameter, megabore) with temperature programmed from 50 to 320°C (10°C/min). C_{10} to C_{22} hydrocarbons were included as internal standards. Mass spectra were recorded between m/z 45 and 750 with an accelerating voltage of 3.5 kV.

Bioassay

The ability of exogenously applied salicylic acid to induce resistance and peroxidase activity was tested by infiltrating cotyledons of 6- to 10-d-old cucumber seedlings with salicylic acid in water (pH adjusted to 6.0). Cotyledons were infiltrated using a syringe with a 25 gauge needle. Buffer was not used because some buffer ingredients (*e.g.* phosphates) are known to induce resistance in cucumber (4). Controls were infiltrated with water. The cotyledons were assayed for resistance by inoculation with *C. lagenarium* (eight 5-μL droplets of a 1×10^5 spores/mL suspension 24 h after infiltration with salicylate). The total number of necrotic lesions and the diameter of each lesion were determined as measures of induced resistance. The diameter of each lesion was used to calculate the total necrotic lesion area. Penetration into the tissue was measured microscopically as described (15). Peroxidase was measured as described above.

RESULTS

Identification of Salicylic Acid in Phloem Exudates

Inoculating leaf 1 of cucumber with *P. s.* pv *syringae* D20 resulted in the expression of enhanced systemic peroxidase activity within 20 h (Fig. 1). This suggested that increases in inducing chemicals must have appeared before 20 h. Examination of phloem exudates at 16 to 18 h after inoculation with *P. s.* pv *syringae* demonstrated the presence of a blue fluorescent compound that had R_F and UV spectral properties identical with those of salicylic acid. This compound was not detected in plants injected with water or buffer. TMS derivatives of the blue fluorescent compound and salicylic acid had the same relative retention time by GC analysis. The mass spectra of the two compounds were also identical with a small molecular ion (M^+) at m/z 282, a small ion at m/z 281 ($M^+ - 1$), and prominent ions at m/z 267 ($M^+ - CH_3$), 209 (M^+ − TMS), 193 (M^+ − TMSO), 149, 135, and 91. Culture

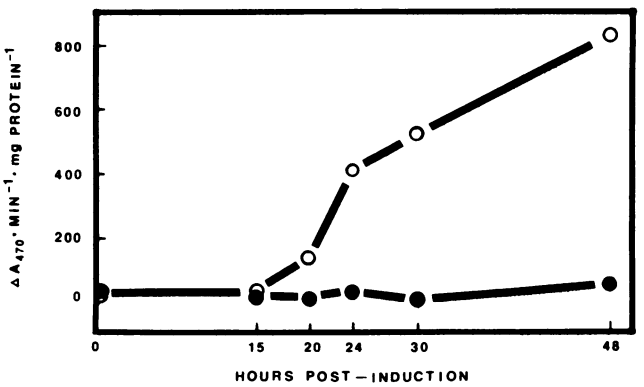

Figure 1. Systemic induction of apoplastic peroxidase in the second leaf of cucumber following inoculation of leaf 1 with *P. s.* pv *syringae* strain D20. (○ = *P. s.* pv *syringae*-inoculated plants; ● = control plants.)

Table I. *Time Course of Salicylic Acid Accumulation in Phloem Exudates of Petioles of Leaves 1 and 2 after Inoculation[a] of Leaf 1 with P. s. pv syringae Strain D20*

Time after Inoculation	Concentration of Salicylic Acid	
	Leaf 1 petiole	Leaf 2 petiole
	μM ± SE	
0	ND[b]	ND
6	ND	ND
12	28.1 ± 11.2	4.4[c]
18	313.4 ± 48.1	212.8 ± 9.3
24	107.3 ± 20.6	68.1 ± 11.8

[a] Plants inoculated with 3×10^7 cfu/mL of *P. s.* pv *syringae*. [b] Not detectable. [c] Only one sample had detectable amounts of salicylic acid.

filtrates of *P. s.* pv *syringae* did not contain detectable levels of salicylic acid (data not shown).

