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Cytological observations of cucumber plants during induced resistance elicited by rhizobacteria

Y.C. Jeun,[a] K.S. Park,[a] C.H. Kim,[b] W.D. Fowler,[c] and J.W. Kloepper[c,*]

[a] *Department of Plant Resource Science, Cheju National University, Juju 690-756, Republic of Korea*
[b] *Plant Pathology Division, National Institute of Agricultural Science and Technology, Suwon 441-707, Republic of Korea*
[c] *Department of Entomology and Plant Pathology, Biological Control Institute, 209 Life Sciences Bldg., Auburn, AL 36949-5409, USA*

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Abstract

Expression of induced resistance was cytologically compared between cucumber plants induced with either plant growth-promoting rhizobacteria (PGPR) or chemicals. Inoculation with PGPR strains *Serratia marcescens* (90–166) and *Pseudomonas fluorescens* (89B61) induced systemic protection in the aerial part of cucumber plants against the anthracnose pathogen *Colletotrichum orbiculare*. Disease development was significantly reduced in these plants compared to control plants that were not inoculated with the PGPR strains. Inoculation with the PGPR strains caused no visible toxicity, necrosis, or other morphological changes. Induction with DL-3-aminobutyric acid (BABA) or amino salicylic acid (ASA) also significantly reduced disease development. Soil drench with 10 mM BABA and 1.0 mM ASA-induced resistance in cucumber leaves without any toxicity to the plants. Higher concentrations of ASA (up to 10 mM) were phytotoxic, resulting in plant stunting and blighted appearance of leaves. Cytological studies using fluorescent microscopy revealed a higher frequency of autofluorescent epidermal cells, which are related to accumulation of phenolic compounds, at the sites of fungal penetration in plants induced with PGPR and challenged by the pathogen. Neither spore-germination rate nor formation of appressoria was affected by PGPR treatments. In contrast, both BABA and ASA significantly reduced spore-germination rate and appressoria formation, while there were no differences from controls in the frequency of autofluorescent epidermal cells at the sites of fungal penetration. Our findings suggest that PGPR and chemical inducers cause different plant responses during induced resistance.
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1. Introduction

Induced resistance is expressed as enhancement of plant defense responses activated by exogenous stimuli (Ryals et al., 1992; Sticher et al., 1997). Over the past decade, research has focused on elucidating the mechanisms of induced systemic resistance such as physiological changes in induced plants, i.e., the signal pathway.

Although the precise mechanisms of induced resistance are not yet clearly understood, some signal pathways have been reported. Published reports indicate that systemically induced resistance can be elicited and expressed in various ways (Kessmann et al., 1994; Pieterse and Van Loon, 1999; Sticher et al., 1997; Van Loon et al., 1998). Elicitation of induced resistance by pathogens is termed systemic acquired resistance (SAR) (Ross, 1961; Sticher et al., 1997). Typically, SAR is associated with the hypersensitive reaction (HR) (Siegrist et al., 2000) in which limited necrosis occurs on the treated part of the plant (Cameron et al., 1994; Van Loon, 1997). SAR also results in accumulation of pathogenesis-related proteins (PR proteins) (Jeun, 2000; Niderman et al., 1995; Woloshuk et al., 1991) and autofluorescence related to the accumulation of phenolic compounds (Hunt et al., 1997). In most cases, signaling of SAR is dependent on the accumulation of salicylic acid (SA) (Malamy et al., 1990; Métraux et al., 1990).

* Corresponding author. Fax: 1-334-844-5067.
E-mail address: jkloeppe@acesag.auburn.edu (J.W. Kloepper).

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For example, SAR is not expressed in transgenic tobacco plants carrying the NahG gene that breaks down SA by catalyzing it to catechol (Gaffney et al., 1993).

SAR can also be induced with chemicals such as DL-3-amino butyric acid (BABA), benzo-(1,2,3)-thiadiazole-7-carbothioic acid *S*-methyl ester (BTH), or 2,6-dichloro-isonicotinic acid (INA) (Kessmann et al., 1994). Cohen (1994a,b) showed that BABA-induced SAR against both *Phytophthora infestans* (Mont.) deBary on tomato and *Peronospora tabacina* D.B. Adam on tobacco. In BABA-treated tomato plants, PR proteins accumulated before challenge-inoculation with *P. infestans* (Cohen et al., 1994; Jeun, 2000). The signal transduction pathway in SAR induced by BABA and tobacco mosaic virus (TMV) in cucumber plants was shown to be dependent on SA (Siegrist et al., 2000). In *Arabidopsis thaliana* (L.) Heynh., SAR induced by BABA against *Peronospora parasitica* (Pers.:Fr.) Fr. did not result in accumulation of SA or in activation of genes encoding PR proteins (Zimmerli et al., 2000).

