

## Rhizobacterial Exopolysaccharides Elicit Induced Resistance on Cucumber

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**The role of exopolysaccharides (EPSs) from a plant growth-promoting rhizobacterium, *Burkholderia gladioli* IN26, on elicitation of induced systemic resistance was investigated. A purified EPS induced expression of *PR-1a::GUS* on tobacco and elicited induced resistance against *Colletotrichum orbiculare* on cucumber. The maximum level of disease protection was noted when seeds were soaked in 200 ppm of the EPS. Our results indicate that EPS from specific rhizobacteria can elicit induced resistance and suggest that bacterial EPS might be a useful elicitor of resistance under field conditions.**

**Keywords:** Plant growth-promoting rhizobacteria, exopolysaccharide, induced systemic resistance

With pathogenic bacteria, induction of defense responses by bacteria cell components has been intensively studied, and these bacterial determinants are referred to as pathogen-associated molecular patterns (PAMPs) [22]. PAMPs that have been shown to induce innate plant defense responses include the O-antigenic side chain of LPS and flagellin [7]. Similarly, induction of systemic resistance elicited by nonpathogenic rhizobacteria has been reported as one mechanism for observed reductions in plant diseases following applications of specific rhizobacterial strains [14–16, 29, 34, 36]. With other rhizobacteria, diverse bacterial determinants including bacterial secreted compounds (*e.g.*, siderophores and salicylic acid (SA)) and cell surface materials (*e.g.*, lipopolysaccharides (LPS)) contribute to ISR [4, 17, 23]. Specifically, siderophores and SA produced by PGPR have elicited ISR in various crop systems including carnation, radish, and tomato [6, 17, 35]. More recently, other compounds produced by *Bacillus* spp. and *Pseudomonas* spp., bacterial volatile organic compounds (VOCs), an *N*-alkylated benzylamine derivative, and the

antibiotic 2,4-diacetylphloroglucinol (DAPG), have been shown to elicit ISR on *Arabidopsis* [11, 23, 28].

Exopolysaccharides (EPSs) are a group of carbohydrates secreted from various bacterial species including pathogenic and symbiotic bacteria and fungi [3, 18]. For pathogenic bacteria, EPSs act as pathogenicity factors [10] and are necessary for developing disease symptoms such as water-soaking or wilting [10, 18]. EPSs and cell-associated polysaccharides also play a critical role in initial colonization and enhancement of survival of pathogenic species of *Agrobacterium*, *Erwinia*, *Pseudomonas*, and *Xanthomonas* [5]. For beneficial bacteria, including the rhizobia, various functions of EPSs have been reported. EPS produced by the alfalfa-symbiotic bacterium *Sinorhizobium meliloti* functions as a signaling molecule that triggers a developmental response in the plant or suppresses host defense responses [20]. EPS produced by the root-associated saprophytic bacterium (rhizobacterium) *Pantoea agglomerans* YAS34 was associated with plant growth promotion of sunflower [1]. EPS from a plant pathogenic *Pantoea agglomerans* elicited a rapid production of active oxygen species in tobacco, parsley, wheat, and rice cell culture [24]. However, elicitation of ISR by EPS from PGPR has not been reported. To our knowledge besides the case of rhizobia, EPS secreted by rhizobacteria has not been reported as a bacterial determinant of ISR upto now. *Burkholderia gladioli* IN-26 is a strain of plant growth-promoting rhizobacteria (PGPR) that reduced bacterial speck on tomato [13]. In tobacco, IN-26 elicited protection against wild fire, caused by *Pseudomonas syringae* pv. *tabaci*, and this protection was associated with activation of the *PR-1a* gene [25]. However, the specific bacterial determinants of IN26 that elicit ISR have not been elucidated.

The objective of this study was to determine whether EPSs isolated from PGPR strain *B. gladioli* IN26 could elicit ISR in cucumber against *Colletotrichum orbiculare*. We also attempted to optimize the concentration of EPS and application methods. Our results comprise the first evidence that EPSs of PGPR can elicit ISR on cucumber.

