

Symposium

The 7th Molecular Plant-Microbe Interactions

December 3, 2004, Seoul, Korea

Invisible Signals from the Underground: Bacterial Volatiles Elicit Plant Growth Promotion and Induce Systemic Resistance

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(Received on January 17, 2005; Accepted on February 11, 2005)

Plant growth-promoting rhizobacteria (PGPR) are a wide range of root-colonizing bacteria with the capacity to enhance plant growth and control plant pathogens. Here we review recent progress 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 released exclusively from the PGPR strains triggered the greatest level of growth promotion and induced systemic resistance. Pharmacological applications of 2,3-butanediol promoted the plant growth and induced resistance, while bacterial mutants blocked in 2,3-butanediol and acetoin synthesis was devoid of growth-promotion and induced resistance capacities. The results suggested that the bacterial VOCs play a critical role in the plant growth promotion and induced resistance by PGPR. Using transgenic and mutant lines of *Arabidopsis*, we provide evidences 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 bacterial VOCs as initiators of both plant growth promotion and defense responses in plants.

Keywords : bacterial volatiles, induced systemic resistance, PGPR, volatile organic compounds

Free-living root colonizing bacteria (rhizobacteria) have been studied for the past century as possible inoculants for

increasing plant productivity and controlling microbial pathogens (Kloepper, 1992). Soil or seed applications with plant growth-promoting rhizobacteria (PGPR) have been used to enhance the growth of several crops (Glick, 1995) as well as to suppress the growth of plant pathogens (Kloepper et al., 2004). PGPR that colonize root systems through seed applications and protect plants from foliar diseases include *Pseudomonas* spp., *Bacillus* spp., *Paenibacillus* spp., and *Serratia* sp. (Kloepper et al., 1999, 2004; Pieterse et al., 2002). The mechanisms for plant growth promotion and induced systemic resistance (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 (Glick, 1999; Kloepper, 1991; Timmusk et al., 1999). Recently, the phenomenon that PGPR elicit plant defense has also been found to lead to a state of 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 (Conrath et al., 2002). ISR is different from systemic acquired resistance (SAR) that triggers systemically plant defense response following hypersensitive response after inoculation of plant pathogens (Durrant and Dong, 2004; Mysore and Ryu, 2004; Ryals et al., 1996; van Loon et al., 1998). Previous works demonstrated that several bacterial determinants such as siderophores, salicylic acid (SA), and lipopolysaccharides (LPS) contributed to ISR (van Loon et al., 1998). In the further studies to define defense signaling components of plant, many researchers found novel plant defense signaling

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using *Arabidopsis* as a model plants (Pieterse et al., 1996; Ryu et al., 2003b, 2004ab; Ton et al., 2002). These signaling pathways turned out to be dependent on jasmonic acid and ethylene dependent but independent on SA, which differed from SAR signaling pathway (Pieterse et al., 2002). More recently, some exceptions of this ISR signaling were reported suggesting that plant defense signaling is more complex than we have elucidated (Iavicoli et al., 2003; Ryu et al., 2003b, 2004a).

Volatile organic compounds as signal molecules

Plants and microorganisms abound with natural chemicals, many of which are volatiles. These molecules are chemically diverse, representing fatty acid derivatives, terpenes, indoles, and molecules from other chemical families (Paré and Tumlinson, 1999). Ethylene, a molecule from other chemical development and defense, was the first gaseous hormone discovered in nature (Bleeker and Kende, 2000). New and fundamental insights could emerge from the study of other plant volatiles that can act as signals in plant and microorganisms or can be released selectively, converting the immediate environment of the producer. Methyl jasmonate (MeJA) is a well characterized airborne signal molecule that can trigger defense responses in plants. The role of MeJA was originally identified with respect to the wound induced expression of proteinase inhibitors that protect plants against digestive Serine proteinases of herbivorous insects (Codero et al., 1994; Farmer and Ryan, 1990). MeJA has also been found to induce phytoalexin accumulation in bean and barley (Croft et al., 1993; Weidhase et al., 1987) as well as indolyl glucosinolates in oilseed rape leaves (Doughty et al., 1995). C_6 -volatiles are released from all green plant tissues with insect or mechanical damage and include unsaturated C_6 -alcohol and aldehydes that are produced from a branch of the LOX pathway catalyzed by the hydroperoxide lyase (HPL) enzyme (Hatanaka et al., 1987). Certain C_6 -components when released proximal to plants can reduce herbivore feeding (Hildebrand et al., 1993) and seed germination frequency (Gardener et al., 1990), as well as phytoalexin induction (Zeringue, 1992). Recently, molecular data demonstrated that aerial treatment of *Arabidopsis* and Lima bean with the synthetic C_6 -volatile (*E*)-2-hexenal induces the transcription of defense related genes including *lox* and *pal* (Arimura et al., 2000; Bate et al., 1998). C_6 -volatile treatment also triggers VOC emissions in tomato (Farag and Paré, 2002) with (*Z*)-3-hexen-1-ol the most potent of the volatiles tested.

