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VOLATILES PRODUCED BY PGPR ELICIT PLANT GROWTH PROMOTION AND INDUCED RESISTANCE IN *ARABIDOPSIS*

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ABSTRACT

Plant growth-promoting rhizobacteria (PGPR) are naturally occurring soil bacteria that colonize roots, enhance plant growth, and activate plant defence against microbial pathogens. Here we present data that indicate some PGPR strains release a blend of volatile organic compounds (VOCs) that promote growth in *Arabidopsis* seedlings and induce resistance against *Erwinia carotovora* subsp. *carotovora*. In particular, the volatile components 2,3-butanediol and acetoin were released exclusively from two PGPR strains that trigger the greatest level of growth promotion and induced disease resistance. Pharmacological applications of 2,3-butanediol induced growth promotion and induced resistance, while bacterial mutants blocked in 2,3-butanediol and acetoin synthesis were devoid in growth-promotion and induced resistance capacities. Using transgenic and mutant lines of *Arabidopsis*, we provide evidence that the signal pathway activated by volatiles from one PGPR strain is dependent on cytokinin activation for growth promotion and dependent on an ethylene-signaling pathway for induced pathogen resistance. This discovery provides new insight into the role of bacteria VOCs as initiators of plant growth promotion and defense responses in plants.

INTRODUCTION

Plant growth-promoting rhizobacteria (PGPR) are a wide range of root-colonizing bacteria with the capacity to enhance plant growth by increasing seed emergence, plant weight, and crop yields (Kloepper, 1992). Soil or seed applications with PGPR have been used to enhance growth of several crops (Glick, 1995) as well as to suppress the growth of plant pathogens. The phenomenon that PGPR elicit plant defense has also been found to lead to a state of induced systemic resistance (ISR) in the treated plant (Kloepper et al., 1999; van Loon et al., 1998). ISR occurs when the plant's defense mechanisms are stimulated and primed to resist infection by pathogens (Van Loon, 1998). PGPR that colonize root systems with seed applications and protect plants against foliar diseases include *Pseudomonas fluorescens*, *P. putida*, *Bacillus pumilus*, and *Serratia marcescens* (Kloepper et al., 1999; Pieterse et al., 2002).

The mechanisms for plant growth promotion and ISR by PGPR have been extensively studied in the past decade. There are several determinants for mechanisms of growth promotion that include bacterial synthesis of the plant hormones indole-3-acetic acid (IAA), cytokinin, and gibberellin, breakdown of plant-produced ethylene

by bacterial production of 1-aminocyclopropane-1-carboxylate (ACC) deaminase, and increased mineral and N availability in the soil (Kloepper, 1992; Glick, 1995). For mechanisms of ISR, previous works demonstrated that several bacterial determinants such as siderophores, SA, and lipopolysaccharides (LPS) contributed to ISR (van Loon et al., 1998).

With regard to bacterial determinants that trigger growth promotion and ISR in plants, the role that volatile emissions from bacteria serve in plant development has not been reported. Here we report that a blend of air-borne chemicals released from specific bacterial strains of PGPR triggers growth promotion and ISR in *Arabidopsis thaliana* seedlings. Several genera of PGPR strains were assessed for eliciting growth promotion and ISR by volatiles under *in vitro* conditions. The PGPR strains were previously shown to elicit ISR on several crops against fungal, bacterial, and viral pathogens under greenhouse and field conditions (Zehnder et al., 2000). The volatiles produced by selected PGPR strains *B. subtilis* GB03 and *B. amyloliquefaciens* IN937a were characterized

MATERIALS AND METHODS

Plant Material

All mutant and transgenic lines were derived from parental *A. thaliana* ecotypes Columbia (Col-0), C24, Wassilewskija (WS) or Landsberg erecta (Ler). These were obtained from the Ohio State University Stock Center. Mutant and transgenic lines are listed on Table 1.

Arabidopsis thaliana seeds were surface-sterilized (2 min 70% ethanol soaking followed by a 20 min 1% sodium hypochlorite soaking), rinsed (4X) in sterile distilled water (SDW), and placed on Petri-dishes containing half-strength MS medium (Murashige and Skoog salt, GIBCOBRL Gaithersburg, MD), which consisted of 0.8% agar and 1.5% sucrose, adjusted to pH 5.7. Two days after vernalization at 4°C, seedlings were transferred to plates for the experimental uses described below.