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Seeds of *Nicotiana tabacum* cv. Xanthi-nc, genetically engineered with a GUS reporter gene fused to the *PR-1a* promoter [33], were provided by J. Ryals (Novartis, Agricultural Biotechnology Research Unit, Research Triangle Park, NC, U.S.A.). Cucumber (*Cucumis sativus* L. cv. Eunsung) and tobacco seeds were surface-sterilized by soaking in 1% NaOCl for 3 min followed by soaking in 75% ethanol for 3 min and then rinsed 3 times with sterile distilled water before seeding in plastic pots (10 cm diameter) filled with soilless potting mix.

PGPR strain *Burkholderia gladioli* IN26 was maintained at  $-80^{\circ}\text{C}$  in tryptic soy broth (TSB) amended with 20% glycerol. Inoculum for experiments was prepared by streaking strains from  $-80^{\circ}\text{C}$  onto tryptic soy agar (TSA) plates, incubating the plates at  $28^{\circ}\text{C}$  for 24 h, and scraping bacterial cells into 0.02 M potassium phosphate buffer, pH 6.8. The final bacterial concentration was adjusted to  $10^9$ – $10^{10}$  colony-forming units (CFU)/ml prior to use.

*Colletotrichum orbiculare* was maintained at  $4^{\circ}\text{C}$  on potato dextrose agar (PDA). Inoculum for plants was grown on green bean agar [9] for 6 days at  $25^{\circ}\text{C}$ . After incubation, conidia were harvested using a small brush. The spore concentration was adjusted to  $10^5$  spores/ml using a hemacytometer. This spore suspension with 0.02% Silwet L-77 (Union Carbide, Tarrytown, NY, U.S.A.), which enhances the adhesion of conidia to the leaf surface, was used as the inoculum for challenge inoculations on cucumber leaves.

We conducted preliminary tests to determine if the bacterial determinants of ISR were bacterial cell wall components or bacterial metabolites. A straightforward approach with a simple separation of bacterial suspension after centrifugation at 7,000 rpm for 20 min was carried out on the *PR-1a::GUS* tobacco. EPS was isolated and purified following the modified methods described by Cerning *et al.* [2]. For isolation of EPS, culture samples were collected from liquid cultures of *B. gladioli* IN26 in King's B agar and centrifuged at  $8,000 \times g$  for 20 min. The supernatant was adjusted to 0.1% with distilled water and added to activated charcoal to allow adsorption of the cell wall enzyme and protein components after adjusting the pH to 7.0 by adding 0.5 N HCL. The samples were centrifuged for 30 min at  $8,000 \times g$  at  $4^{\circ}\text{C}$ . The supernatants were filtered through a  $0.2 \mu$  cellulose filter. For elimination of LPS, the 100-ml supernatants were added to 100 ml of buffer that contained 0.1 M Tris-HCl (pH 8.0), 6.0% sodium citrate, and 1.0% deoxycholic acid. After washing the supernatant 3 times with pure ethanol, 10 g of nonionic HP-20 resin (DIAION 8L501; Mitsubishi Chemicals, Japan) was added for absorbing the EPS from the supernatant and then stirred for 1 h in order to remove lipopolysaccharide (LPS). After eliminating LPS from the bacterial supernatant, pure acetone was added for precipitation of the pure EPS. The purified EPS sample was tested by an Endotoxin LAL

detection kit (Limulus Ameobocyte Lysate test, Endosafe EP2523CT, Charles River, MA, U.S.A.) to measure for LPS contents. The samples were stored at  $4^{\circ}\text{C}$  until use in bioassays.

For *PR-1a* promoter expression test, GUS activity was measured in leaflets of the transgenic tobacco plant using a fluorometric assay described by Jefferson [12] and Park and Kloepper [25]. Different concentrations of EPS were infiltrated into the bottom leaf of 4-week-old tobacco seedlings under greenhouse conditions. Treatments included 10, 50, and 100 ppm EPS along with 0.5 mM salicylic acid (positive control) and water (negative control). Six replications of each treatment were used, and GUS activity was determined 3 days after infiltration of EPS.

