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Soil Bacteria Elevate Essential Oil Accumulation and Emissions in Sweet Basil

ERIKA BANCHIO,[†] XITAO XIE,[‡] HUIMING ZHANG,[‡] AND PAUL W. PARÉ*,[‡]

Departamento de Biología Molecular, FCEFQyN, Universidad Nacional de Río Cuarto, Campus Universitario, 5800 Río Cuarto, Argentina, and Department of Chemistry and Biochemistry, Texas Tech University, Lubbock, Texas 79409-1061

Plant growth-promoting rhizobacteria ameliorate environmental conditions for plants by facilitating nutrient uptake and mitigating disease susceptibility. While volatile chemicals from certain soil microbes are sufficient to elicit growth and defense responses in *Arabidopsis*, whether such volatile signals can induce essential oil accumulation and chemical emissions has yet to be reported. Here, we provide biochemical evidence that the plant growth-promoting soil bacterium *Bacillus subtilis* GB03 releases volatile chemicals that elevate fresh weight essential oil accumulation and emissions along with plant size in the terpene-rich herb sweet basil (*Ocimum basilicum*). The two major essential oil components from sweet basil, α-terpineol and eugenol, increased ca. 2- and 10-fold, respectively, in plants exposed to GB03 volatiles or with root inoculation as compared to water controls. On a fresh and dry weight basis, shoot and root biomass increases of ca. 2-fold were observed with GB03 volatile exposure or GB03 media inoculation as compared with controls. In testing the efficacy of GB03 volatiles to trigger plant growth and secondary compound production, a physical partition separating roots from bacterial media was provided to preclude nonvolatile microbial elicitors from contributing to GB03-stimulated basil responses. These results demonstrate that volatile bacterial elicitors can concomitantly increase essential oil production and biomass in an herbaceous species rich in commercially valued essential oils.

KEYWORDS: Aromatic herbs; *Bacillus subtilis*; basil; essential oils; *Ocimum basilicum*; plant growth-promoting rhizobacteria (PGPR); volatile organic compounds (VOCs)

INTRODUCTION

Plant growth-promoting rhizobacteria (PGPR) are naturally occurring soil microorganisms that colonize roots and stimulate plant growth. Such bacteria are applied to a wide range of agricultural crops for the purpose of growth enhancement, including increased seed germination, plant weight, and harvest yields (*1, 2*). PGPR colonization is proposed to trigger growth by bacterial synthesis of plant hormones including indole-3-acetic acid, cytokinin, and gibberellins as well as by increased mineral and nitrogen availability in the soil (*3*−*7*). In the absence of physical contact with plant roots, blends of volatile chemicals emitted from specific strains of PGPR can also trigger growth promotion and induced resistance in the model plant *Arabidopsis* (*8*−*10*). *Bacillus subtilis* GB03, a commercially available saprophytic symbiont, is one such strain that emits a complex blend of volatile components that activates plant growth promotion. A bouquet of over 25 bacterial volatile odors has been identified (*8, 11*) that triggers differential expression of approximately 600 *Arabidopsis* transcripts related to cell wall modifications, primary and secondary metabolism, stress responses, hormone regulation, and other expressed proteins (*12*).

Ocimum basilicum L. (sweet basil) is rich in stored essential oils and is commonly utilized in the spice industry (*13*). The abundant essential oils located in leaf trichomes are lipophilic volatiles that consist mostly of monoterpenes, sesquiterpenes, and phenylpropanoid metabolites. *O. basilicum* essential oil contains approximately 40 different metabolites, although two essential oil components, α-terpineol and eugenol, account for almost 60% of the total volatile blend (*14, 15*). While plant inoculation with PGPR as well as exposure to bacterial volatiles can substantially enhance *Arabidopsis* growth, the impact that PGPR have on secondary metabolite production has yet to be investigated. Environmental conditions and agricultural practices can significantly alter overall yield as well as the composition in sweet basil (*16*). Although all plants produce and store some level of volatile hydrocarbons, increases in essential oil synthesis and accumulation for basil as well as other commercial spices that are harvested specifically for robust aromas have direct economic benefits. The objective of this study is to examine how sweet basil growth and odor production are influenced by exposure to air-borne volatiles from the beneficial soil bacterium *B. subtilis* (GB03); plant growth and odor production have also

* To whom correspondence should be addressed. Tel: 806-742-3062. Fax: 806-742-1289. E-mail: Paul.Pare@ttu.edu.
[†] Universidad Nacional de Río Cuarto.
[‡] Texas Tech University.