Bacterial Cultures

Seven strains of PGPR (Auburn University) that lead to significant reduction in foliar diseases in *A. thaliana* were tested for their capacity to elicit ISR. The strains were *Pseudomonas fluorescens* 89B61, *Bacillus pumilus* T4, *B. pasteurii* C-9, *B. subtilis* GB03, *B. amyloliquefaciens* IN937a, *Serratia marcescens* 90-166, *Enterobacter cloacae* JM22, and *B. pumilus* SE34. *Escherichia coli* DH5a (QIAGEN Inc. Valencia, CA), which does not trigger growth promotion and ISR in *A. thaliana*, was used as a control. Other bacteria used included *B. subtilis* 168 (2,3-butanediol-producing) (*Bacillus* Genetic Stock Center, Ohio State University), *B. subtilis* strains BSIP1171 (2,3-butanediol-over-producing), BSIP1173 (2,3-butanediol-non-producing), and BSIP1174 (2,3-butanediol-non-producing) provided by D. Jahn (Braunschweig University, Braunschweig, Germany). The *Arabidopsis* pathogen *Erwinia carotovora* subsp. *carotovora* SCC1 was obtained from Dr. E. Tapio Palva, University of Helsinki, Finland. For experimental use, all bacteria were streaked onto tryptic soy agar (TSA) plates (Difco Laboratories, Detroit, MI) and incubated at 28°C in the absence of light for 24 h. For disease assay, ISR was chemically initiated in

plants by applying 20 μ l salicylic acid [1 mM in sterile distilled water (SDW)] at the crown of each seedling.

Bacterial volatile assay

One day prior to plant experiments, the bacterial strains were cultured on TSA plates and scraped into SDW. Plastic Petri-dishes (100 x 15 mm) that contained a center partition (I-plates; Fisher Scientific; Pittsburgh, PA) were prepared with MS solid media, and two-day-old emerging *A. thaliana* seedlings (5-6 seedlings/plate) were transferred to one side of the I-plates. The non-plant sides of the I-plates were inoculated with 20 μ l (10^9 CFU ml⁻¹) of a given PGPR strain or SDW applied drop-wise. The volatile bacterial extract, diluted 2, 3-butanediol (99+% purity; Aldrich), or solvent alone (CH₂Cl₂) was mixed with lanolin (Sigma) in a ratio of 0.08 g lanolin/ml test solution and 20 μ l of the resulting suspension was applied to a sterile paper disk (d = 1cm) (Whatman) on the opposite side of the I plate from the plant seedlings. Plates were covered and sealed with parafilm to minimize air and VOC exchange and arranged in a randomized design within the growth cabinets. Treated I-plates were incubated at 22°C with a 12-/12-hr light/dark photoperiod.

Plant Growth Measurements

Fourteen days after inoculation total leaf surface area (TLSA) was measured by an integrated digital video image analysis system, AGVISION system (AgImage Plus Version 1.08, Decangon Devices, Inc. and Panasonic CCTV camera MODEL WV-BL200 both Pullman, WA).

Disease Assay

Fourteen days after initial media inoculation, 5 μ l-suspensions of *E. carotovora* subsp. *carotovora* (10^8 CFU ml⁻¹) were drop-inoculated onto five leaves per *A. thaliana* seedling. Leaves exhibiting soft rot symptoms were determined by visual inspections 24 hrs after inoculation. Numbers of symptomatic leaves per seedling were counted as a measure of disease severity. Population densities of *E. carotovora* subsp. *Carotovora* were measured at 24 hr after inoculation by weighing plant leaf tissue macerated in SDW (1 ml) and plated on TSA plates containing 20 μ g/ml kanamycin as a selection marker. The strain of *E. carotovora* used is kanamycin resistant, which reduces the risk of contamination by other bacterial species.