Ten mg of plant tissue from each replication of all treatments was removed by a cork borer from the plant and ground in an Eppendorf tube with 20  $\mu$ l of extraction buffer [12]. Extracts were centrifuged twice at  $8,000 \times g$  for 5 min at  $4^{\circ}\text{C}$ , and 20  $\mu$ l of the resulting supernatant was incubated with 20  $\mu$ l of 2 mM 4-methylumbelliferyl- $\beta$ -D-glucuronide (MUG) at  $37^{\circ}\text{C}$  for 1 h. The reaction was terminated by adding 960  $\mu$ l of 0.2 M sodium carbonate solution, and fluorescence was measured with a TKO 100 fluorometer (Hoefer Scientific Instruments, San Francisco, CA, U.S.A.). GUS activity was expressed as nM of MU/mg of sample/h. Data were analyzed with ANOVA in SAS JMP software (SAS Institute, 1995). Significant differences among treatment means on each sample date were determined using LSD at  $P=0.05$ . The experiments were repeated three times with similar results.

The purified EPS of IN26 was adjusted to three different concentrations (50, 100, and 200 ppm) with sterile distilled water, and 100  $\mu$ l of each treatment was infiltrated into cotyledons of 1-week-old cucumber seedlings. Controls included infiltration of water (negative control) and 0.5 mM salicylic acid (positive control). There were six replicate plants for each treatment. Seven days after infiltration of EPS, the plant height was measured. For the challenge inoculation, spore suspensions of *C. orbiculare* ( $1.0 \times 10^5$ /ml) supplemented with 0.02% Silwet L-77 were sprayed on the cucumber plant until run-off, and plants were then placed at 100% relative humidity for 24 h. After incubation, all treatments were transferred to the greenhouse at  $20$ – $28^{\circ}\text{C}$  under natural light.

Lesions were counted on second leaves of each plant at 7 days after inoculation. The mean number per plant was analyzed with ANOVA using SAS JMP software (SAS Institute, 1995). The experiments were conducted two times. After confirming the homogeneity of variances with Bartlett's test, combined data were analyzed with ANOVA and significant differences in treatment means were detected with LSD at  $P=0.05$ .

For treatment of cucumber seeds with EPS, seeds were treated with 100  $\mu$ l of 200 ppm EPS immediately after

**Table 1.** Preliminary screening of bacterial determinant to associate with induced resistance by *PR-1a::GUS* tobacco.

Treatment	Relative GUS activity compared with control
<i>B. gladioli</i> IN26 supernatant	-
<i>B. gladioli</i> IN26 whole cell	++
Dead+supernatant	-
Dead+pellet cells	++
Autoclaved+supernatant	-
Autoclaved+pellet cells	++

All treatments were infiltrated into the lower leaf of a transgenic tobacco plant for induction of *PR-1a::GUS* expression (see Materials and Methods).

sowing in soilless potting mix. Plant growth was measured 2 or 3 weeks after seed treatment. For challenge inoculation of pathogens, the spore suspension of *C. orbiculare* ( $1 \times 10^5$  spores/ml) in 0.02% Silwet L-77 and was sprayed on cucumber plants 14 days after EPS treatment. All plants were maintained at 100% relative humidity for 24 h and then transferred to the greenhouse. For spray application of EPS, 14-day-old cucumber plants were sprayed with each treatment. Spore suspensions of *C. orbiculare* were sprayed on plants 7 days after the foliar spray of EPS.

#### Isolation of EPS from *B. gladioli* IN26

Infiltration of only pelleted whole cells, dead cells, and autoclaved cells into the *PR-1a::GUS* tobacco induced GUS activity, relative to the buffer control. In contrast, GUS activity was not induced with supernatants from the same treatments (Table 1). These results indicate that ISR determinant(s) from strain IN26 are localized on the cell wall and are heat-stable.