Another form of resistance, termed induced systemic resistance (ISR), has been reported (Pieterse et al., 1996; Van Loon et al., 1998). ISR is mostly induced by plant growth-promoting rhizobacteria (PGPR). Application of PGPR strains *Serratia marcescens* Bizio (90–166) and *Pseudomonas fluorescens* Migula 89B61 has been shown to reduce the severity of cucumber anthracnose, angular leaf spot, and cucurbit wilt diseases (Kloepper et al., 1996; Liu et al., 1995a,b,c; Press et al., 1997; Raupach et al., 1996; Wei et al., 1996; Zehnder et al., 1997a,b).

ISR is distinguished from SAR by a different signal pathway (Knoester et al., 1999; Pieterse et al., 1996; Press et al., 1997; Van Wees et al., 1997). In contrast to SAR, the signal pathway of ISR is usually independent of SA accumulation (Van Loon et al., 1998). In *Arabidopsis* plants that are insensitive to ethylene or jasmonic acid, ISR was not triggered after induction by PGPR (Pieterse et al., 1998). This finding indicates that ethylene and jasmonic acid have a role in the signal pathway of ISR. Typically, PR proteins are not induced during ISR as they are during SAR (Hoffland et al., 1995; Pieterse et al., 1996; Van Wees et al., 1997). However, some PGPR strains did induce the PR-la gene in tobacco (Park and Kloepper, 2000). Moreover, some PGPR strains have antifungal activity while chemical inducers of SAR do not (Van Loon et al., 1998). Other than comparing the expression of PR proteins and dependence or independence of SA, jasmonic acid, and ethylene, few studies have systematically compared ISR and SAR based on plant defense reactions. A comparison of biochemical and cytological characteristics of plants expressing SAR and ISR would add useful information regarding the similarities and differences between the two.

In the present study, we compared aspects of induced resistance against cucumber anthracnose elicited by PGPR strains *S. marcescens* 90–166 and *P. fluorescens* 89B61 to SAR induced by BABA and amino salicylic acid (ASA). Infection structures of the pathogen and defense responses of the plants were cytologically examined on leaf surfaces of cucumber plants induced with the two PGPR strains and the two chemicals.

2. Materials and methods

2.1. Plant and pathogen

Cucumber seeds (*Cucumis sativus* L. cv. Eunsung) were sown in 10-cm-diameter plastic pots filled with a commercial soilless mix (TKS 2, Floragard, Oldenburg, Germany) containing 10% perlite. Cucumber seedlings were grown in the greenhouse at 28 °C during the day and 25 °C at night. Plants were watered daily and fertilized weekly with 1% Wuxal Super (12:4:6; Aglukon, Duesseldorf, Germany).

Colletotrichum orbiculare (Berk. & Mont.) Arx, which causes cucumber anthracnose, was grown on green-bean agar medium (Goode, 1958) for 5 days. After incubation at 28 °C, 10 ml distilled water was poured onto the fungal mycelia, and conidia were harvested using a small brush. The conidial concentration was adjusted to 2.5×10^5 conidia per ml. One liter of conidial suspension was mixed with 100 μl Silwet L-77 (Loveland Industries, Greeley, CO), which enhances the penetration of conidia into the leaf and serves as inoculum for challenge-inoculations on cucumber leaves.

2.2. PGPR strains and chemicals

PGPR strains *S. marcescens* (90–166) and *P. fluorescens* (89B61) were selected as inducers for this study because they had previously induced resistance to cucumber pathogens (Liu et al., 1995a,b,c; Raupach et al., 1996; Wei et al., 1996). The bacterial strains were grown on tryptic soy agar and incubated at 28 °C for 24 h. Ten milliliters of distilled water was poured onto the surface of the medium and the bacterial cells were scraped from the plates. The concentration of each PGPR strain was adjusted to 1×10^8 colony forming units (cfu) per ml. Cucumber plants at the second-leaf stage were induced with PGPR by adding 30 ml of bacterial suspension to the soil of each pot. Noninduced controls received drenches of water without bacterial cells. Plants were challenge-inoculated with *C. orbiculare*, and disease was assessed as described below. The experiment was designed as a randomized complete block and was conducted three times. Significant treatment effects were identical among the three experimental trials, and representative data from one trial are shown in the results section.

In a separate experiment, BABA and ASA were tested at 1.0 and 10.0 mM concentrations. Thirty milliliters

of each chemical solution were applied as soil drenches to each plant 3 days before challenge-inoculation with *C. orbiculare*. Controls received drenches of distilled water after challenge. Plants were observed daily for signs of phytotoxicity. This experiment was also conducted three times, and representative data from one of the trials are shown in the results.

