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Volatile Organic Compound Profiles of Plant Growth-Promoting Bacterial Strains Isolated from Root Nodules of Vegetable Legumes

A. Sarath Babu[1], G. Selvakumar[2]*, A. Vijaya Gopal[1], D. Kalaivanan[2], Sunil Kumaraswamy[2], A.N. Lokesha[2] and K.S. Shivashankara[2]

[1]Department of Microbiology, ANGRAU Agricultural College, Bapatla, Andhra Pradesh, India.
[2]ICAR-Indian Institute of Horticultural Research, Bengaluru, Karnataka, India.

Abstract

The volatile organic compounds (VOCs) produced by eight plant growth-promoting bacterial strains originating from surface-sterilized root nodules of cowpea and garden pea were examined using headspace-solid phase microextraction-gas chromatography–mass spectrometry (HS-SPME-GC-MS). One hundred and fifty-nine VOCs were detected in the control and bacterial inoculated flasks. Among the VOCs produced by the bacterial isolates, esters (14.46%), ketones (13.83%), alcohols (12.57%) and hydrocarbons (9.4%) were the most prevalent, while other classes of VOCs were detected at lower frequencies. While 33.13% of the compounds were exclusive to cowpea nodule isolates, 38.50% of the compounds were exclusive to garden pea nodule isolates. The compounds that were common between both groups accounted for 28.40% of the total compounds detected. The major VOCs with plant growth promotion potential produced by *Enterobacter* sp. CPH64, *Enterobacter* sp. CPK42, *Chryseobacterium* sp. CPM11, and *Stenotrophomonas* sp. CPH62, originating from cowpea root nodules were palmitic acid, dimethyl silanediol, hexanol 2-ethyl, 2-methyl 1- butanol and 2-tridecanone, while *Enterobacter* sp. GP44, *Enterobacter* sp. GP71, *Enterobacter* sp. GP84, and *Bacillus* sp. GP102, from garden pea root nodules, produced the VOCs, viz. 3-methyl-1-butanol, 2-methyl-1-butanol, 2,3,5-trimethyl pyrazine, dimethyl silanediol, 2-tridecanone, butane 1-methoxy 3-methyl, hexanol 2-ethyl- and palmitic acid. These compounds have been previously shown to promote plant growth, improve plant defence, leaf chlorophyll content, enhance fruit ripening and mitigate stress effects. This is an early report on the VOC profile of bacterial endophytes isolated from root nodules of two vegetable legumes, viz. cowpea (tropical) and garden pea (temperate).

Keywords: Vegetable Legumes, Root Nodule-associated Bacteria, Volatile Organic Compounds: HS-SPME-GC-MS, Plant Growth Promotion

***Correspondence:** gselva74@rediffmail.com

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INTRODUCTION

Vegetable legumes are a diverse and nutritionally rich group of plants that are crucial for the human diet and sustainable agriculture due to their nitrogen-fixing abilities. This category includes peas, beans, lentils, chickpeas, and soybeans. Cowpea (*Vigna unguiculata*) and garden pea (*Pisum sativum*) are two vital food legumes. In 2019, global cowpea production reached 8.9 Mt[1] while garden pea cultivation covered 2.18 Mha, yielding 21.77 Mt at a productivity rate of 9.99 t ha^{-1}.[2] It was previously believed that only rhizobial strains could colonize the interiors of root nodules. However, later studies have indicated that diverse bacteria belonging to different genera, viz.: *Agrobacterium*, *Arthrobacter*, *Acinetobacter*, *Bacillus*, *Bosea*, *Enterobacter*, *Micromonospora*, *Mycobacterium*, *Paenibacillus*, *Pseudomonas* and *Stenotrophomonas,* are also associated with legume root nodules and play important roles in promoting the growth and development of their hosts.[3,4] But research on their functional metabolites remains limited. Most studies on bacterial volatile organic compounds have focused on rhizosphere-associated strains or well-characterized PGPR such as *Bacillus* and *Pseudomonas*,[5-7] while the VOC profiles of root nodule bacteria of cowpea and gardenpea nodules remains to be deciphered, despite increasing evidence that nodule-associated non-rhizobial bacteria play important roles in plant growth and stress adaptation. This study therefore addresses a clear gap by providing the first VOC profile of bacterial endophytes isolated from root nodules of two vegetable legumes, viz. cowpea (tropical) and garden pea (temperate).