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Table 1. *B. subtilis* Growth Promotion in *O. basilicum* (Sweet Basil) as Measured by Shoot and Root Length (Means ± SE; *n* Is Indicated by the Number in Parentheses) for 14 Day Old I-Plate-Grown Plants[a]

	shoot length (cm/plant)	root length (cm/plant)	*N* leaf/plant
	volatile exposure		
GB03	4.0 ± 0.3 (11) a	12.4 ± 0.5 (8) ± a	6.4 ± 0.4 (11) a
DH5α	3.1 ± 0.2 (11) b	8.2 ± 0.3 (10) b	5.3 ± 0.4 (11) a, b
H_2O alone	3.2 ± 0.1 (10) b	8.8 ± 0.8 (9) b	5.0 ± 0.4 (10) b
F values	5.8	18.1	3.5
	inoculation		
GB03	5.7 ± 0.3 (8) a	19.4 ± 1.5 (11) a	8.0 ± 0.0 (8) a
DH5α	4.1 ± 0.2 (10) b	15.2 ± 1.3 (11) b	6.7 ± 0.3 (10) b
H_2O alone	4.1 ± 0.1 (9) b	14.4 ± 1.5 (10) b	6.3 ± 0.3 (9) b
F values	20.2	4.2	16.1

[a] Values followed by the same letter in a column with a given treatment are not significantly different according to a Fisher LSD test ($p < 0.05$).

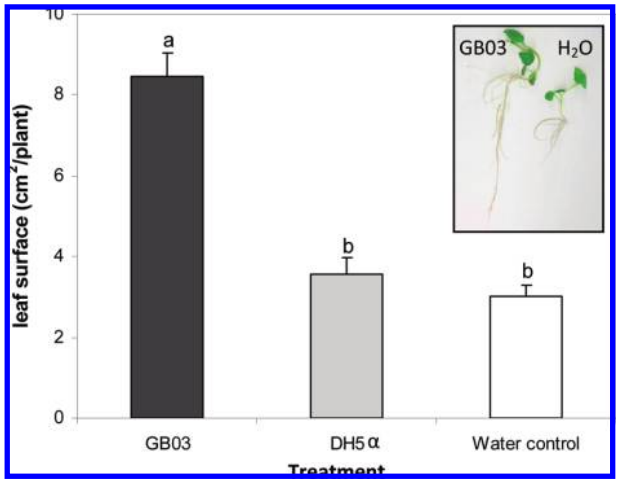

Figure 1. Leaf area of *O. basilicum* 14 day old plants exposed to *B. subtilis* GB03 volatile emission. Different letters indicate significant differences between treatments according to a Fisher LSD test at $p = 1 \times 10^{-7}$ and $f = 48.6$ ($n \geq 10$, means ± SDs); the inset is representative of images of 15 day old *O. basilicum* plants exposed to GB03 VOCS (left) or water treatment alone (right).

been measured with direct bacterial-root inoculation to allow for comparisons between the two elicitation protocols, air-borne bacterial exposure and media inoculation.

MATERIALS AND METHODS

Bacterial Cultures. *B. subtilis* GB03 and *Escherichia coli* DH5α were streaked onto tryptic soy agar (TSA) plates (Difco) and incubated in the absence of light at 28 °C for 24 h. Bacteria cells were harvested in double-distilled water (DDW) to yield 10^9 CFU mL^{-1} as determined by optical density measurements and serial dilutions combined with plate counts.

