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GC–MS SPME profiling of rhizobacterial volatiles reveals prospective inducers of growth promotion and induced systemic resistance in plants

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Abstract

Chemical and plant growth studies of Bacilli strains GB03 and IN937a revealed that the volatile components 2,3-butanediol and acetoin trigger plant growth promotion in Arabidopsis. Differences in growth promotion when cytokinin-signaling mutants are exposed to GB03 versus IN937a volatiles suggest a divergence in chemical signaling for these two bacterial strains. To provide a comprehensive chemical profile of bacterial volatiles emitted from these biologically active strains, headspace solid phase microextraction (SPME) coupled with software extraction of overlapping GC-separated components was employed. Ten volatile metabolites already reported from GB03 and IN937a were identified as well as 28 compounds not previously characterized. Most of the newly identified compounds were branched-chain alcohols released from IN937a, at much higher levels than in GB03. Principal component analysis clearly separated GB03 from IN937a, with GB03 producing higher amounts of 3-methyl-1-butanol, 2-methyl-1-butanol and butane-1-methoxy-3-methyl. The branched-chain alcohols share a similar functional motif to that of 2,3-butanediol and may afford alternative structural patterns for elicitors from bacterial sources.

Keywords: *Bacillus subtilis*; *B. amyliquefaciens*; Ehrlich pathway; 3-Methyl-1-butanol; Plant growth promoting rhizobacteria (PGPR); Principal component analysis (PCA); Solid-phase microextraction (SPME); Volatile organic compound (VOC)

1. Introduction

While solvent extraction of biological materials may be effective in obtaining an appreciable fraction of natural products present in plant tissue, headspace analysis provides a more representative sampling of volatile components that can provide chemical signals via airborne transmission. Mounting chemical and biochemical data indicate that plants perceive and respond to VOCs present in the environment (Farmer, 2001; Paré et al., 2005). Plant VOCs can serve as signals between neighbors, whereby defense mechanisms are induced in undamaged plants in response to volatiles produced by near by infested plants. Specific plant volatiles, methyl jasmonate, jasmone and C6-volatiles have been implicated in such defense mechanisms (Arimura et al., 2001; Weber, 2002; Farag et al., 2005). In the case of plant bacteria interactions we recently, reported that a blend of air-borne chemicals released from specific bacterial strains of plant growth promoting rhizobacteria (PGPR) can trigger growth promotion and induced systemic resistance (ISR) in neighboring Arabidopsis seedlings (Ryu et al., 2003). In particular, two of seven tested PGPR strains, *Bacillus subtilis* GB03 and *B. amyloliquefaciens* IN937a elicited growth promotion and ISR *via* bacterial VOC emissions, suggesting that synthesis of bioactive VOCs is a strain-specific phenomenon. Volatile components 2,3-butanediol and 3-hydroxy-2-butanone (acetoin) were detected at comparable levels from GB03 and IN937a strains, while these metabolites were not

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detected from other non-growth promoting bacterial strains. Pharmacological application of acetoin and 2,3-butanediol triggered growth promotion and ISR response in Arabidopsis, suggestive that these C4-components could be in part bacterial components responsible for airborne chemical signaling in GB03 and IN937a. Further screening of signaling pathway mutants and transgenic plants for regulatory control of growth promotion and ISR mediated by GB03 and IN937a volatiles displayed striking differences among both strains. While volatiles released from GB03 triggered growth promotion and ISR through a cytokinin and ethylene–signaling pathways, IN937a volatiles appeared to operate through a cytokinin and ethylene–independent pathways. These findings cannot be explained in view of their similar volatile profiles (Ryu et al., 2004) and implicate the presence of other undetected or identified volatiles that can operate via different signaling pathways in plants.

Head space volatiles can be extracted from an air flow onto an absorbent filter and then released by rinsing with organic solvent or collected in the absence of air flow by solid phase micro extraction (SPME) and directly released into a heated GC injector. The first analysis of PGPR volatile emissions involving growth promotion was preformed using absorbent filters (Ryu et al., 2003), although several factors may limit the suitability of using adsorbent filters for the routine analysis of bacterial VOC emissions. A continuous air flow over bacteria cultures may not closely mimic low oxygen partial pressures usually present in a root environment where PGPR naturally resides (Kloepper et al., 1999) and low adsorption affinity of certain resins for low molecular weight metabolites can reduce collection yields. SPME, in contrast can extract volatiles from bacterial cultures in a relatively short space of time, usually <30 min in a no flow, low oxygen environment, and has been established as effective in the collection of bacterial VOCs for several reported systems (Goupry et al., 2000; Marilley and Casey, 2004). Despite such advantages of SPME, fiber coatings can limit sensitivity by preferentially absorbing or excluding particular analytes based on polarity or size. For example, PDMS fiber preferentially adsorbs non-polar metabolites while divnylbenzene/carboxen/PDMS (DCP) fiber favors short chain polar compounds (Jelen et al., 2000; Doleschall et al., 2003). Fiber surface area can also limit analyte absorption capacity (Ishikawa et al., 2004).