Bacterial volatiles characterization

Volatiles were collected from strains GB03, IN937a, and *E. coli* DH5a as well as from uninoculated media. The strains were grown on MS medium for 24 h at 28°C before collection of volatiles. Individual plates were placed on a sliding glass plate inside a closed Teflon-framed chamber (18 x 18 x 3 cm). Charcoal-purified air was humidified by bubbling through a supersaturated NaCl solution and passed over the bacterial culture at a rate of 1 L min⁻¹. A sterile cotton plug was placed at the inlet of each chamber. Bacterial volatiles were collected by pulling 0.5 L min⁻¹ by vacuum through Super-Q adsorbent traps located at the other end of the chamber. Collection chambers were placed under metal halide and sodium lamps for a 16-h/8-h light/dark photoperiod with a total intensity of 700 μ mole/m²/sec and kept at 28°C. Volatiles were collected at intervals of 24 h over a period of 6 days. Compounds were extracted from filters with 150 μ l of dichloromethane. Nonyl acetate (800 ng) was added as an internal standard. Extracts to be used for biological testing were pooled without the addition of the internal standard.

Chemical extracts were analyzed by capillary GC. For comparisons of the same compound under different treatments, response factors for individual compounds were assumed to be equal. Selected samples were also analyzed by GC-MS on a (ion trap) mass spectrometer (GCQ plus, Thermoquest, Austin, TX) interfaced to a gas chromatograph (Trace GC2000) and operated in the electron impact mode. The components of the bacterial volatile emission were identified by comparison of GC retention times with those of authentic standards and by comparison of mass spectra with spectra of an EPA/NIH database.

RESULTS

Growth Promotion and ISR by Air-Borne Bacterial Signal(s)

Inoculation with two of six strains, GB03 and IN937a, significantly promoted growth and ISR against *E. carotovora* compared to the water and DH5 α controls (data not shown). Growth promotion and ISR activated in *Arabidopsis* by PGPR VOCs was assayed in the laboratory by physically separating seedlings from PGPR on divided Petri-dishes (referred to as I-plates) so as to allow only airborne signals to be transmitted between bacterial cultures and the plant seedlings.

Bacterial VOCs Mimic Growth Promotion and ISR Triggered by PGPR

Compounds 2,3-butanediol and 3-hydroxy-2-butanone (also referred to as acetoin) were consistently released from the GB03 and IN937a strains, while these metabolites were not released from DH5a, 89B61, or MS media alone (data not shown). Dodecane, 2-undecanone, 2-tridecanone, and 2-tridecan-1-ol were produced only from the GB03 strain, while tetramethyl pyrazine was detected at significantly higher levels from GBO3 than that released from IN937a and DH5a. Decane and undecane were released at low levels from all bacterial strains. Decanal was detected in the medium even without bacterial exposure (Fig. 1A)

The role of 2,3-butanediol in Growth Promotion and ISR

The *B. subtilis* mutants BSIP1173 and BSIP1174 do not produce acetoin or 2,3-butanediol due to an insertional knockout of the acetolactate synthase operon that controls the penultimate step in acetoin formation (pyruvate to acetolactate conversion) as well as acetolactate dehydrogenase, the enzymatic step that converts acetoin to 2,3-butanediol (Ramos et al., 2000). The absence of acetoin and butanediol VOC emissions was tested directly against the wild-type strain 168 that is fully functional in acetoin and 2,3-butanediol synthesis. With comparable growth for all strains on MS media, volatiles of strain GB03, from wild-type (2,3-butanediol-producing) strain 168, exhibited growth promotion and ISR, while no disease protection occurred with two mutants lacking production of 2,3-butanediol (BSIP1173 and BSIP1174) or with *E. coli* DH5 α . Significant reduction in disease severity resulted from treatment with the chemical control, SA, that is a signaling molecule known to activate disease resistance in *Arabidopsis* (Delaney et al., 1995).

Volatile extracts collected from strains GB03 and IN937a were tested for biological activity and were found to reduce disease severity significantly compared to the dichloromethane (solvent) control (Fig. 2). Exposure of *A. thaliana* to volatile extracts collected from DH5a had no effect on ameliorating disease severity, which was comparable to the solvent control.

Screening Signaling-Pathway Mutants and Transgenic Plants for Regulatory-Control of Growth Promotion and ISR

To begin to elucidate the signal pathway(s) that relates to growth promotion and ISR, a series of mutant and transgenic plant lines were exposed to PGPR VOCs that we found to trigger growth promotion and ISR. Disease severity was reduced by exposure to VOCs from both strains GB03 and IN937a for mutant lines including a coronatine/JA insensitive line *coil*, a SA-degrading line NahG, a constitutively producing PR line *crp1*, and a line that is SA-insensitive or non-expresser of PR genes *npr1*. Of the mutants tested, only in the ethylene-insensitive line *ein2*, when exposed to VOCs from strain GB03, was the severity of disease symptoms not ameliorated (Table 1).