Growth Chambers. *O. basilicum* plants were grown in a closed chamber (75 mm × 75 mm × 100 mm; Magenta box, size GA-7; Sigma Chemical Co., St. Louis, MO) in which the two chamber halves were coupled together by a plastic collar (5 mm × 75 mm × 20 mm). In the base of the chamber, ca. 120 mL of half-strength Murashige and Skoog (MS) media containing 0.8% (w/v) agar and 1.5% (w/v) sucrose adjusted to pH 5.7 was added. A 4 dram glass vial containing ca. 5 mL of the same media was placed in the chamber when the media was poured. The vial was inoculated with the specified bacterium (20 μL) and served as the source for bacterial volatiles. Alternatively, sweet basil was grown on specialized plastic Petri dishes (100 mm × 15 mm) that contained a center partition (I-plates; Fisher Scientific, Pittsburgh, PA); both sides contained half-strength MS media with 0.8% (w/v) agar and 1.5% (w/v) sucrose adjusted to pH 5.7. To the nonplant side of the Petri dish, 20 μL of GB03-, DH5α-suspension culture or DDW was added. By positioning plants and bacteria in separate regions of the partitioned Petri dish or Magenta box, plants were exposed to bacterial volatile organic compounds (VOCs) without physical contact between the bacterial cultures and the plants. In treatments in which bacteria were in physical contact with the roots, referred to as media inoculation, glass vials were not introduced into the Magenta box, but instead, the bacterial suspension (20 μL) was added directly to the media containing the seedlings.

Planting. Seedlings were surface sterilized [2 min, 70% (v/v) ethanol soaking followed by a 20-min 1% (v/v) sodium hypochlorite soaking], rinsed (4×) in sterile-distilled water (SDW), and planted 1 per chamber or 3 per Petri dish on the surface of the solid media. All plants were grown under metal halide and high-pressure sodium lamps on a 16 h light/8 h dark cycle with a total light intensity of 200 μmol m^{-2} s^{-1}, a temperature of 25 ± 4 °C, and a relative humidity of 40 ± 10%. Plants were grown for 14 or 30 days with Petri dish or Magenta box setups, respectively. Each experiment consisted of 10−15 replicates per treatment with plants arranged randomly on growth-room tables.

Plant Growth Measurement. Plants were removed from the growth container, roots were water rinsed to remove MS media, and growth-promoting effects of bacterial treatments were evaluated by shoot and root length, leaf number, shoot and root fresh and dry weights, and leaf area. The leaf area was measured by an integrated digital video image analysis system including a CCTV camera (Panasonic WV-BL200, Secaucus, NJ) with AGIMAGE PLUS (version 1.08) software (Decangon Devices, Pullman, WA).

Solid-Phase Microextraction (SPME) Analysis. Plant volatile emissions were measured for I-plate-grown plants employing poly-(dimethylsiloxane) SPME fibers that were suitable for volatile analysis (PDMS 7 μm; Supelco, Bellefonte, PA). Petri dishes were transferred to glass chambers, and an internal standard (7.5×10^{-5} μmol *p*-cymene) was added on a 1 cm^2 filter paper disk. The SPME fiber was inserted above the plant material, and the chamber was water bath-heated (22 ± 2 °C); headspace volatiles were allowed to collect for 30 min. SPME fibers were desorbed at 210 °C for 1 min in the gas chromatography (GC) injection port as described previously (*11*).

Essential Oil Extraction and Analysis. Thirty day old shoot samples from Magenta box-grown plants were weighed and subjected to continuous CH_2Cl_2 extraction in a micro-Soxhlet extractor (Fisher Scientific) for 30 min; *p*-cymene (1.49×10^{-4} μmol) was added as an internal standard. Chemical analyses were performed using a HP 5890A GC-MS (Hewlett-Packard, Palo Alto, CA) with a DB5 column (J & W Scintific, Folsom, CA) column (60 m × 0.25 mm; film thickness, 0.25 μm) and a mass-selective detector. Analytical conditions: injector and detector temperatures, 250 and 270 °C, respectively; oven temperature programmed from 60 (3 min) to 240 °C at 4°/min; the carrier gas was helium at a constant flow of 0.9 mL/min; source, 70 eV.

The two major volatile components, α-terpineol and eugenol, were quantified based on GC-flame ionization detection (FID) peak areas in comparison with the internal standard. FID response factors for each compound generated equivalent areas with negligible difference (<5%). For comparison of the same compound under different treatments, response factors for individual compounds were assumed to be equal.

Selected samples were analyzed by GC-MS on an ion trap-mass spectrometer (Thermo-Finnegan GC-Q, Austin, TX) and operated in the electron impact mode. Injections were made in the splitless mode for 30 s, and samples were analyzed on a 30 m × 0.25 mm (i.d.) DB5 column (J & W Scientific) under the same conditions describe in GC-FID analysis. The transfer line and ion source temperature were adjusted to 220 and 180 °C, respectively. The oil components α-terpineol and eugenol were initially identified based on mass spectral and retention time data and confirmed by direct comparisons with commercial standards from Sigma-Aldrich Co. (St. Louis, MO) (*17*).