We then assessed if the determinant contained any proteins. In general, many proteins are anchored in the bacterial cell wall or membranes. To separate the anchored proteins, we used a proteinase K digestion kit. Intriguingly, GUS expression was still sustained even after protease treatment, indicating that ISR determinant(s) from strain IN26 are on the cell wall or membrane rather than metabolites or proteins. Additional fraction analysis revealed that the active determinant could be similar characteristics with extracellular polysaccharides (data not shown).

**Table 2.** Minimization of LPS contamination from extracted EPS of IN26.

Purification steps	Endotoxin unit (EU/ml) <sup>a</sup>	Relative LPS contents from total EPS (%)
Crude EPS	240,000	1.091
Charcoal-eluted EPS	30,863	0.140
HP-20 column-eluted EPS	7,849	0.036

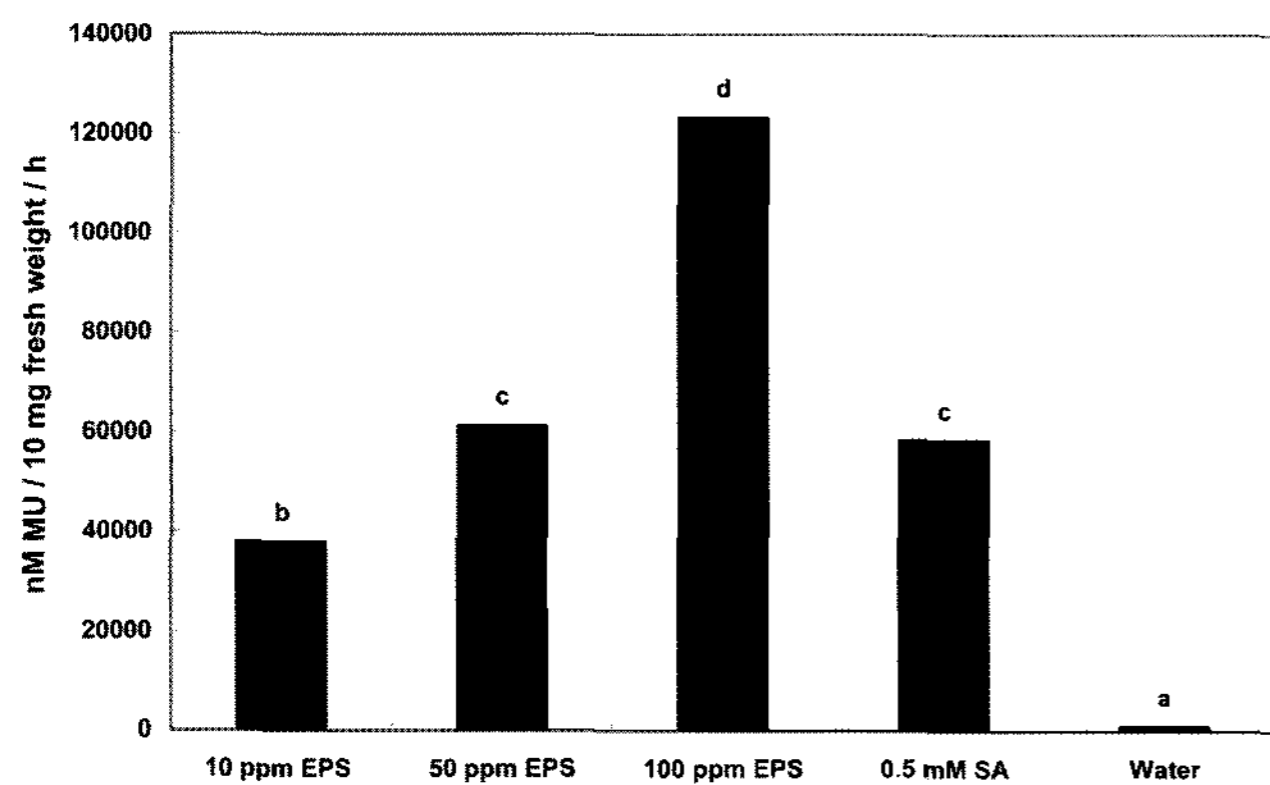
<sup>a</sup>L1 internal unit=1 endotoxin unit; Endotoxin standard: 11 EU/ng endotoxin.