2.3. Challenge-inoculation and disease assessment

The conidial suspension of *C. orbiculare* (2.5×10^5 conidia per ml) was sprayed on cucumber leaves 3 days after treatment with the chemicals and 5 days after treatment with PGPR. Control and pathogen-inoculated plants were kept in the dark in a humid chamber at 100% relative humidity at 25 °C for 24 h and then transferred to a greenhouse maintained at 60% relative humidity and at 28 °C during the day and 25 °C at night.

The development of lesions on the inoculated leaves was observed daily. The number of anthracnose lesions on the inoculated leaves was recorded 7 days after challenge-inoculation. The rate of protection was calculated as described by Cohen (1994a), where the rate $(\%) = 100\ (1 - x/y)$ in which x and y are the number of lesions on the leaves of treated and nontreated plants, respectively.

2.4. Light microscopy of infection structures

Leaves of the inoculated cucumber plants were detached at 1, 3, and 5 days after challenge-inoculation. Leaf disks, 5-mm-diameter were removed with a cork borer. Three leaf disks from the 2nd leaf were collected from one plant per 12 replications. The leaf samples were stained as described by Jeun et al. (2000). Leaf samples were fixed with 2% glutaraldehyde in 0.05 M phosphate buffer (pH 7.2) for 2 h. Samples were washed in the phosphate buffer three times for 10 min each, stained with 0.02% Uvitex 2B (w/v) (Diethanol) to facilitate observation of fungal structures, and then mounted on glass slides in 50% glycerin. The infection structures of *C. orbiculare* at the penetration sites were observed using a fluorescent microscope (Zeiss) equipped with filter set 05 (BP 400-440, FT 460, LP 470). The numbers of germinated conidia, appressoria, and autofluorescent plant cells on the leaf surfaces of the plants from all treatments were recorded.

2.5. Data analysis

The numbers of lesions, appressoria formation, and autofluorescent plant cells at the penetration sites in the inoculated leaves were subjected to analysis of variance using JMP (SAS, Cary, NC). When significant F values were obtained, treatment means were separated using LSD at $P = 0.01$ or $P = 0.001$.

3. Results

3.1. Effects of PGPR strains and chemicals on anthracnose severity

Lesions became apparent 5 days after challenge-inoculation. The number of lesions was significantly less on leaves of plants treated with PGPR strains compared with those of the noninduced control plants (Table 1) Fewer lesions were seen on plants treated with PGPR strain 90–166 than 89B61 ($P = 0.05$). The size of lesions on leaves of control plants increased rapidly at 7 days after challenge-inoculation (data not shown). In contrast, the development of lesions was restricted on leaves of plants treated with the PGPR strains.

At a concentration of 10 mM, BABA-induced resistance against anthracnose in the aerial parts of the plants (Table 1 and Fig. 1). Resistance was not induced (data not shown) at concentrations of 0.1 and 1 mM. The protection values elicited by 100 mM BABA were comparable with those by 10 mM BABA (data not shown). Treatment with 1 mM ASA on the root system also reduced lesion numbers on leaves (Table 1 and Fig. 1). At a concentration of 0.1 mM but not at 1 mM, ASA did not induce resistance. At 10 mM, ASA was phytotoxic to the drenched plants (data not shown). In general, the two chemicals reduced the number of lesions to a greater degree than treatments with the PGPR strains (Table 1).

3.2. Microscopic observations of the leaf surface

3.2.1. Infection structures on the leaves of control plants

At 24 h after inoculation, 20.6% of germinated conidia of *C. orbiculare* formed appressoria on the leaf surfaces. Most appressoria formed melanin, which was identified by the black color (Fig. 2A). No visible responses were found in the leaf tissues at 1 day after inoculation (data not shown). At three days after inoculation, some epidermal cells showed weak autofluorescence at the penetration sites; however, the frequency of appressoria formation did not increase compared to leaves that were examined one day after inoculation (data not shown). Intercellular hyphae were detected at some penetration sites of the inoculated leaves 3 days later (Fig. 2B). Most of the penetration sites were not brightly fluorescent, indicating a weak defense reaction of the host cells (Fig. 2A). The intercellular hyphae spread broadly into the plant tissues 5 days after inoculation (data not shown).

3.2.2. Infection structures on the leaves of PGPR-treated plants

At 1 day after inoculation, the infection structures of *C. orbiculare* on the leaves of plants treated with PGPR strains were not different from those of control

Table 1
Reduction of lesion number and protection rate on the leaves of cucumber plants treated with PGPR strains *S. marcescens* 90–166 and *P. fluorescens* 89B61 or chemicals DL-3-aminobutyric acid (BABA) and amino salicylic acid (ASA) 7 days after inoculation with *C. orbiculare*