Statistical Analysis. Data were subjected to analysis of variance (ANOVA) followed by comparison of multiple treatment levels with the control, using the posthoc Fisher's least significant difference (LSD) test. Infostat software version 2.0 (Group Infostat, Universidad Nacional de Córdoba, Argentina) was used for all statistical analyses.

RESULTS

Sweet basil growth as measured by shoot and root length was enhanced with the commercial PGPR stain *B. subtilis* GB03

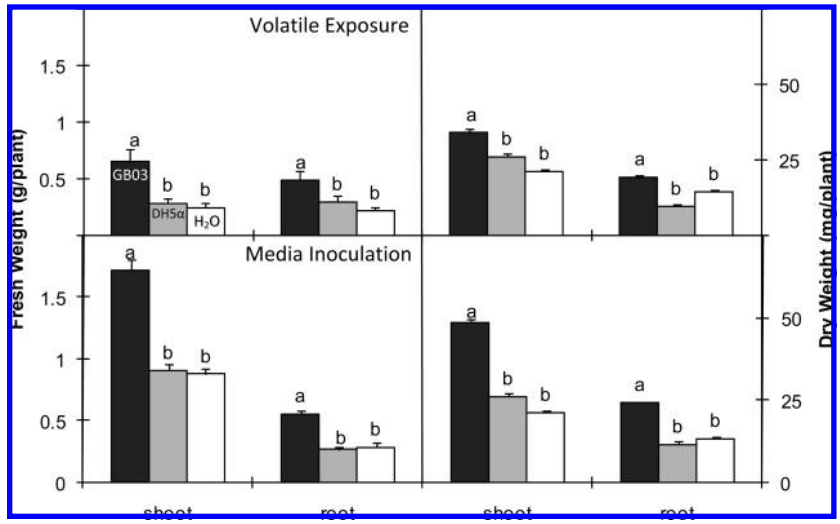

Figure 2. Fresh and dry weight on a tissue weight basis for 30 day old *O. basilicum* exposed to volatile treatment (**top**) or media inoculation (**bottom**). Black, gray, and white bars represent GB03, DH5α, and H_2O treatments, respectively. Different letters indicate significant differences between treatments according to a Fisher LSD test at $p < 0.05$ ($n \geq 10$, means ± SDs).

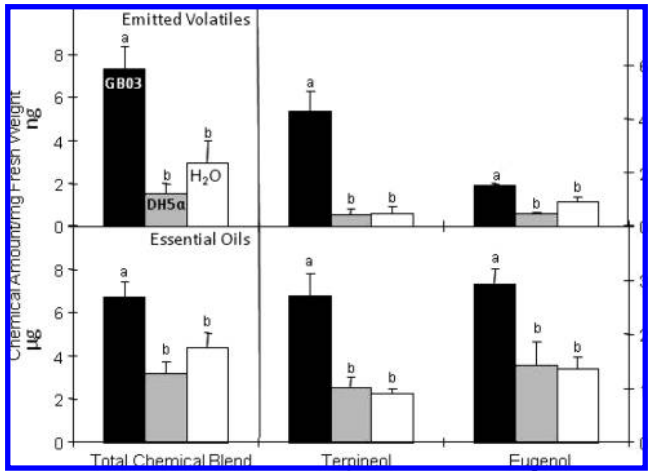

Figure 3. Comparison of volatiles emitted (**top**) with essential oil extracts (**bottom**) from 14 day old, I-plate-grown *O. basilicum* plants; black, gray, and white bars represent GB03, DH5α, and H_2O exposures, respectively. Different letters indicate significant differences between treatments according to a Fisher LSD test at $p < 0.05$ ($n \geq 5$, means ± SDs).

by either volatile exposure or root inoculation exhibited when compared to the non-PGPR stain DH5α or water treatment alone (**Table 1**). A greater shoot length was observed in GB03-treated plants, increasing for 14 day old plants 7.0 mm with volatile treatment and 16 mm for inoculated plants. The root length was also significantly greater for GB03-treated plants ($p < 0.05$) with 40 and 35% longer roots with volatile GB03 exposure or direct root inoculation, respectively, as compared to water control. A similar growth promotion trend with an increased leaf number was observed for GB03 treatment as compared to DH5α and water controls ($p < 0.05$). The leaf area increased over two times ($p < 0.05$) in plants exposed to GB03 VOCs as compared to DH5α and water controls (**Figure 1**).