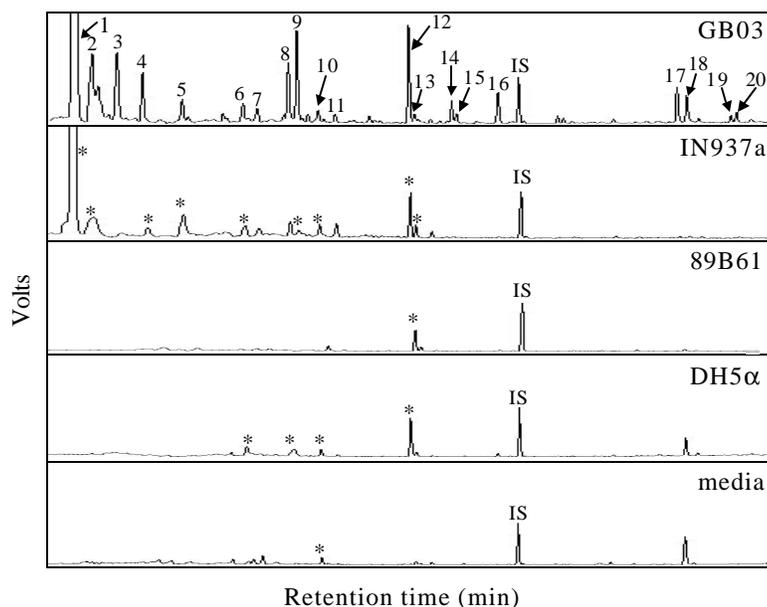


Fig. 1. Chromatographic profiles of volatiles from bacteria strains IN937a and GB03, both of which promote growth by the emission of volatile chemicals, compared with a growth promoting strain 89B61 that does not trigger promotion by volatile emissions, a non-growth promoting bacterial strain DH5 α , and an uninoculated media control. Compounds positively identified include 3-hydroxy-2-butanone [1], 2,3-butanediol [2], decane [6], tetramethyl pyrazine [9], undecane [10], decanal [13], dodecane [14], 2-undecanone [16], 2-tridecanone [17], and 2-tridecanol [18]; nonyl acetate was added as an internal standard [IS]. Asterisks in the lower chromatograms designate compounds that align with numbered peaks above.

DISCUSSION

The discovery that bacterial-produced VOCs trigger plant growth enhancement and induced systemic resistance constitutes a novel mechanism for rhizobacteria – plant interaction. Of the PGPR tested, two of seven strains (*Bacillus subtilis* GB03 and *B. amyloliquefaciens* IN937a) elicited constitutively growth promotion and ISR of *Arabidopsis* seedlings, suggesting that synthesis of bioactive VOCs is a strain-specific phenomenon.

Table 1. Growth promotion response of *A. thaliana* mutants planted on I-plates with airborne exposure to GB03 and IN937a strains.

Strain	Description	Growth promotion		Induced resistance	
		GB03	IN937a	GB03	IN937a
Col-0, C24, WS, Ler	wild-types	++ ¹	++	++	++
<i>ein2</i>	cytokinin- and ethylene-insensitive	--	++	--	++
<i>cbb1</i>	brassinosteroid-insensitive	++	++	++	++
<i>gai2</i>	gibberellic acid-insensitive	++	++	++	++
<i>eir1</i>	auxin-transport deficient and ethylene-insensitive	++	++	++	++
<i>etr1</i>	ethylene-insensitive	++	++	++	++
<i>cre1</i>	cytokinin-receptor deficient	--	++	--	++
<i>coil</i>	Jasmonate insensitive	++	++	++	++
<i>NahG</i>	Salicylic acid deficient	++	++	++	++
<i>NPRI</i>	Salicylic acid insensitive	++	++	++	++

¹Symbols indicate that VOCs from the particular bacterial strain did (++) or did not (--) result in significant growth promotion or induced resistance of *Arabidopsis* seedlings in I-plate assays relative to a water (control) treatment.