Treatment[a]	Number of lesions[b]					Protection (%)[c]
	EXP 1	EXP 2	EXP 3	EXP 4	EXP 5	
Control	121.0	58.8	164.2	120.8	93.8	
SA (0.1 mM)	127.8	68.2	–	–	–	12.3
SA (1 mM)	37.0*	38.0	23.6*	2.3*	19.3*	78.5
SA (10 mM)	0.0*	0.0*	–	–	–	–
SA (100 mM)	0.0*	0.0*	–	–	–	–
BABA (0.1)	104.2	112.2	–	–	–	3.2
BABA (1)	58.5*	57.8	–	–	–	48.0
BABA (10)	25.67*	8.17*	25.0*	1.6*	61.2*	78.2
BABA (100)	24.67*	32.3	–	–	–	74.5
$LSD_{0,05}$	52.8	44.3	38.1	34.5	34.7	
Control	109.9	134.7	91.7			
90–66	42.9*	118.3	28.3*			43.7
89B61	64.1*	90.8*	57.1*			37.0
$LSD_{0,05}$	34.1	42.5	31.8			

[a] Inoculation with 10^8 cfu/ml of PGPR strains *S. marcescens* strain 90–166 and *P. fluorescens* strain 89B61 or treatment with amino salicylic acid (1 mM) and DL-3-aminobutyric acid (10 mM) were carried out 5 and 3 days before the challenge-inoculation, respectively.

[b] Values represent means of numbers by counting the lesions on the leaves of 12 plants per replications.

[c] Percentage were calculated by the formula, protection $(\%) = 100(1 - x/y)$ in which x and y are number of lesions on the leaves of treated and nontreated control plants, respectively.

* Indicates significant reduction in lesion numbers compared to that of the water control at $P = 0.05$.

plants. Three days after challenge-inoculation, the frequency of conidial germination on the leaves of the plants treated with PGPR strain 90–166 was not different from that of the control plants (Fig. 3A). Likewise, the germination rate on leaves treated with 89B61 did not decrease compared to that of the control plants (Fig. 3A). No differences in appressoria formation were found between PGPR treatments and control plants (Fig. 4A). However, callose-like structures (β-1,3-glucan polymer) were frequently deposited at the penetration sites on the leaves of plants treated with either 90–166 or 89B61 (Fig. 2C, arrow). The frequency of autofluorescent cells at the fungal penetration sites was significantly higher with both PGPR treatments than the control (Fig. 5A).

3.2.3. Infection structures on the leaves of chemical-treated plants

Like PGPR treatments, the infection structures on the leaves of plants treated with BABA or ASA were not different from those of control plants at 1 day after challenge-inoculation. However, at 3 days after inoculation, the conidial germination of *C. orbiculare* and the appressoria formation were greatly reduced on the leaves of plants treated with BABA or ASA (Figs. 3B and 4B). This reaction was different from that observed on plants treated with PGPR (Fig. 4A). Although some epidermal cells of BABA- and ASA-treated plants were brightly autofluorescent (Figs. 2E and F, arrows), the frequency of those cells was not significantly different compared with those of control plants (Fig. 5B).

4. Discussion

The results presented here support the hypothesis that SAR and ISR are expressed differently in plant reaction and pathogen development. Specifically, systemic induced resistance may be expressed differently on the leaf surface and in the epidermal cells of cucumber plants treated with PGPR strains or with BABA and ASA. The plants pre-inoculated with PGPR showed a decrease in the number of lesions by approximately 40% compared to nonbacterized controls. Those plants receiving applications of BABA or ASA had approximately an 85% reduction in lesion numbers (Table 1). The different protection values between treatment with PGPR and chemicals may be due in part to a soil environment that was unfavorable for PGPR to colonize and survive in the rhizosphere, while the activity of the chemicals may be less affected by environmental conditions of the soil.

Our cytological observations indicate chemical inducers and PGPR affect the early steps of infections differently. Application of chemicals greatly decreased conidial germination of *C. orbiculare* on the leaf surface, while preinoculation with PGPR strains did not reduce the conidial germination of the fungus (Figs. 3A and B). Treatment with both BABA and ASA resulted in strong suppression of appressoria formation on the leaf surfaces (Fig. 3B), which likely explains the decrease of lesion numbers (Table 1). A reduction of spore germination has also been demonstrated in several incompatible interactions (Doke et al., 1987; Kovats et al.,