In this paper, SPME combined with gas chromatography–mass spectrometry (GC–MS) was utilized for profiling of volatile blends in GB03 and IN937a. Volatiles were also profiled in two non-growth promoting strains DH5α and 89B61 serving as a negative control. Results from this study led to the detection of apparent VOCs differences between GB03 and IN937a. Major branched-chain alcohols 3-methyl-1-butanol, 2-methyl-1-butanol and their conjugates were detected in IN937a, albeit not in GB03. Differences in volatile profiles might account for the dissimilarity observed among both strains in triggering growth promotion via volatile chemical signals, and suggests the existence of diverse VOC metabolism existing in two closely related *Bacillus* species.

2. Results

2.1. Method development

The SPME coatings 7 μm PDMS and 50/30 μm DCP were initially tested for the extraction of chemical standards including: 2,3-butanediol, penten-2-1-ol, and β-caryophyllene prepared at concentrations of 0.25 mg μl^{-1}, 6 μg μl^{-1} and 0.1 μg μl^{-1}. DCP fibers were selected over PDMS fibers since detection of 2,3-butanediol and penten-2-1-ol was only possible with DCP fibers at a low concentration of 100 ng μl^{-1}. Further optimization was performed using DCP fibers by varying exposure time and extraction temperature, for a optimal setting of 1 h and 60 °C, respectively (data not shown).

Using SPME to analyze volatiles from 4 PGPR strains GB03, IN937a, DH5α and 89B61, a total of 38 volatile components (Table 1) were recovered, of which 10 were previously identified (Ryu et al., 2004). Increase in identification was due in part to a higher sensitivity of the DCP fiber towards low molecular weight volatiles and processing of MS files with AMDIS software that assists in adjacent peak deconvolution, background subtraction and increased detection limit (Halket et al., 1999). Identified VOCs belonged mostly to six classes, including 4 aldehydes, 4 esters, 7 alcohols, 3 acids, 5 ethers, 5 ketones, 3 sulphur compounds, 6 hydrocarbons and carbon dioxide (Table 1). Most of the compounds were produced from the four strains, albeit at different amounts and with few compounds specific only for strain IN937a. IN937a was among the strains producing VOCs in the largest concentrations followed by GB03.

2.1.1. Alcohols

Compounds 2,3-butanediol, ethanol, 1-propanol-2-methyl and 1-butanol were consistently released at comparable levels from growth promoting strains GB03 and IN937a. Conversely, 3-methyl-1-butanol and 2-methyl-1-butanol were produced from IN937a at significant higher levels 200- and 40-fold, respectively, than that detected from GB03. A representative gas chromatogram is shown in Fig. 1 and indicates the differences in volatile emission patterns between the two strains (Note that because SPME fiber is more sensitive to lighter compounds, the peaks to the right in the GC traces are considerably larger than would occur if all compounds were samples with equal sensitivity). 3-methyl-1-butanol and 2-methyl-1-butanol (also referred to as fusel alcohols) are produced by enzymatic conversion of branched chain amino acids (BCAAs) i.e., leucine and isoleucine via the Ehrlich pathway (Dickinson et al., 1997; Marilley and Casey, 2004). The pathway involves the concerted action of a transaminase, a

Table 1
VOC profile of PGPR strains: *Bacillus amyloliqefaciens* (IN937a), *B. subtilis* (GB03), *Escherichia coli* (DH5α) and *Pseudomonas fluorescens* (89B61)