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. Recently Ryu and his coworkers discovered 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 tested in the study were previously shown to elicit growth promotion and ISR on several crops against fungal, bacterial, and viral pathogens under greenhouse and field conditions (Lui et al., 1995; Murphy et al., 2003; Ryu et al., 2003b, 2004b; Wei et al., 1991; Zehnder et al., 1999). Current review focuses on the volatiles produced by selected PGPR strains *B. subtilis* GB03 and *B. amylo-quefaciens* IN937 and describes the role of the compounds during plant development and defense responses.

Bacterial volatiles elicited growth promotion and ISR

Initially, we found that bacterial volatiles are probably involved in plant growth promotion in a process of developing an assay system to assess growth promotion capacity of rhizobacteria *in vitro* (Ryu et al., 2004c). Assessment of growth promotion induced by bacterial volatiles in *Arabidopsis* revealed that inoculation with GB03 and IN937a strains significantly promoted the growth of *Arabidopsis* and induced systemic resistance against *E. carotovora* compared to that by water or DH5a treated controls. The growth promotion and ISR activated in *Arabidopsis* by PGPR VOCs were assayed in a laboratory condition physically separated 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 (Ryu et al., 2003a, 2004a). 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 (Fig. 1). Dodecane, 2-undecanone, 2-tridecanone, and 2-tridecan-1-ol were produced only from the strain GB03, while tetramethyl pyrazine was detected at significantly higher levels from strain GB03 than that released from strains 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. 1). Following the discovery that bacterial-produced VOCs trigger plant growth enhancement, we tested bacterial VOCs elicited ISR in *Arabidopsis*. Of the PGPR tested, two of seven strains (*B. subtilis* GB03 and *B. amyloquefaciens* IN937a) elicited constitutively growth promotion and ISR of *Arabidopsis* seedlings, suggesting that synthesis of bioactive VOCs is a strain-specific phenomenon. Results of our chemical and biochemical

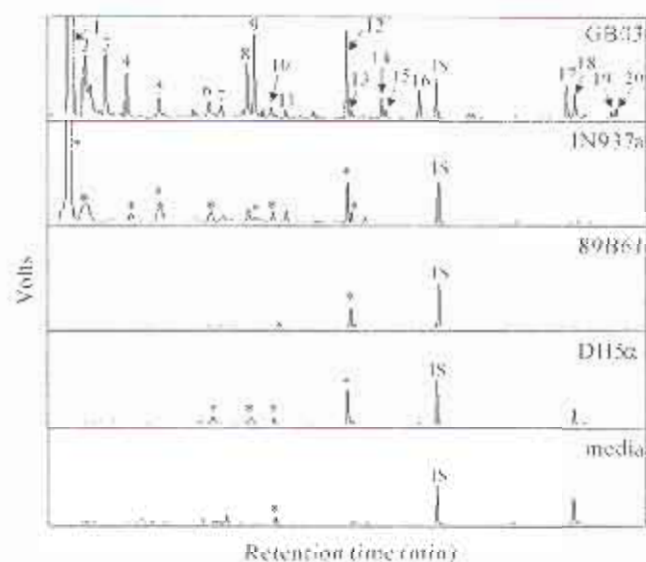


Fig. 1. Chromatographic profiles of volatiles from bacteria strains IN937a and GB03, both of which promote growth by the emission of volatile chemicals, compared with a growth promoting strain 89B61 that does not trigger promotion by volatile emissions, a non-growth promoting bacterial strain DH5 α , and an un inoculated 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 [15], 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.

studies indicated 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. Bacterial volatiles were further identified by mass spectral and gas chromatography analysis in comparison with synthetic standards (Ryu et al., 2004a). The stereochemistry of (2*R*,3*R*)-(-)-2,3-butanediol was determined by retention time comparisons with authentic standards (2*S*,3*S*)-(+)-2,3-butanediol, (2*R*,3*R*)-(-)-2,3-butanediol and the meso (*R*,*S*)-2,3-butanediol on a chiral GC column. The *S,S* and *R,R* isomers were separated by *ca.* 1 min while the meso form came over 7 minutes later. Acetoin, the oxidized precursor of (2*R*,3*R*)-(-)-butanediol is converted in *Bacillus* sp. by a NADH mediated acetoin reductase reaction (Ramos et al., 2000). Assuming an absence of a racemase enzyme, the acetoin intermediate would also be present in the *R* conformation as

(3*R*)-hydroxy-2-butanone. The biological activities of stereoisomer and regioisomers of 2,3-butanediol as well as other low molecule weight bacterial volatiles and synthetic analogues in triggering plant growth promotion/ISR are yet to be established.