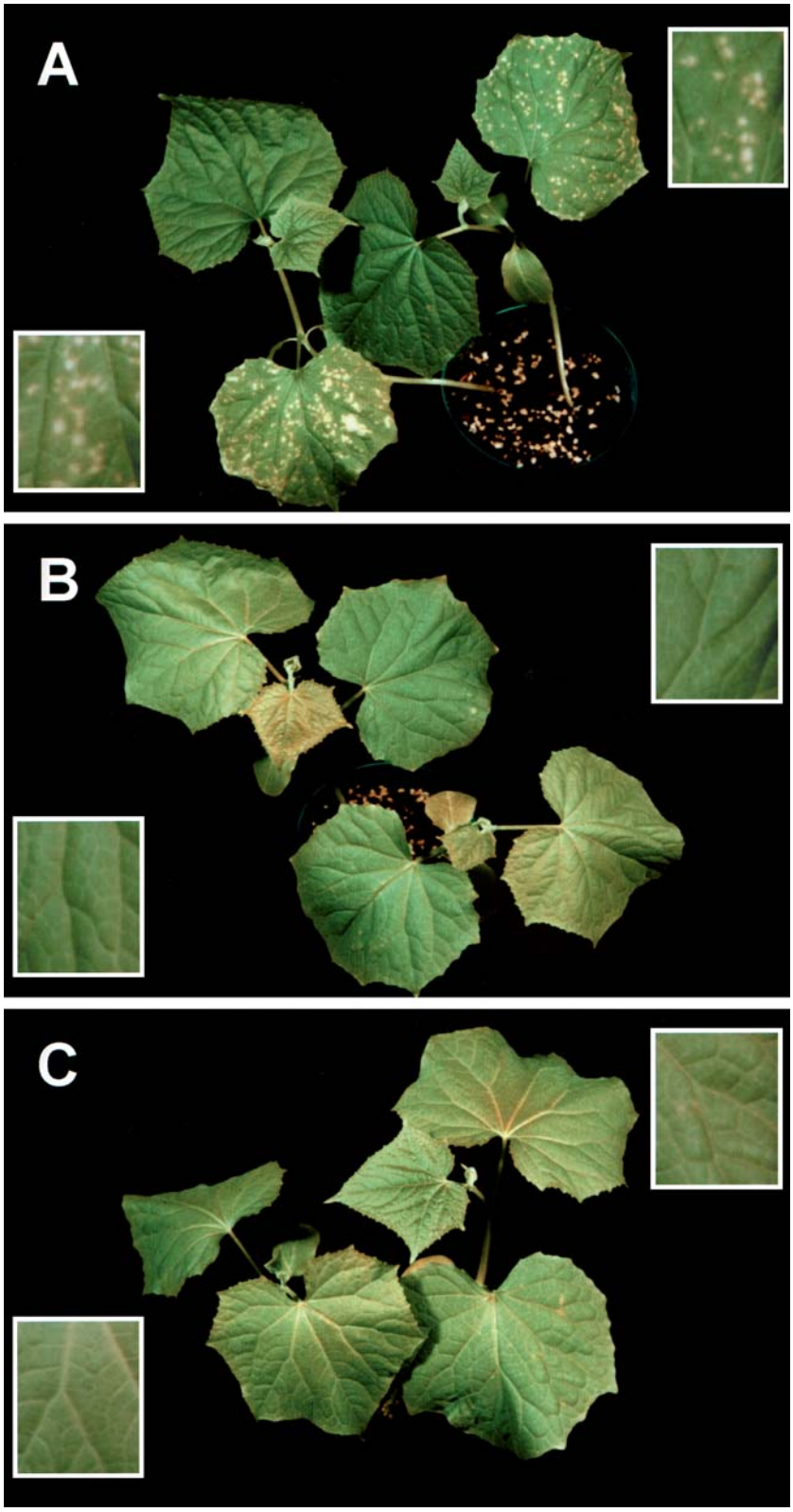

Fig. 1. Systemic acquired resistance in cucumber plants against anthracnose disease 7 days after inoculation with *C. orbiculare* (1.0×10^8 conidia/ml). (A) Nontreated control, (B) drenched with 30 ml of 10 mM BABA, and (C) 1 mM ASA 3 days before the challenge-inoculation. Square boxes on left and right sides of each figure are magnified parts of inoculated leaves. (A) Well-developed anthracnose lesions on nontreated control; and suppression of lesions on leaves of (B) BABA and (C) ASA pretreated plants.