RT (min)	Compound	IN937a	GB03	DH5α	89B61
Alcohols (a.u. or μg)					
4.49	Ethanol *$	10[a]	7[a]	2[b,c]	3.6[c]
6.36	1-propanol-2-methyl *$	29[a]	62[a]	6[b]	3.5[b]
7.13	1-Butanol *$	3.6[a]	3.4[a]	0.5[b]	2.2[c]
8.49	1-Pentanol *$	66[a]	1[b]	ND	0.4[b]
8.56	1-Butanol-3-methyl *$	3961[a]	20[b]	19[b]	9[b]
8.61	1-Butanol-2-methyl	290[a]	8[b]	0.9[b]	4[b]
9.64	2,3-Butanediol *$	119[a]	257[a]	12[b]	4[b]
Aldehydes (a.u. or μg)					
6.87	Butanal-3-methyl	29[a]	77[b]	15[c]	25[c,a]
7.04	Butanal-2-methyl *$	0.3[a]	0.06[a]	ND	0.8[a]
12.37	2,4-Hexadienal	TR	TR	0.01[a]	0.01[a]
13.45	Benzaldehyde *$	0.003[a]	0.002[a]	ND	0.0005[b]
Acids (a.u. or μg)					
9.74	Glyoxylic acid	1[a]	0.8[a]	0.7[a]	0.94[a]
11.1	Butanoic acid-3-methyl *$	0.06[a]	0.3[a]	0.1[b]	ND
10.8	Acetic acid diethyl	6[a]	36[b]	2[a,c]	3[c]
Esters (a.u. or μg)					
6.11	Ethyl acetate *$	21[a]	49[a]	12[a,b]	3[b]
10.12	Acetic acid butyl ester *$	2.8[a]	0.1[b]	4[a,c]	ND
11.4	Butanol-3-methyl-acetate*$	127	TR	TR	TR
12.35	2-Butene-1-ol-3-methyl-acetate	0.15[a]	0.13[a]	0.1[a]	0.04[b]
Ethers (a.u. or μg)					
6.08	Furan-2-methyl *$	0.04[a]	0.1[b]	TR	TR
7.6	Furan-2-ethyl *$	0.03	ND	ND	ND
7.5	Butane-1-methoxy-3-methyl *$	7530	ND	ND	ND
8.83	Furan-tetrahydro-2,5-dimethyl	0.2	TR	TR	TR
13.7	Furan-2-pentyl *$	0.0005[a]	0.0006[a]	0.0003[a]	ND
18.52	Hexadecane	0.37[a]	0.6[a]	0.14[a]	0.4[a]
Hydrocarbons (a.u. or μg)					
4.86	Isoprene *$	483[a]	614[a,b]	125[a]	28[c]
7.53	Acetylene	9[a]	0.6[b]	0.12[c]	2[d]
9.78	Cyclohexane	0.1[a]	0.2[a]	ND	0.15[a]
15.42	1-Undecene	0.8[a]	1.2[a]	0.4[a,b]	0.2[b]
15.53	1-Undecane	1.5[a]	3[b]	0.2[a,c]	0.5[a]
17.1	Dodecane	0.7[a]	0.9[a]	0.15[b]	0.6[a]
Ketones (a.u. or μg)					
4.75	Acetone *$	0.2[a]	0.3[a]	0.5[a,b]	1.3[b]
5.74	2,3-Butanedione *$	38[a]	77[a]	14[b]	7[c]
5.85	2-Butanone *$	24[a]	44[b]	12[c]	2.5[d]
8.17	Acetoin *$	153[a]	226[a]	74[a]	0.1[b]
10.16	2-hydroxy-3-pentanone *$	3.5[a]	3.5[a]	0.04[b]	0.07[b]
S-containing compounds (a.u. or μg)					
4.37	Methanethiol *$	0.6[a]	0.1[a]	0.3[a]	0.04[b]
8.67	Dimethyl disulfide *$	4[a,d]	2.4[b]	2[b,c]	3.6[c,d]
13.48	Dimethyl trisulfide	0.12[a]	0.18[a]	0.1[a]	0.7[b]
Inorganic compound					
4.03	Carbon dioxide	1912[a]	3907[a]	1277[b]	600[c]

[a] Compounds marked with * were identified by comparison of RT and mass spectral data with those of authentic compounds, the others by comparison of mass spectral data with those of NIST library, ND not detected, TR traces.
[b] Results are means of triplicate experiments; ND, not detected; TR, traces.
[c] Values for compounds marked with $ are concentrations expressed in μg 24 h^{-1}, others unmarked are expressed as relative peak areas to (Z)-3-hexenyl acetate (IS) expressed in arbitrary units (a.u.).
[d] Values followed by the same letter within the same row are not significantly different ($P > 0.05$).