Time Course of Salicylic Acid Appearance

The first leaf of cucumber plants was inoculated with *P. s.* pv *syringae,* and samples of petiole phloem exudate were collected at 0, 6, 12, 18, and 24 h after inoculation. Increased quantities of salicylic acid were detected in the exudates of the phloem of both leaf 1 and 2 at 12 h after inoculation (Table I). Phloem exudates from leaf 1 petiole contained about six times more salicylic acid than leaf 2 at this time. In another experiment (Table II), enhanced salicylic acid was detected in the phloem exudates of the petiole of leaf 1 as early as 8 h. Salicylic acid was not detected in plants at 48 h or more postinoculation (data not shown).

Effect of Detaching Leaf 1 at Intervals after Inoculation

Leaf 1 was detached at 2, 4, 6, 8, and 24 h after inoculation with *P. s.* pv *syringae* to more precisely determine the time of appearance of salicylic acid (Table II). Salicylic acid was not detected in the phloem exudates of leaf 1 at 2, 4, or 6 h after inoculation (even when up to the equivalent of 250 μL of exudate was analyzed). Increased quantities of salicylic acid were detected in phloem exudates of leaf 2 at 24 h after inoculation with *P. s.* pv *syringae* in all plants except those that had leaf 1 detached at 2 h after inoculation. Hydrolysis of the ethanol-soluble fraction of the phloem exudates collected from leaf 1 at 4 to 5 h after inoculation indicated that there was no increase in any conjugates of salicylic acid at this time (data not shown). Analysis of the acidic peroxidase isozymes that are correlated with the induction of systemic resistance and total peroxidase activity of leaf 2 at 24 h after inoculation with *P. s.* pv *syringae* revealed that peroxidase levels were increased in the plants that had leaf 1 on the plant for 6 h or longer (Fig. 2). No increase in peroxidase was found in plants that had leaf 1 detached at 4 h, even though there was an increase in salicylic acid.

Table II. *Effect of Detaching Leaf 1 at Intervals after Inoculation[a] with P. s. pv syringae Strain D20 on Appearance of Salicylic Acid in Petiole of Leaf 2*

Time Leaf 1 Detached	Salicylic Acid in Leaf 1 Petiole Phloem Exudate at Time Leaf 1 Detached	Salicylic Acid in Leaf 2 Petiole Phloem Exudate 24 h after Inoculation of Leaf 1
h	*μM ± SE*	
2	ND[b]	ND
4	ND	71.4 ± 20.7
6	ND	235.8 ± 6.3
8	30.5 ± 2.2	350.2 ± 19.9
24	—[c]	257.2 ± 3.3

[a] Plants inoculated with 1×10^8 cfu/mL of *P. s.* pv *syringae*. [b] Not detectable (below limit of detection). [c] Not determined.