In many previous studies, separation of EPS and LPS from bacterial fractions has been reported to be difficult [27]. Thus, we cannot exclude possible contamination of our fraction by LPS, which has already been reported as a bacterial determinant of ISR in tomato and *Arabidopsis* [17, 32]. To rule out LPS contamination from EPS purification, elution with charcoal and nonionic resin LP-20 columns provided to minimize LPS contents from our EPS extracts. The most purified EPS was obtained from usage of the nonionic LP-20 column. We failed to eliminate LPS from purified EPS entirely. However, the amount of LPS (0.036% out of total EPS) in the EPS extraction is likely too low to affect ISR capacity *in planta* (Table 2).

Even though we employed a purification process for obtaining pure EPS by eliminating LPS contamination from the extract, the EPS extract contained LPS as low as 0.036% of total EPS, indicating that the purity of EPS used in our study was 99.964%. We could not exclude the possibility that a small amount of EPS could be a factor to be implicated in ISR against cucumber anthracnose. However, such LPS treatment with the concentration itself (0.036% of total EPS) did not affect ISR against cucumber anthracnose (data not shown). We have not determined the structure of the EPS from strain IN26. Structural studies of EPSs produced by *Burkholderia* spp. have been reported for *B. cepacia*, *B. pseudomallei*, and *B. brasiliensis*. *B. brasiliensis* is an endophyte isolated from the interior rice root of healthy plants [8]. The role of EPS from *Burkholderia* spp. has been suggested to include interactions between host and bacteria, such as plant colonization and pathogenesis in mammalian host [18]. Until now, EPSs produced by PGPR strains have not been reported as a bacterial determinant of ISR. To our knowledge, a 0.036% content of LPS from the EPS sample is unlikely to have any effect on the physiological change of the cucumber plant.

#### *PR-1a* Gene Expression by Bacterial EPS

We evaluated the effect of different concentrations of the EPS from strain IN26 on the expression of *PR-1a* as a marker gene on induced resistance. The statistical analysis indicated that each GUS expression on the *PR-1a::GUS* tobacco plant infiltrated with three concentrations of EPS from strain IN26 significantly differed between treatment, suggesting that EPS induces the GUS gene following the *PR-1a* promoter. The effect of 0.5 mM SA application was similar to that of 50 ppm EPS, whereas water treatment induced only basal level of GUS expression (Fig. 1). The expression of GUS activity gradually increased with increasing EPS concentrations of 10, 50, and 100 ppm. These results further support the conclusion that EPS is a direct elicitor of ISR. Leaf infiltrations with even 200 ppm EPS did not induce any visible lesions, indicating that