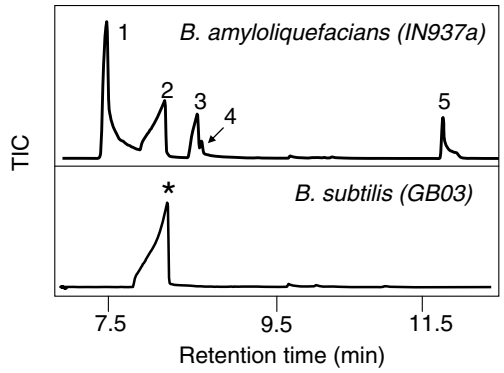

Fig. 1. Sections of the total ion current chromatograms of volatiles from IN937a and GB03 inoculated on MS media, both of which promote growth by the emission of volatile chemicals. Peaks: 1 = butane-1-methoxy-3-methyl; 2 = acetoin; 3 = 3-methyl-butanol; 4 = 2-methyl-butanol; 5 = 1-butanol-3-methyl-acetate. Asterisks in the lower chromatogram indicates compound that align with numbered peak above.

decarboxylase, and an alcohol dehydrogenase as in yeast and lactic acid bacteria (Fig. 2). In IN937a, products originating from leucine catabolism (3-methyl-1-butanol) were found at higher concentrations than corresponding products of isoleucine (2-methyl-1-butanol) and or valine (2-methyl-1-propanol) see Table 1.

2.1.2. Aldehydes

In contrast to alcohols, aldehyde peaks associated with bacterial VOCs did not show the same regimented emissions from growth-promoting or non-growth-promoting bacterial strains. 2-methyl-1-butanal, 2,4-hexadienal and benzaldehyde were all released at low levels from most bacterial strains. An exception to this pattern was for 3-methyl-1-butanal formed in significant higher amounts by GB03 than other bacterial strains.

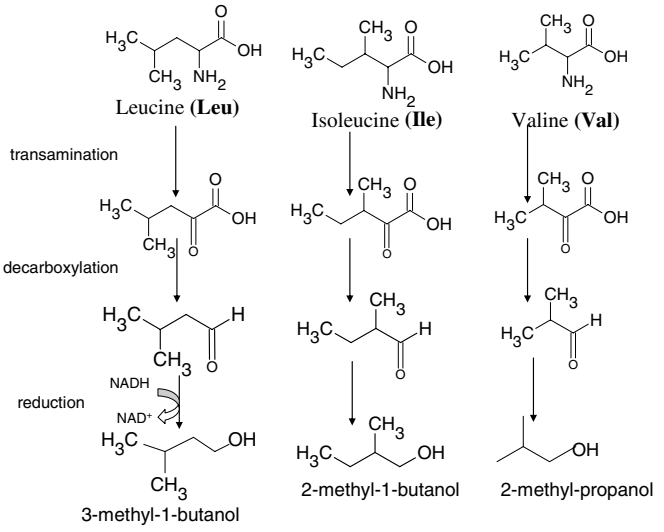

Fig. 2. Proposed catabolic pathway for the production of 3-methyl-1-butanol, 2-methyl-1-butanol and 2-methyl-1-propanol from L-leucine, L-isoleucine and L-valine, respectively (modified from Marilley and Casey, 2004).

2.1.3. Acids

3-Methyl-butanoic acid, oxidative product of 3-methyl-1-butanol was detected from both strains GB03 and IN937a. The presence of acid at very low levels in PGPR volatile blends versus its alcohol might reflect the redox state of the cells to favor the reductive branch of the Ehrlich pathway over the oxidative one as in yeast (Vuralhan et al., 2003).

2.1.4. Esters

Ethyl acetate, butyl acetate and 2-butene-1-ol-3-methyl acetate were released at significantly higher levels from GB03, IN937a and DH5α than from 89B61, where as butanol-3-methyl acetate was only produced from IN937a strain. Butanol-3-methyl acetate is likely to arise from acetylation of 3-methyl-1-butanol derived from leucine catabolism.

2.1.5. Ethers

Butane-1-methoxy-3-methyl was detected as a major volatile component in strain IN937a, not detected in all other strains. The prevalence of 3-methyl-1-butanol in IN937a volatile blend implicates its likelihood to provide its corresponding ether butane-1-methoxy-3-methyl via a methyl transferase activity. Other detected ethers include: furan-2-methyl, furan-2-ethyl, furan-2-pentyl and tetrahydro-2, 5-dimethyl furan, released from both GB03 and IN937a at very low levels.