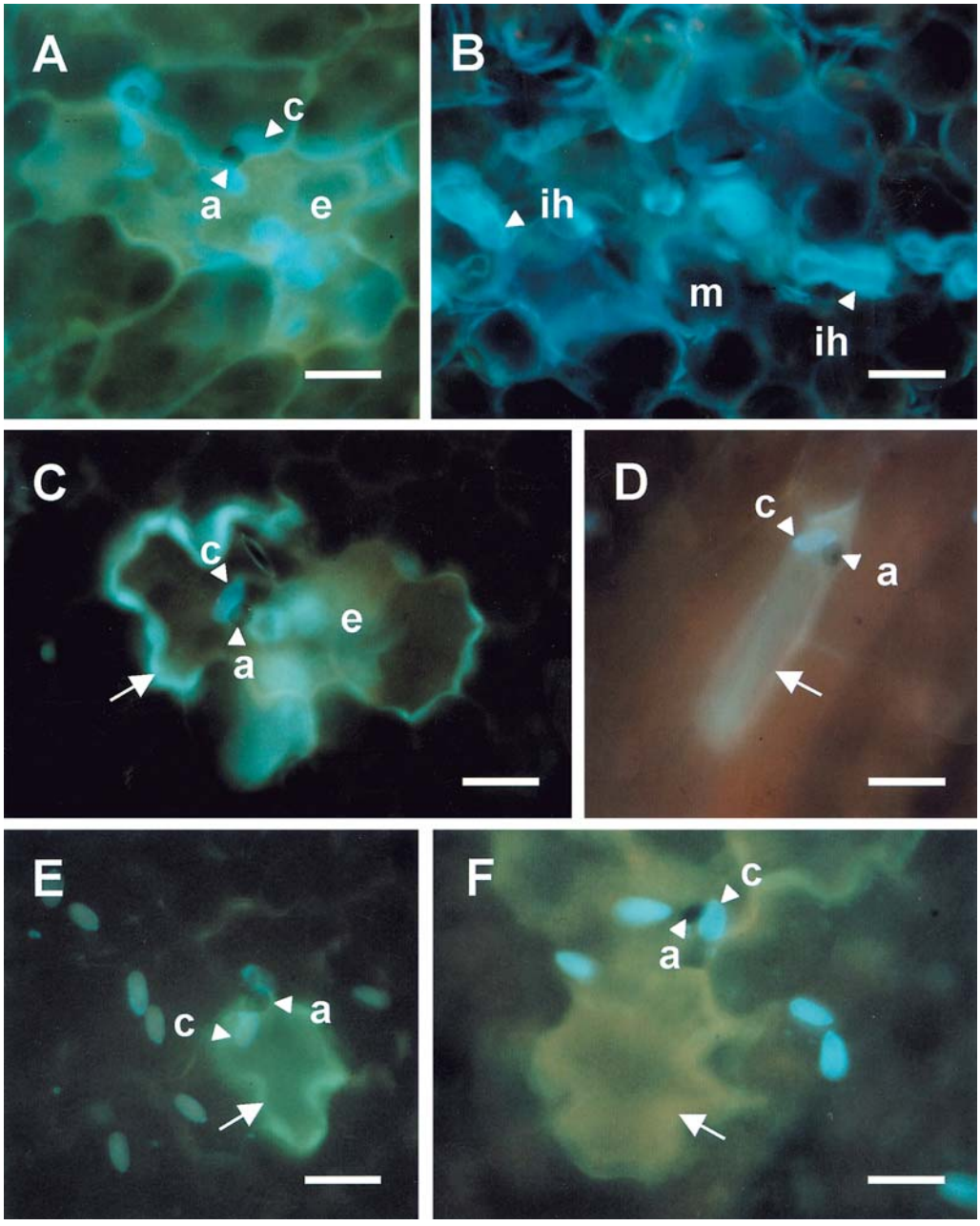

Fig. 2. Fluorescent microscopy of infection structures and resistance responses on leaves of cucumber plants 3 days after challenge-inoculation with *C. orbiculare*. (A and B) Nontreated; (C) inoculated with PGPR strain 90–166; (D) inoculated with PGPR strain 89B61; (E) treated with BABA; and (F) treated with ASA. Treatments with BABA (10 mM) and ASA (1 mM) and inoculation with PGPR strains (10^8 cfu/ml) were applied 3 and 5 days before challenge-inoculation, respectively. Bars = 20 μm. *Abbreviations:* a, appressorium; c, conidium; e, epidermal cell; ih, intercellular hypha; and m, mesphyll cell. Arrows point to the response of epidermal cells to the pathogen.

1991b). The cause of reduction in spore germination has not been clearly illustrated. One explanation may be a thigmotropic-differentiation (i.e., conditioned by different properties of surfaces, such as hardness or feature) of the leaf surfaces mediated by the application of inducers (Deising et al., 1996). These morphological changes in leaf surfaces may be significant in suppressing germination of conidia in the treated plants. However, other studies have shown no reduction of germination of fungal or bacterial pathogens on the resistance-expressing leaves (Jeun et al., 2000; Kovats et al., 1991a; Zimmerli et al., 2000).

In some plant–pathogen interactions, a certain compound excreted by the host may enhance the formation of appressoria, which are structurally different from a conidium. For example, wax isolated from the avocado fruit surface-induced appressoria formation by *Colletotrichum gloeosporioides* (Penz.) Penz. & Sacc. (Hwang