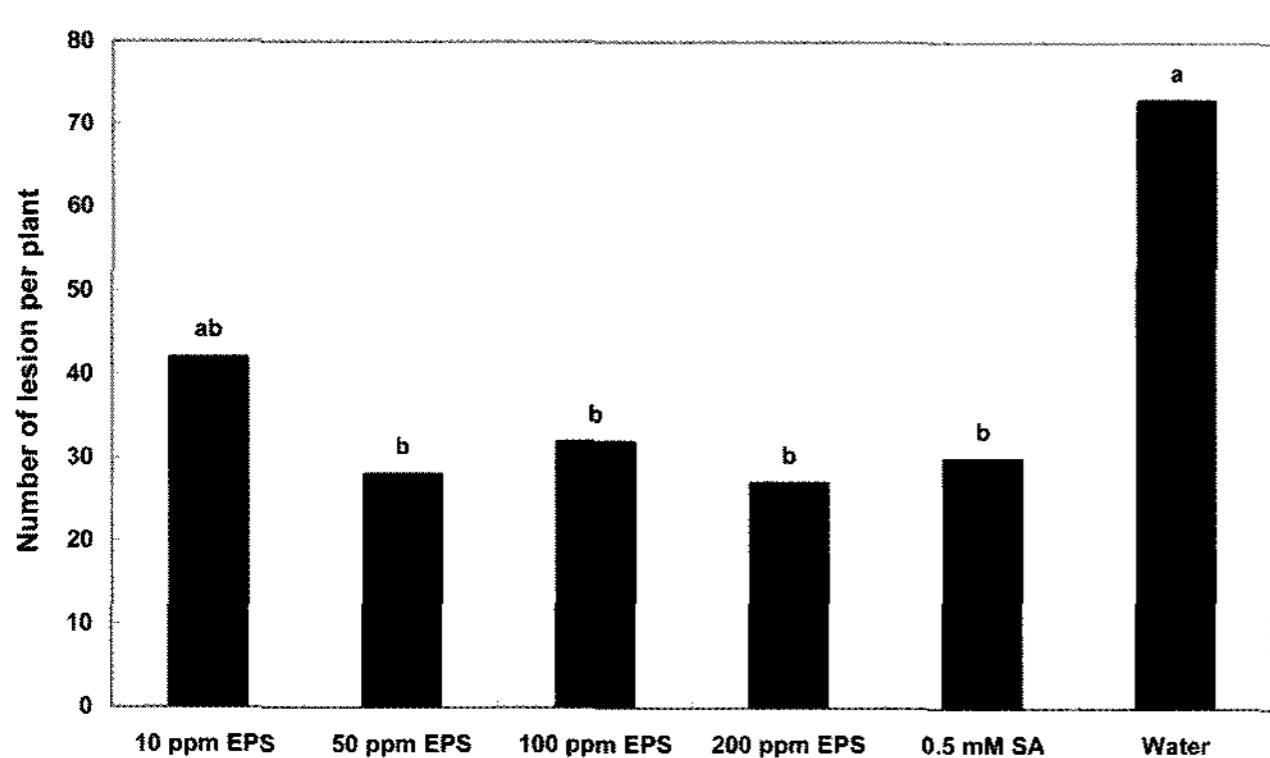
**Fig. 1.** Induction of *PR-1a* promoter by infiltration of different concentrations of EPS from *Burkholderia gladioli* IN26.

The experimental design was a randomized complete block with 12 replications of tobacco plants per treatment. All treatments were infiltrated into the lower leaf of a transgenic tobacco plant for induction of *PR-1a::GUS* expression. Applications with salicylic acid (positive) and water (negative) controls were used (see Materials and Methods). MU, 4-methylumbelliferone. Different letters indicate significant difference on Fisher's LSD test at  $P=0.05$ .

200 ppm EPS can be a concentration to elicit ISR without causing any visible damage to the plant.

### Induced Systemic Resistance on Cucumber by Bacterial EPS

To evaluate the elicitation of ISR by EPS, we conducted experiments with cucumber as a model plant against the cucumber anthracnose caused by *C. orbiculare*. Pretreatment of four different concentrations of EPS significantly reduced the number of lesions per plant, whereas treatment with water had no effect. When the treatment of a much higher concentration of EPS (200 ppm) was infiltrated on the



**Fig. 2.** Induced systemic resistance against *Colletotrichum orbiculare* by infiltration of different concentrations of EPS from *Burkholderia gladioli* IN26.

Leaves of 14-day-old cucumber plants were infiltrated on the lower side leaf with 0.1 ml of each treatment of elicitor or 0.5 mM SA. Each experiment was a randomized complete block with six replications of single plants per treatment. Different letters indicate significant difference on Fisher's LSD test at  $P=0.05$ .

cucumber leaf, the ISR capacity was saturated. SA application did not differ with EPS treatment. However, cucumber treated with water control developed anthracnose lesions twice as fast as treatment with EPS or SA. Our results indicate that even 50 ppm EPS elicited plant resistance against cucumber anthracnose (Fig. 2).