2.1.6. Hydrocarbons

Hexadecane, cyclohexane, 1-undecene, 1-undecane and dodecane were released at low levels from all bacterial strains, whereas isoprene was detected as a major component in both strains GB03 and IN937a. Isoprene is formed via the methylerythritol phosphate pathway in GB03 (Wagner et al., 2000).

2.1.7. Ketones

Acetoin was released consistently from GB03 and IN937a at significant higher level than from other non-growth-promoting strains 89B61 and DH5α as previously recorded (Ryu et al., 2004). From our work, two new compounds showed similar emission patterns 2-hydroxy-3-pentanone and 2,3-butanedione. 2,3-Butanedione is produced chemically by oxidative decarboxylation of the intermediate α-acetolactate, a precursor to acetoin (Hugenholtz et al., 2000).

2.1.8. Sulphur containing compounds

Low levels of dimethyl disulfide and dimethyl trisulfide were found in all strains, whereas methanethiol showed significantly higher emission levels in strain IN937a. This class of compounds has not been previously identified in PGPR VOCs (Ryu et al., 2003, 2004) and is thought to derive from methionine and cysteine catabolism as in lactic acid bacteria (Seefeldt and Weimer, 2000).

2.2. Principal component analysis (PCA) of PGPR volatiles

To identify strain differences regarding volatiles production, PCA was performed on the VOCs data (relative abundances of a total of 38 identified compounds). PCA is a useful tool to clarify patterns in a complex data matrix. In PCA, a number of principal components (PC) are extracted, representing the largest variation through a swarm of data points (Wold et al., 1987). PCA analysis showed the separation of GB03 and IN937a from each other mostly along factor 1 and 3 overall explaining 80% and 59% of the variance. Whereas strain DH5α and 89B61 were both clustered together (Fig. 3). The scores plot shows that the reproducibility of the triplicate bacterial samples was good. Metabolite loadings, which define the most important components with respect to the clustering behavior, revealed that fusel alcohols 3-methyl-1-butanol, 2-methyl-1-butanol and their conjugates exhibited a large contribution to the segregation of strain IN937a from GB03. These compounds were consistently released as major VOCs from IN937a, however at very low levels in GB03 (Table 1).

2.3. In vivo detection of fusel alcohols from IN937a inoculated potato headspace

The media composition in which the bacteria are cultured has been shown to impact the VOC emission profile for several bacterial strains (Sprecher and Hanssen, 1982; Fiddaman and Rossall, 1994). To identify whether fusel alcohols can still be detected from IN937a cultured on plant roots, VOCs were collected from the head space of inoculated potato tubers. Unlike bacterial VOCs that can be easily sampled by head-space collections from bacteria growing on MS media, rhizosphere emissions by PGPR present the complication of de-adsorbing low molecular weight compounds from the soil matrix. By inoculating PGPR on sterile sliced potato tubers, the production of bacterial volatiles in a clean matrix without interference from the soil was possible. Very low emission levels were detected from uninoculated potato (Fig. 4). Of the three fusel alcohols detected from IN937a growing on MS media, only 3-methyl-1-butanol and its corresponding ether butane-1-methoxy-3-methyl were detected in the head space of IN937a inoculated potato (Fig. 4). The two growth promoting volatiles 2,3-butanediol and acetoin were consistently detected from GB03 and IN937a inoculated potato emissions, while these metabolites were not released from control potato tubers. 2,3-Butanediol and acetoin have only been identified from GB03 and IN937a cultured on MS media (Ryu et al., 2003, 2004). To our knowledge, these results provide the first evidence that PGPR bioactive VOCs, 2,3-butanediol and acetoin can be produced on plant roots where it naturally resides.

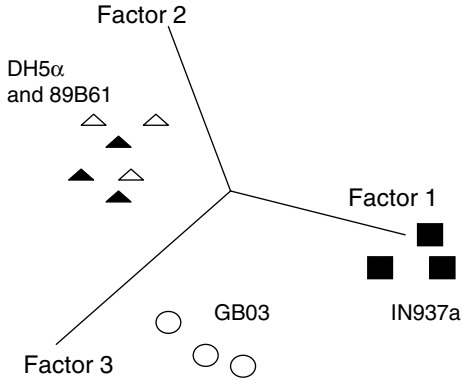

Fig. 3. PCA scatter plot showing the separation of strain GB03 (○) from IN937a (■). PGPR strains were inoculated on MS media and the produced VOC (38 compounds) were used in the analysis.