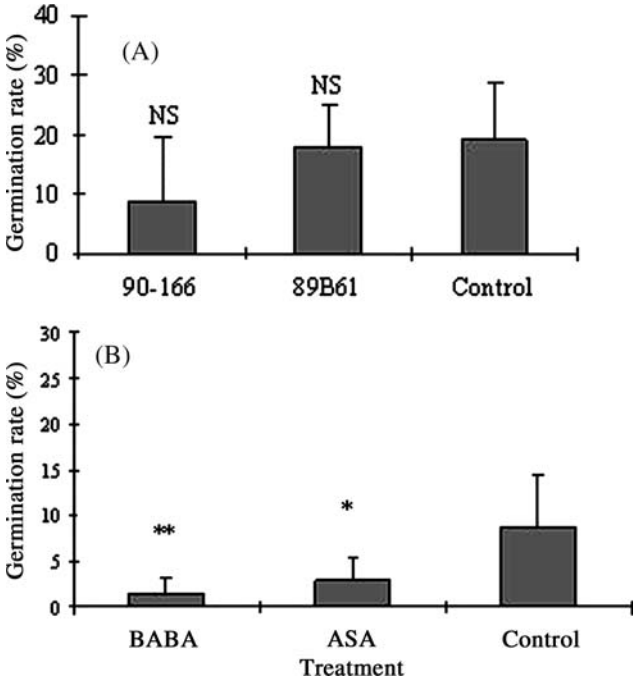

Fig. 3. Germination of conidia of *C. orbiculare* on the leaves of cucumber plants 3 days after inoculation with the fungal pathogen. (A) Plants inoculated with PGPR strains 90–166 and 89B61; (B) plants receiving chemical treatments of DL-3-aminobutyric acid (BABA) and amino salicylic acid (ASA). Treatments with BABA (10 mM) and ASA (1 mM) and inoculation with PGPR strains (10^8 cfu/ml) were applied 3 and 5 days before challenge-inoculation, respectively. The vertical bars indicate standard deviation from three separate experiments, each containing 4 leaf discs from 4 plants per treatment. NS, no significant difference; *, significant difference, $P = 0.01$; **, significant difference, $P = 0.001$.

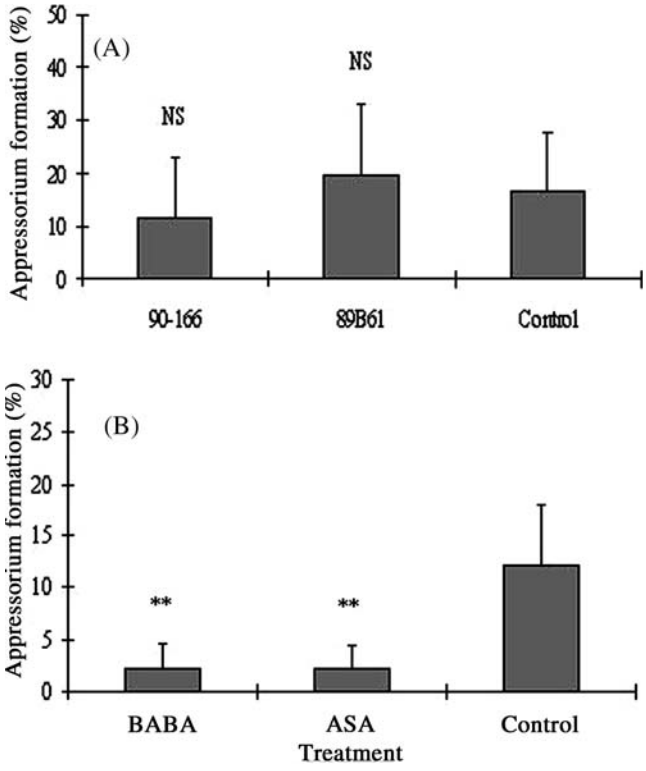

Fig. 4. Frequency of appressorium formation on the leaves of cucumber plants at 3 days after inoculation with *C. orbiculare*. Data are expressed as the percentage of conidia that form appressoria on leaf discs. (A) Plants inoculated with PGPR strains 90–166 and 89B61; (B) plants receiving chemical treatments of DL-3-aminobutyric acid (BABA) and amino salicylic acid (ASA). Treatments with BABA (10 mM) and ASA (1 mM) and inoculation with PGPR strains (10^8 cfu/ml) were applied 3 and 5 days before challenge inoculation, respectively. The vertical bars indicate standard deviation from 3 separate experiments, each containing 4 leaf discs from 4 plants per treatment. NS, no significant difference; *, significant difference, $P = 0.01$; **, significant difference, $P = 0.001$.

and Kolattukudy, 1995; Podila et al., 1993). Similarly, cAMP may have acted as an initial signal for formation of appressorium of *Magnaporthe grisea* (Hebert) Barr (Lee and Dean, 1993). The reduction of appressoria formation has also been demonstrated in the resistance-expressing leaves of cucumber plants preinoculated with the anthracnose pathogen on the lower leaves (Kovats et al., 1991a). However, suppression of appressoria formation is not found in all cases of SAR expression. The leaves of tomato plants pretreated with either BABA or tobacco necrosis virus (TNV) did not show a decrease in appressoria formation by late blight pathogen *P. infestans* (Mont.) de Bary (Jeun et al., 2000). Similarly, in our study preinoculation with PGPR strains did not suppress appressoria formation on cucumber leaves (Fig. 3A). Nevertheless, resistance against cucumber anthracnose was triggered by the treatment with PGPR strains (Table 1). This finding indicates that some resistance mechanisms other than the suppression of appressoria formation must be involved in the expression of resistance induced by the PGPR strains.