Growth promotion was sustained for 30 days when sweet basil was grown in larger Magenta boxes in the presence of GB03 volatiles (**Figure 2**). On a fresh weight basis per plant, GB03 exposure doubled the shoot and root mass as compared to DH5α or water-treated controls. The dry weight increase with GB03 treatment was also significantly enhanced in shoots and roots by 30−50%, as compared to controls ($p < 0.05$). Directly inoculating the media in which plants were grown with GB03 likewise induced growth promotion in comparison with DH5α and water controls.

To examine the impact of GB03 exposure on plant volatile emission, head space volatiles were collected by SPME with GB03, DH5α, or water exposure. Quantification of headspace volatiles revealed an increase ($p < 0.05$) in the total chemical blend as well as the individual components α-terpineol and eugenol as compared with DH5α or water-treated controls (**Figure 3**). Not surprisingly, essential oils extracted from 14 day old I-plate-grown plants exhibited substantially greater total essential oil content as well as greater amounts of α-terpineol and eugenol as compared with emitted volatiles (ca. 1000 times), although the same pattern of greater emissions with GB03 than DH5α or water control was observed.

In comparing microbial elicitation by bacterial volatile emissions vs media inoculation, greater essential oil production was observed with plant exposure to bacterial volatiles absent of direct bacterial contact with roots (**Figure 4**). In quantifying the induction of secondary compound production by volatile bacterial elicitors vs direct root−rhizobacterium contact, the mean total essential oil measured on a fresh weigh basis was 2-fold less with GB03−media inoculation than with GB03 volatile exposure.

DISCUSSION

Increased growth and development in plants inoculated with PGPR have been reported for a number of species (*18*−*20*). Effects of GB03 inoculation on sweet basil evaluated in the present study are consistent with previous reports with shoot and root length and the number of leaves positively affected by GB03. With reports that GB03 volatiles trigger growth promotion in *Arabidopsis* (*8*), the question as to whether such bacterial volatiles also trigger growth promotion in other species as well as whether secondary metabolism can be induced with GB03 volatiles has not been previously examined. By measuring growth promotion and essential oil production in sweet basil treated with bacterial volatiles or inoculation of the media, direct comparisons with specific bacterial stimuli on growth and metabolite production are possible. Because rhizobacteria are usually in contact with the roots, it is relevant to compare plant responses triggered by bacterial volatiles vs the complex response of full bacterial interaction with the plant. The media-

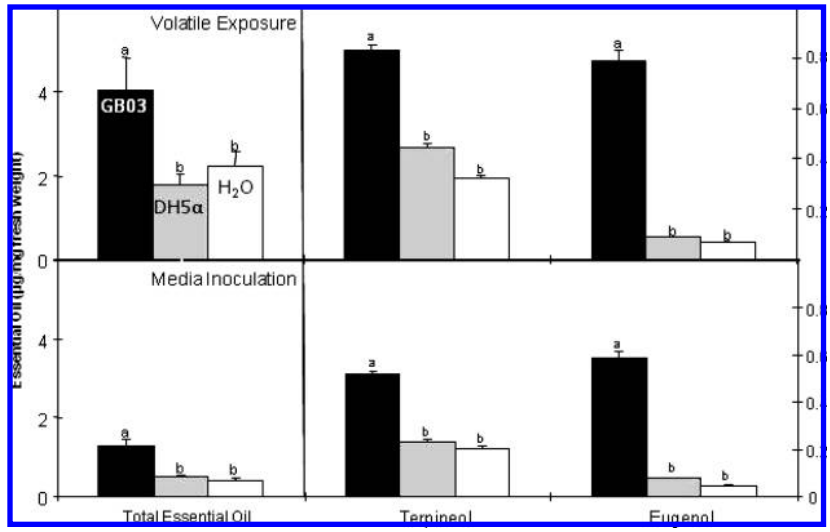

Figure 4. Total essential oil and individual components (α-terpineol and eugenol) collected by Soxhlet extraction for Magenta box grown, 30 day old *O. basilicum*; plants were exposed to volatiles (**top**) or media inoculation (**bottom**), with black, gray, and white bars representing GB03, DH5α, and H_2O treatments, respectively. Different letters indicate significant differences between treatments according to a Fisher LSD test at $p < 0.05$ ($n \geq 5$, means ± SDs).

inoculated plants show a greater growth promotion than those exposed to bacterial volatiles alone (**Figure 2**), while induction of essential oil production is reversed with greater metabolite accumulation with exposure to bacterial volatiles than media inoculation (**Figure 4**).