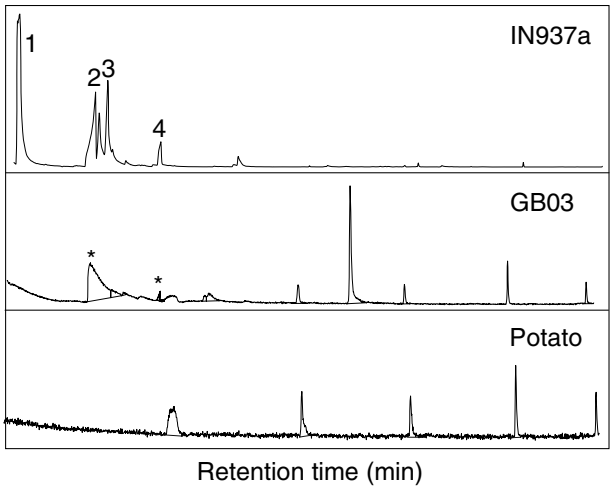

Fig. 4. SPME gas chromatograms of volatiles from bacteria strain IN937a and GB03 inoculated on potato tuber disc and uninoculated potato. Peaks: 1 = butane-1-methoxy-3-methyl; 2 = acetoin; 3 = 3-methyl-1-butanol; 4 = 2,3-butanediol. Asterisks in the lower chromatogram indicates compound that align with numbered peak above.

3. Discussion

Chemical and pharmacological studies with GB03 and IN937a have implicated 2,3-butanediol as being biologically in airborne signaling of growth promotion in Arabidopsis (Ryu et al., 2003). However, differences in growth promotion responses observed in Arabidopsis cytokinin signaling mutants *cre1* and *ein2* with GB03 and IN937a suggest that GB03 and IN937a produce different chemical profiles. To more thoroughly examine specific differences in VOC emission profiles, the two strains that trigger plant growth promotion by volatile signaling, GB03 and IN937a, as well as two control strains that do not trigger plant growth promotion via airborne signaling 89B61 and DH5α were chemically sampled by SPME and the GC–MS files were processed by AMDIS software allowing for resolution of complex volatile blends. Thirty-eight components were identified, of which 10 were previously detected.

The observed variation between GB03 and IN937a volatile profiles suggest that a diverse VOC metabolism exists among *Bacillus* species and supports the idea that VOCs can serve as taxonomic markers in microbial systems (Scholler et al., 2002). Four major VOCs, 2-methyl-1-butanol, 3-methyl-1-butanol, butanol-3-methyl acetate and butane-1-methoxy-3-methyl were released almost exclusively from strain IN937a, albeit at extremely low levels from GB03 when cultured on MS media. 3-Methyl-1-butanol was also identified as a major volatile component in the headspace of IN937a inoculated potato tubers, although not from GB03. The accumulation of 3-methyl-1-butanol, 2-methyl-1-butanol and 2-methyl-1-propanol in *Bacillus* species headspace volatiles suggest that they are formed as degraded products of leucine, isoleucine and valine amino acids, respectively, via the Ehrlich pathway (Fig. 2). There biogenetic origins can be unambiguously determined by feeding amino acids isotopes to IN937a cultures and monitoring for label enrichment in released fusel alcohols. Coupling these differential VOC profiles data in *Bacillus* PGPR strains (IN937a and GB03) with genes transcript levels can assist as well in probing involved genes and ultimate biosynthetic pathways. The correlative analysis of differential metabolic profiling and gene expression profiling in two different strains or cultivars has proven a powerful approach for the identification of candidate genes and enzymes, particularly those in secondary metabolism (Fridman and Pichersky, 2005).

Differences between GB03 and IN937a volatile emissions were observed at the plant signal-response level. While GB03 volatiles triggered growth promotion and ISR in Arabidopsis through a cytokinin-ethylene signaling pathway, IN937a volatiles appeared to be cytokinin-ethylene independent (Ryu et al., 2003). Whether the release of fusel alcohols and its conjugates only in IN937a volatile blend are directly responsible for the dissimilar activation of hormone respones is still under investigation. By exposing wild-type and cytokinin-ethylene mutant plants to these individual volatiles and probing for enhanced growth or ISR induction (Ryu et al., 2003), the role of these bacterial VOCs will be clarified.