Plant cells become autofluorescent during fungus-induced HR (Yu et al., 2001). Autofluorescence is associated with accumulation of phenolic compounds during lesion formation (Koga et al., 1980) and has been used to assess SAR in *Arabidopsis* (Hunt et al., 1997). Using fluorescent microscopy, we confirmed that the epidermal cells from plants treated with PGPR strains 90–166 and 89B61 became autofluorescent more frequently at the penetration sites compared to those of nontreated control plants (Fig. 5A), indicating increased occurrence of phenolic compounds. Callose structures were also often observed in the epidermal cells of PGPR-treated plants (Fig. 2C, arrow). Callose formation is a well-known resistance mechanism in many host–parasite interactions (Kovats et al., 1991b; Strömberg and Brishammar, 1993). These results suggest that the resistance mediated by PGPR strains 90–166 or 89B61 is expressed mainly through the active responses of plant cells at the penetration sites of the anthracnose pathogen.

Unlike plants treated with PGPR strains, the frequency of autofluorescent cells on leaves of plants

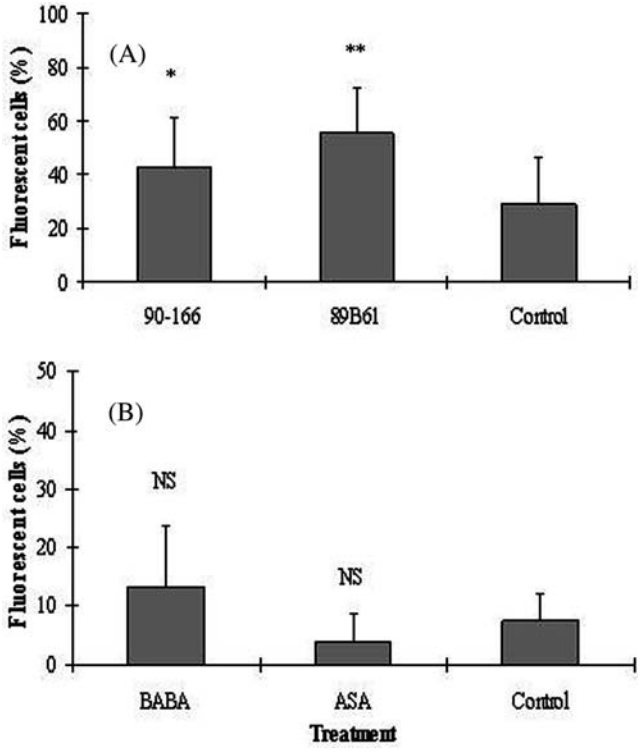

Fig. 5. Frequency of fluorescent cells at the penetration sites on the leaves of cucumber plants 3 days after inoculation with *C. orbiculare*. Data are expressed as the percentage of penetration sites that contain fluorescent cells. (A) Plants inoculated with PGPR strains 90–166 and 89B61; (B) plants receiving chemical treatments of DL-3-aminobutyric acid (BABA) and amino salicylic acid (ASA). Treatments with BABA (10 mM) and ASA (1 mM) and inoculation with PGPR strains (10^8 cfu/ml) were applied 3 and 5 days before challenge inoculation, respectively. The vertical bars indicate standard deviation from 3 separate experiments, each containing 4 leaf discs from 4 plants per treatment. NS, no significant difference; *, significant difference, $P = 0.01$; **, significant difference, $P = 0.001$.

treated with chemicals was not different from the non-treated control (Fig. 5B). These results indicate that plant responses may not play an important role in cucumber for expression of resistance mediated by the tested chemicals. Although the plants pretreated with both BABA and ASA showed weak responses, the protection mediated by the chemicals against anthracnose was much higher compared to those treated by the biotic PGPR strains (Fig. 4B and Table 1).

Production of melanin by appressoria may be important for penetration of *C. orbiculare* into host cells (Howard and Ferrari, 1989; Howard et al., 1991). Therefore, suppression of melanin biosynthesis would be expected to play a significant role in an incompatible interaction between cucumber and *C. orbiculare* during pathogen infection. In the present study, we could not clearly distinguish the rates of melanin formation among the treatments because fluorescent microscopy was inadequate for the qualitative and quantitative analyses of melanin formation.

Our results suggest that ISR elicited by PGPR is associated with fewer cytological changes in the plant than those that occur with SAR elicited by BABA or ASA. Further work is needed to identify mechanisms by which PGPR-treated plants restrict lesion formation caused by *Colletotrichum*.

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