2,3-butanediol is a major bacterial volatile in growth promotion and ISR

To study the role of 2,3-butanediol as a major bacterial determinant on growth promotion and ISR in *Arabidopsis*, we conducted an experiment with bacterial mutants that were defective production of 2,3-butanediol. 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 synthesis (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 growth promotion/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 the treatment with a control chemical, SA that is a signaling molecule known to activate disease resistance in *Arabidopsis* (Ryals et al., 1995). Volatile extracts collected from strains GB03 and IN937a were tested for biological activity and they reduced disease severity significantly compared to the dichloromethane (solvent) control. Exposure of *A. thaliana* to volatile extracts collected from DH5 α had no effect on ameliorating disease severity, which was comparable to the solvent control. Furthermore, pharmacological applications of 2,3-butanediol triggered growth promotion and ISR, while bacterial mutants blocked in 2,3-butanediol and 3-hydroxy-2-butanone synthesis were devoid of growth promotion and ISR capacities (Ryu et al., 2003a, 2004a).

Signal pathways for growth promotion and ISR by bacterial volatiles

To elucidation of 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. The rationale for testing various mutant lines of *Arabidopsis* was to probe already characterized biosynthetic pathways as potential

regulatory sites for triggering growth promotion and ISR. Mutant lines included a jasmonic acid-insensitive line (*coi1*), an ethylene-insensitive line (*ein2*), a salicylic acid-degrading line (NahG), a salicylic acid constitutively expression of pathogenesis related proteins line (*cpr1*), and a line that lacks synthesis of salicylate and activation of the regulatory gene *npr1*. The observation that VOCs from strain IN937a induced growth promotion on all mutant lines tested 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. 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.

Disease severity was reduced by exposure to VOCs from both strains GB03 and IN937a for mutant lines including a coronatine/IA insensitive line *coi1*, a SA-degrading line NahG, a constitutively producing PR line *cpr1*, and a line that is SA-insensitive or non-expresser of PR genes *npr1*. Among 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. Mutant lines included a jasmonic acid-insensitive line (*coi1*), an ethylene-insensitive line (*ein2*), a salicylic acid-degrading line (NahG), a salicylic acid constitutively expression of pathogenesis related proteins line (*cpr1*), and a line that lacks synthesis of salicylate and activation of the regulatory gene *npr1*. VOCs from strain IN937a elicited ISR on all of these lines. Hence, elicitation of ISR by VOCs of IN937a is independent of jasmonic acid, ethylene, salicylic acid, and *npr1*. Such a pattern of signal pathway has not been previously reported with ISR elicited by bacteria and therefore, it is likely that VOCs of IN937a elicit a distinct, and uncharacterized, signaling pathway in *Arabidopsis*.

Perspectives

To date, application of chemicals to enhance plant growth or induced resistance in plants is limited due to some negative effects of chemical treatment and difficulty in determining the optimal concentrations to benefit the plant. For alternative means to solve these problems, biological applications have been extensively studied. Collectively, our description on bacterial volatiles eliciting growth promotion and ISR suggested that the bacterial volatile producers could be an environmentally sound means to grow and protect plants under greenhouse or field condi-

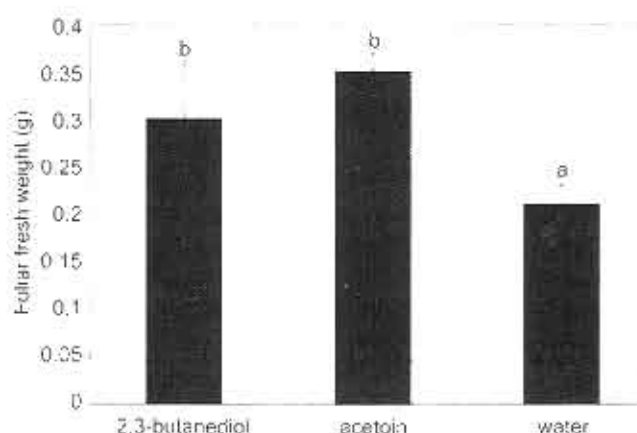


Fig. 2. Enhanced fresh weight in *Arabidopsis thaliana* - 3 weeks subsequent to soil drenching with indicated synthetic growth promoters, respectively. Different letters indicate significant differences using Fisher's LSD test at $P = 0.05$.

tions better. From the whole plant perspective, it still remains to be determined whether growth promotion by PGPR VOCs occurs in soil or soil-less media. Recently, we found that exogenous application of racemic 2,3-butanediol or acetoin directly to soil grown *Arabidopsis* seedlings elicited growth promotion and induced resistance. Rhizosphere drenching with either acetoin or 2,3-butanediol increased foliar fresh weight of *Arabidopsis* by 66% and 43%, respectively, over water treated controls (Fig. 2). We have yet to determine if acetoin directly promotes plant growth or is serving as a substrate for the production of 2,3-butanediol by bacteria present in the rhizosphere. In *Nicotiana benthamiana* a similar pattern of enhanced growth was observed with soil drenching of 2,3-butanediol when compared to those plants treated with pure water (unpublished data). From these preliminary results, we hypothesize that 2,3-butanediol synthesized by the plant could also serve as a stimulant triggering growth promotion. The two cheap chemicals can be applied directly cultivation system in the field. However, many questions still remain unanswered. To understand the nature of bacterial volatiles, analysis of derivatives of 2,3-butanediol and gene expression profiling of *Arabidopsis* genes responding bacterial volatiles can be conducted. 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 O_2 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 near future.

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