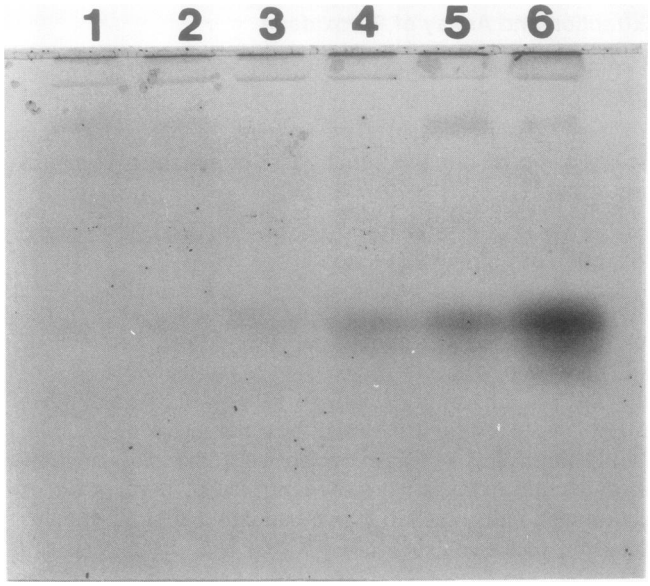

Figure 2. Effects of detaching leaf 1 at interval after inoculation with *P. s.* pv *syringae* on systemic induction of acid peroxidase in leaf 2. The time of detachment of leaf 1 (h after inoculation) followed by the total peroxidase activity (Δ A 470/min·mg protein) in leaf 2 30 h after inoculation is given for each lane. Lane 1: (control, uninoculated), 0.906 ± 0.126; lane 2: 2 h, 0.906 ± 0.167; lane 3: 4 h, 0.901 ± 0.085; lane 4: 6 h, 2.43 ± 0.54; lane 5: 8 h, 8.57 ± 1.79; lane 6: (no detachment), 13.8 ± 4.73. Data for total peroxidase activity are presented as the average of five determinations ± SD.

Effect of Inoculation with the Tn5 Mutant *P. s.* pv *syringae* D21 on Salicylic Acid Accumulation

Inoculation with strain D21 of *P. s.* pv *syringae,* a Tn*5* mutant unable to induce systemic resistance or peroxidase activity (17), did not result in the systemic appearance of detectable quantities of salicylic acid in the phloem exudates of the petiole of leaf 2 at 24 h after inoculation. Inoculation of a separate set of plants with the wild-type strain of *P. s.* pv *syringae* (D20) at the same time, however, did result in the systemic appearance of salicylic acid (196.3 ± 11.1 μM).

Table III. *Effect of Detaching Leaf 1 at 5 h after Inoculation with P. s. pv syringae Strain D20 on Systemic Appearance of Salicylic Acid*

Treatment[a]	Petiole[b]	Salicylic Acid Concentration in Petiole Phloem Exudate at 24 h after Inoculation of Leaf 1
		μM ± SE
Leaf 1 detached[c]	Leaf 2	346.5 ± 87.1
	Leaf 4	346.6 ± 23.1
Leaf 1 on plant	Leaf 2	568.2 ± 98.4
	Leaf 4	240.4 ± 11.0

[a] Plants inoculated on leaf 1 with 2×10^8 cfu of *P. s.* pv *syringae*. [b] Phloem exudate from the petioles of leaves 2 and 4 were sampled at 24 h after inoculation of leaf 1. [c] No salicylic acid detected in phloem exudate of leaf 1 at time of detachment (based on analysis of 200 μL of phloem exudate).

Systemic Accumulation of Salicylic Acid

Plants with four true leaves were inoculated on leaf 1 with *P. s.* pv *syringae*. Leaf 1 was removed from one-half of each set of plants 5 h after inoculation. No detectable amounts of salicylic acid were found in the petiole phloem exudates of leaf 1 collected at this time. All plants were assayed 24 h after inoculation for salicylic acid levels in the phloem exudates of leaves 2 and 4. Both sets of inoculated plants contained higher quantities of salicylic acid in the petiole phloem exudates of both leaves 2 and 4 (Table III). Uninoculated plants did not contain detectable levels of salicylic acid.

Direction of Salicylic Acid Induction

Because the resistance-inducing signal is known to move both down and up the plant, we inoculated cucumber plants with three leaves on leaf 2 with *P. s.* pv *syringae*. After inoculation with *P. s.* pv *syringae,* the concentrations of salicylic acid in the phloem exudates of the petioles of leaves 1, 2, and 3 were determined. Elevated concentrations of salicylic acid were found in petiole exudates of all three leaves. (Leaf 1 = 151.8 ± 17.4 μM; leaf 2 = 138.8 ± 13.2 μM; leaf 3 = 107.1 ± 17.5 μM. Eight plants were used in this experiment. Exudates were collected 30 h after inoculation. Data are from a representative experiment.)