4. Experimental

4.1. Bacterial samples cultured on MS media

Bacillus amyloliqefaciens IN937a, *B. subtilis* strain GB03, *Pseudomonas fluorescens* strain 89B61 and *E. coli* DH5α were grown in 20 ml vials on Murashige and Skoog medium containing 1.5% (w/v) agar, 1.5% (w/v) sucrose, and 0.4% (w/v) TSA for 24 h at 37 °C before collection of volatiles. Vials were sealed with a steel crimp cap fitted with a Teflon/silicon septum that was previously conditioned at 100 °C for 30 min. Strains were stored at −80 °C in TSA containing 20% glycerol. (Z)-3-Hexenyl acetate absent from bacterial cultures VOCs was used as an internal standard (IS), dissolved in water and injected at a concentration of 1 μg/vial.

4.2. Bacterial samples cultured on potato tubers

Solanum tuberusom potato slices were surface-sterilized (2-min, 70% ethanol soaking followed by a 20-min, 1% sodium hypochlorite soaking) and rinsed (four times) with sterile, distilled water in a sterile hood. Both plant materials were placed in a 20 ml vial and inoculated with different bacterial strains: *Bacillus subtilis* strain GB03, *B. amyloliqefaciens* IN937a, *Pseudomonas fluorescens* strain 89B61 and *E. coli* DH5α cultures.

4.3. Chemical analysis of volatiles

4.3.1. SPME analysis

Two commercially available SPME fibers suitable for volatile analysis available from Supelco (Bellefonte, PA) were examined for this study. These were poly(dimethylsiloxane) (PDMS; 7 μm) and stable flex divnylbenzene/carboxen/PDMS (DCP, 50/30 μm). Vials containing samples were placed in a heating block (Gerstel MultiPurpose Sampler MPS 2, Baltimore, MD) at 50 °C with the SPME fiber inserted into the headspace above the bacterial sample. Adsorption was timed for 30 min.

4.3.2. GC–MS parameters and analysis

SPME fibers were desorbed at 210 °C for 1 min in the injection port of an HP 5890A GC/MS(Hewlett–Packard, Palo Alto, CA) with a DB5 column (J& W Scientific, Folsom, CA) column (60 m, 0.25 mm i.d., 0.25 μm film thickness). GC–MS runs were 25 min, and the fiber remained in the injection port for 10 min after each run. The injection port was operated in splitless mode with a constant He flow of 1.0 ml/min. The initial oven temperature was 33 °C, held for 3 min, ramped at 10 °C min^{-1} to 180 °C, and ramped at 40 °C min^{-1} to 220 °C and held for 5 min. The HP quadrupole mass spectrometer was operated in the electron ionization mode at 70 eV, a source temperature of 200 °C, quadrupole temperature of 150 °C, with a continuous scan from m/z 40 to 500.

Positive identification of each chemical constituent was performed by comparison of its retention time and mass spectrum with that of authentic standard (when available). Tentatively identified compounds were uniquely identified on the basis of EPA/NIH database. Peaks were quantified by selected abundant fragments (m/z) to overcome the problem of co-eluted compounds (Halket et al., 1999). Twenty one compounds were quantified using calibration curves of authentic standards. High purity chemicals (generally higher than 99%) were purchased from Sigma–Aldrich (St Louis, MO): Ethanol, 2-methyl-1-propanol, 1-butanol, 1-pentanol, 3-methyl-1-butanol, 2,3-butanediol, 2-methyl-1-butanal, benzaldehyde, 3-methyl-butanoic acid, butyl acetate, 3-methyl-butyl acetate, 2-methyl-furan,

2-ethyl-furan, 2-pentyl-furan, isoprene, 2,3-butanedione, 2-butanone, acetoin, 2-hydroxy-3-pentanone, methanethiol, dimethyl disulfide. Samples were run in triplicate, and integrated areas were normalized on (*Z*)-3-hexenyl acetate and averaged.

4.4. Data and statistical analysis

Relative volatile abundances were calculated using a custom PEARL script to extract peak areas of individual ions characteristic of each component (Broeckling et al., 2006). Principal component analysis (PCA) was performed on normalized data sets with Pirouette (InfoMetrix, Woodinville, WA) software. Analysis of variance was run using JMP5 (SAS Institute Inc, Cary, NC) statistical software. Means were separated using Duncan's multiple range test at P value less than 0.05.

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