Plant growth promotion observed in agronomic crops following rhizobacteria soil inoculation may result from increased bacterial production of growth hormones, solubilization of phosphates, oxidation of sulfur, an increase in nitrate availability, or sn increase in root permeability (*21*). With an absence of bacterial contact with roots as in the I-plate assays, only low molecular weight volatiles can serve as elicitors to trigger plant responses. Indeed, two volatile components have been identified in headspace collections from GB03 with potential biological activity in triggering growth promotion in *Arabidopsis*: 3-hydroxy-2-butanone (acetoin) and (2*R*,3*R*)-butanediol (*8*). Transcriptional and histochemical data indicate that GB03 volatiles trigger growth promotion by regulating auxin homeostasis specifically elevating auxin accumulation in the roots (*12*). In addition, microarray data reveal coordinated regulation of photosynthesis by GB03 down-regulation of sugar signaling (*22*). Similar proportional increases with wet and dry weight tissue analyses for the different VOC treatments exclude the possibility that growth promotion is simply due to increased plant hydration. The physical and chemical measurements reported here with sweet basil agree with previous studies in which growth is augmented by plant exposure to GB03 volatiles (*8*); the participation of auxin synthesis and/or transport in such growth promotion can only be speculated at this time.

Because plant volatiles are utilized for flavor enhancement in foods as well as for natural defense against herbivore pests, how bacterial-induced plant growth promotion influences essential oil production on a fresh weight basis is of particular interest in the growing of sweet basil. In this study, accumulation of essential oils has increased over 2-fold with GB03 bacterial volatiles or media inoculation with GB03 as compared to DH5α or water controls. Such increases in essential oil accumulation suggest induction of terpenes biosynthesis levels, although direct measurements have yet to be made. Induction of secondary metabolite responses has been reported with other beneficial microbe−plant interactions. For example, an increase in the abundance of glandular hairs and essential oil yield is observed in sweet basil by PGPR induction (*23*). *Mentha arvensis* cultivars inoculated with the fungus *Glomus fasciculatum* trigger increased plant height, shoot growth, and oil content (*24*), while in *Oreganum* sp., increases in overall essential oil concentration on a fresh weight basis are observed with mycorhizae interactions (*25*). It is proposed that arbuscular mycorrhizal (AM) fungi increase plant growth and essential oil accumulation by extending the plant's root system that in turn allows for an extension of the root zone as well as greater production of extracellular acid phosphatases, allowing for the acquisition of organic phosphates (*26*).

From an ecological perspective, accumulation of essential oils in plant tissues can provide a direct microbial defense response, as many components of such blends have antimicrobial activity (*27*). In addition to plant−GB03 interactions enhancing stored essential oils as measured by essential oil extraction, phenylpropenoid and terpene volatile emissions from sweet basil increase as measured by SPME (**Figure 3**). Emitted headspace volatiles provide chemical signaling potential for plants in the case of herbivore damage (*10*). Induction of plant volatile emissions in response to herbivore damage via biotic elicitors can serve in the attraction of natural enemies of herbivores, resulting in an indirect defense against herbivore pests (*10, 17, 28*).

The results presented here establish that volatiles emitted by GB03 significantly increase biomass and essential oil production in laboratory-grown sweet basil. Follow-up field studies will provide insight into whether soil introduction of GB03 increases the productivity and/or reduces fertilizer applications for sweet basil.

ABBREVIATIONS USED

ANOVA, analysis of variance; AM, arbuscular mycorrhizal; GC-FID, gas chromatography-flame ionization detection; LSD, least significant difference; PGPR, plant growth-promoting rhizobacteria; MS, Murashige and Skoog; SPME, solid-phase microextraction; TSA, tryptic soy agar; VOCs, volatile organic compounds.

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