Effect of Exogenous Application of Salicylic Acid on Resistance and Peroxidase Induction

Several concentrations of salicylic acid that were at or higher than the maximum concentration measured in phloem exudates were used to test the resistance- and peroxidase-inducing activities of salicylic acid. Young (10-d-old, approximately 3 cm long) cotyledons were used. Exogenously applied salicylic acid induced an increase in peroxidase activity within 24 h over the water-injected control (Table IV). A small increase in resistance, as measured by total necrotic area and by the penetration of *C. lagenarium* into leaf tissue, was observed when the infiltrated cotyledons were challenged 24 h after application of 250 μM or higher concentrations of salicylic acid (Table IV). Salicylic acid did not have a visible effect on spore germination or appressorium formation on the surface of the inoculated cotyledons. Concentrations of 10 mM or higher were toxic to the plant tissues.

DISCUSSION

Research by Dean and Kuć (1, 2) has previously demonstrated that the source of the translocated signal for systemic induced resistance in cucumber is the inoculated leaf. They clearly demonstrated that removing this leaf prior to the onset of lesion formation would effectively block the systemic induction of resistance. Other work by Kuć's group has demonstrated that the systemic signal is most likely translocated in the phloem (5, 6). We have recently demonstrated that systemic resistance can be induced in cucumber plants within 24 h after inoculation of leaf 1 with *P. s.* pv *syringae,* which causes an HR (17). Because we know that the signal for induced resistance is generated by 6 h after inoculation with *P. s.* pv *syringae* (17), we reasoned that salicylic acid might also be mobilized out of the inoculated source leaf during this time. However, despite the fact that detaching leaf 1 only 4 h after inoculation with *P. s.* pv *syringae* resulted in the induc-

Table IV. *Effect of Exogenous Salicylic Acid on Induction of Resistance*

Concentration of Salicylic Acid Injected into Cotyledons	Average Number of Lesions per Cotyledon[a]	Average Necrotic Lesion Area per Cotyledon[a]	Penetration from Appression[b]	Total Peroxidase Activity[c]
μM		mm^2	%	
0	7.5 ± 0.3	49.0 ± 6.2	29.3 ± 2.4	2.34 ± 0.07
250	7.6 ± 0.2	16.8 ± 3.7	17.3 ± 2.0	8.31 ± 3.80
500	4.5 ± 0.7	9.1 ± 2.2	14.3 ± 2.2	8.32 ± 1.09
1000	3.4 ± 0.5	2.1 ± 0.5	5.6 ± 1.0	11.3 ± 0.65

[a] Measured 96 h after inoculations with *C. lagenarium*. Cotyledons inoculated at eight sites 24 h after injection with water or salicylic acid. Eight cotyledons per treatment. Data from one representative experiment. Values are means ± SE. [b] Measured 66 h after inoculation. Five cotyledons used per treatment. Data from one representative experiment. Values are means ± SE. [c] Measured 24 h after injection of cotyledons with salicylic acid or water. Activity expressed as Δ A 470/min·mg protein. Values are means of two samples ± the range.

tion of a systemic increase in the concentration of salicylic acid (Table II), we did not observe detectable amounts of salicylic acid in the exudates from leaf 1 until 8 h after inoculation. Previous experiments have demonstrated that a statistically significant increase in resistance is obtained if leaf 1 is allowed to remain attached for 6 h after inoculation with *P. s.* pv *syringae* (17). If salicylic acid is the primary systemically translocated signal of induced resistance, detectable amounts should begin to appear in leaf 1 within this 6-h period. Thus, the systemically accumulating salicylic acid does not appear to originate from the inoculated leaf, but rather appears to be induced by yet another putative systemically translocated signal. Based on the detached leaf experiments, the translocated signal that elicits salicylic acid accumulation is generated by 4 h after inoculation. The concentration of salicylic acid that is induced within 4 h, however, is apparently not sufficient to induce the resistance-associated peroxidases. This is consistent with our previous report (17), which demonstrated that leaf 1 needed to remain on the plant for at least 6 h for subsequent systemic induction of the peroxidase isoforms and a small increase in resistance. Although our data suggest that salicylic acid is not the primary signal for induced resistance in cucumber, salicylic acid may still be involved in the cascade of events that begins with the induction of HR and culminates with the manifestation of induced systemic resistance.