Application of bacterial EPS to elicit ISR on crops has not been previously attempted. Among cell wall components, LPS from nonpathogenic *Pseudomonas putida* WCS358 was considered as a main bacterial determinant that elicits ISR, other than flagella [6, 17, 21]. SA production of *P. fluorescens* strain CHA0 and *S. marcescens* strain 90-166 appeared not to be critical to ISR in tobacco [19, 26]. More recently, antifungal compounds including phenazine and 2,4-diacetylphloroglucinol (DAPG) from *Pseudomonas* spp. played as important determinants for elicitation of ISR on bean and *Arabidopsis* systems [11, 26]. The involvement of EPS from bacteria on reduction of disease symptoms has not been elucidated to date. Our results clearly demonstrated that the EPS from a rhizobacterium can be a major factor to trigger ISR in cucumber against *C. orbiculare*.

### Different Application Methods of Bacterial EPS for Induction of Systemic Resistance

To optimize the elicitation of ISR by EPS, the different application methods were examined. The ISR capacity by bacterial EPS was not differed depending on treatment methods or application concentrations. Fifty ppm EPS was not enough to elicit full ISR in all three methods. One hundred and 200 ppm EPS treatments, except 100 ppm EPS with the seed soaking method, effectively reduced lesion number in the three methods but were not different. Leaf infiltration, foliar spray, and seed soaking of 200 ppm EPS and 0.05 mM SA resulted in a significantly lower number of lesions compared with water control. If comparing

**Table 3.** Effect of different application methods on induced systemic resistance against *Colletotrichum orbiculare* by EPS of *Burkholderia gladioli* IN26 in cucumber.

Treatments	Number of lesions per plant		
	Leaf infiltration	Foliar spray	Seed soaking
50 ppm EPS	39ab	73ab	39ab
100 ppm EPS	30b	53b	43ab
200 ppm EPS	26b	46b	20b
0.5 mM SA	32b	40b	19b
Water	72a	101a	65a

Leaves of 14-day-old cucumber plants were infiltrated on the lower side leaf with 0.1 ml of each treatment of elicitor or 0.5 mM SA. Each experiment was a randomized complete block with six replications of single plants per treatment. The experiment was repeated three times, with similar results.

Means followed by different letters indicate significant difference on Fisher's LSD test at  $P=0.05$ .

**Table 4.** Effect of different application methods of EPS of *Burkholderia gladioli* IN26 on growth of cucumber.

Treatments	Plant height (cm)		
	Leaf infiltration	Foliar spray	Seed soaking
200 ppm EPS	29.2a	18.1a	11.1a
Water	24.4b	18.2a	8.2c

Leaves of 14-day-old cucumber plants were infiltrated on the lower side leaf with 0.1 ml of 200 ppm EPS. The experiment was a randomized complete block with six replications of single plants per treatment. The experiment was repeated three times, with similar results.

Means followed by different letters indicate significant difference on Fisher's LSD test at  $P=0.05$ .

the three methods at 200 ppm, the seed soaking method was relatively more efficient (59% inhibitory capacity) than the other two methods (Table 3). The seed soaking method can be a useful way to apply EPS onto crops. If this method is to be made available, the lasting time and effectiveness need to be tested. In the mid-growing season, we can also try to drench with EPS through the drip-irrigation system.

#### Effect of EPS on Cucumber Growth

The most effective concentration screened by a previous study was determined to be 200 ppm, and we used this concentration for further tests. Some SAR-inducing chemicals such as SA, BTH, and BABA inhibited plant growth at certain concentrations, which provides strong induced resistance capacity [15, 29]. We investigated the appropriate method to elicit ISR without affecting plant growth or even with promoting plant growth. Overall, the three treatments did not exhibit any inhibitory effects on plant growth (Table 4). Among the three methods, the seed soaking method promoted cucumber height, 35% greater than that of the control plant. In contrast, foliar spray application did not change plant growth. The leaf infiltration method also enhanced plant growth by 20% (Table 4). However, the procedure will be laborious. Collectively, 200 ppm EPS with the seed soaking method will be the relevant protocol to use in a large-scale application.

In conclusion, EPS purified from a rhizobacterium strain, IN26, efficiently elicited ISR against cucumber anthracnose at 200 ppm. Seed treatment and foliar sprays would be practical application methods to use in field trials. To our knowledge, these results are the first reported that EPS is a bacterial determinant for ISR.

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