Results of our chemical and biochemical studies indicate that 2,3-butanediol is an essential bacterial component responsible for airborne chemical signaling triggering growth promotion and ISR in *Arabidopsis* based on several experimental results. By comparative analysis of volatile profiles of growth promoting and non-growth-promoting bacterial strains, we found that the release of 2,3-butanediol and acetoin was distinct from other VOCs in that these C4 components were detected exclusively in strains GB03 and IN937a that triggered plant growth promotion by VOC emissions (Fig. 2).

The rationale for testing various mutant lines of *Arabidopsis* was to probe already characterized biosynthetic pathways as potential regulatory sites for triggering growth promotion. The observation that VOCs from strain IN937a induced growth promotion on all mutant lines tested (Table 1) indicates that the physiological basis for growth promotion was not associated with the gaseous plant regulator ethylene. While the VOCs from the second PGPR strain, GB03, stimulated growth for several of the mutants, there were exceptions with the cytokinin/ethylene-insensitive mutant, *ein2*, and the cytokinin-receptor deficient mutant *cre1*. We confirmed the lack of growth promotion of *ein2* by VOCs from GB03 in subsequent greenhouse tests (data not shown). Based on the results with *ein2* and *cre1*, the cytokinin and ethylene signaling pathway appears to play some role in growth promotion and ISR with exposure to GB03 VOCs.

From a whole plant perspective, it remains to be determined whether growth promotion by PGPR VOCs occurs in soil or soil-less media. It is possible that volatiles produced by PGPR while colonizing roots are generated at sufficient concentrations to trigger plant responses. Indeed, with the low partial pressure of O₂ in the root environment, activation of the acetoin pathway is certainly possible. Measures of VOCs from PGPR present in soil systems will be studied in due course.

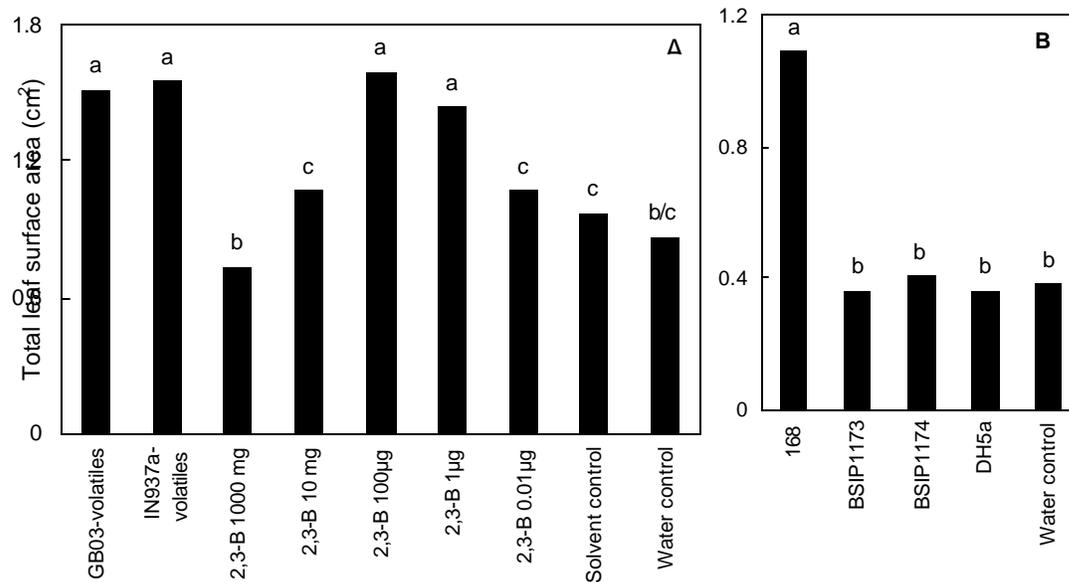


Fig. 2. Growth promotion of *A. thaliana* ecotype Col-0 with (A) exposure to extracted bacterial volatiles from growth promoting (GB03 and IN937a) and non-growth promoting bacteria (DH5 α) and synthetic 2,3-butanediol; (B) exposure to volatiles released from *B. subtilis* wild-type (168) and mutant strains defective in the production of 2,3-butanediol (BSIP1173 and BSIP1174). Different letters indicate significant differences between treatments according to LSD at $P = 0.05$.

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