The results of this paper confirm the results presented by Metraux *et al.* (12) and Malamy *et al.* (10), which demonstrated that inoculation of cucumber and tobacco, respectively, with resistance-inducing pathogens results in a systemic increase in salicylic acid just prior to the expression of induced resistance. Similarly, we detected salicylic acid in phloem exudates by 12 h after inoculation, which was several hours prior to the induction of increased peroxidase activity and resistance. The detached leaf experiments also provide evidence for the association of increased concentrations of salicylic acid and increased resistance and peroxidase activity. The presence of salicylic acid in phloem exudate and the high concentrations of salicylic acid in exudates collected both above and below the point of inoculation with *P. s.* pv *syringae* also are consistent with the observations that the signal for induced resistance is probably translocated in the phloem.

We have found much higher concentrations of salicylic acid in phloem exudates than was reported by Metraux *et al.* (12). This is most likely due to the fact that *P. s.* pv *syringae* induces expression of a near maximum level of resistance in leaf 2 by 24 h after inoculation, whereas induction by *C. lagenarium* or tobacco necrosis virus takes 3 to 5 d. Higher concentrations of salicylic acid may be needed to induce resistance in a shorter period of time. In addition, the bacterial HR, unlike disease, does not continue to develop. This could explain why the amount of salicylic acid declines by 24 h after inoculation with *P. s.* pv *syringae,* while symptoms induced by *C. lagenarium* or the slowly developing tobacco necrosis virus lesions cause a more sustained but lower amounts of salicylic acid (12).

Inoculation of cucumber plants with high concentrations of bacterial cells ($>10^8$ cfu/mL) frequently results in the appearance of veinal chlorosis and stunting of leaves that are less that one-third fully expanded at the time of inoculation. This symptom does not appear in the inoculated leaf, in the fully expanded leaves above or below the inoculated leaf, or in leaves that develop after the time of inoculation. However, similar to the induction of the systemic accumulation of salicylic acid, the chlorosis and stunting can be seen in plants in which the inoculated leaf 1 was detached 4 h after inoculation. This symptom is possibly due to the high concentration of salicylic acid in the phloem tissues of the expanding leaves. Salicylic acid is known to have toxic effects on plant tissue.

Injection of salicylic acid at 250 μM or higher concentrations induced resistance to challenge by *C. lagenarium* if the challenge and injection times were separated by 24 h. The decrease in lesion size and penetration efficiency observed in the salicylic acid-treated cotyledons is consistent with the expression of resistance induced by *C. lagenarium* (15). Acidic peroxidases were also enhanced. The induction of peroxidase by salicylic acid is similar to observations of Metraux *et al.* (11), who found that salicylic acid also induces the message for chitinase, an enzyme that has also been closely associated with the systemic expression of induced resistance in cucumber. We also found that salicylic acid induction of resistance was most pronounced in young cotyledons (prior to first leaf expansion). Older cotyledons (those in which the plants had one or more true leaves) responded slowly to salicylic acid (data not shown). This is consistent with the observations of Mills and Wood (13), who found that salicylic acid induced resistance very slowly in cotyledons of cucumber plants with true leaves, and with the observations that younger leaves are more strongly induced by pathogen inoculation than are older leaves (5).

Although our results indicate that salicylic acid is not the translocated signal (*i.e.* a systemically translocated factor that is generated in the inoculated leaf used in the induction of resistance), our results are consistent with the hypothesis that salicylic acid is an important factor in the expression of induced resistance (10, 12). This hypothesis is supported by our observation that Tn*5* mutants of *P. s.* pv *syringae,* which do not induce resistance, also do not elicit the systemic accumulation of salicylic acid. We hypothesize that a primary signal is generated by the interaction of a leaf with a resistance-inducing pathogen. This signal, in turn, is translocated throughout the plant, where it elicits the production of salicylic acid that, alone or in combination with other signals, induces resistance. Work is now being directed at the events that precede the appearance of salicylic acid.

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