Rhizospheric microbes produce a wide range of volatile organic and inorganic chemical compounds that play a definite role in signalling among bacterial communities, promoting host growth and inducing tolerance to biotic and abiotic stresses.[8,9] Inorganic compounds include carbon dioxide (CO_2), carbon monoxide (CO), nitric oxide (NO), hydrogen sulfide (H_2S), ammonia (NH_3) and hydrogen cyanide (HCN), while volatile organic compounds (VOCs) include a range of terpenes, aromatic compounds, hydrocarbons, ketones, alcohols, aldehydes, acids, and metabolites containing sulfur and nitrogen.[6,10] The plant growth-promoting properties of VOCs emitted by rhizospheric bacteria have been established previously.[11] VOCs are known to stimulate photosynthesis, root growth and the uptake of specific nutrients such as iron and sulfur.[12-15] They are also known to directly inhibit the growth of fungal and bacterial phytopathogens, thereby protecting their hosts.[16,17]

Gas chromatography–mass spectrometry (GC-MS) is particularly well suited for the analysis of VOCs produced by bacterial strains under various conditions due to its sensitivity, selectivity and ability to analyse a wide range of compounds. Prior to their detection by GC-MS, bacterial volatile organic compounds (VOCs) can be efficiently extracted and concentrated by headspace (HS)-solid-phase microextraction (SPME).[18-20] The data generated by GC-MS not only aids in compound identification but also facilitates their quantitative analysis. This study was carried out to profile the VOCs produced by eight plant growth-promoting bacterial isolates obtained from the root nodules of vegetable legumes, viz. cowpea and garden pea, by HS-SPME-GC-MS and to elucidate their putative plant growth-promoting roles from previously published literature.

MATERIALS AND METHODS

Bacterial strains

The bacterial strains used in this study were originally isolated from the surface-sterilized root nodules of cowpea and garden pea cultivated in different parts of the states of Andhra Pradesh and Karnataka, India, and selected for further study based on their superior plant growth-promoting abilities, viz. ammonia production, phosphate solubilization and growth hormone production.[21] The elite bacterial isolates, viz. *Chryseobacterium* sp. CPM11, *Enterobacter* sp. CPH64, *Enterobacter* sp. CPK42, and *Stenotrophomonas* sp. CPH62 from cowpea root nodules, and *Enterobacter* sp. GP44, *Enterobacter* sp. GP71, *Enterobacter* sp. GP84, and *Bacillus* sp. GP102 from garden pea root nodules were subjected to volatile profiling by HS-SPME-GC-MS.

Sample preparation and headspace-solid-phase micro-extraction

Individual bacterial isolates were inoculated in nutrient broth in 250 ml Erlenmeyer flasks with a headspace of 60%. The flasks were tightly sealed with plastic film wrap to prevent any loss of volatile compounds and incubated at 30 °C for 48 hrs. An uninoculated control was included to determine the volatile emissions from the sterile media. After incubation, volatile compounds were trapped with a fused-silica SPME fibre coated with a mixed stationary phase of polydimethylsiloxane (PDMS), divinylbenzene (DVB), and carboxen (CAR) on a stainless-steel holder. The DVB/CAR/PDMS fibre (50/30 µm; highly cross-linked) was pre-conditioned at 300 °C for 1 hour.[20] The fibre was inserted into the flask through a small opening in plastic film and exposed to the headspace for 2 hours, with gentle stirring. After adsorption, the fibre was transferred to the GC-MS injector for thermal desorption and subsequent analysis of the volatile compounds.

GC-MS analysis of volatile organic compounds

The GC-MS analysis was carried out using a Shimadzu Nexis GC-2030 system connected to a Shimadzu TQ 8040 NX mass detector. After sampling, the SPME fibre was left in the injector during the entire run. The VOCs were separated using a fused-silica capillary column (SH-I-5Sil MS; 30 m × 0.25 mm, 0.25 µm film thickness). The injector was set at 255 °C, and samples were injected in split mode (1:5) for 0.2 min. The detector was set at 270 °C. The oven program started at 40 °C for 3 min, increased by 3 °C/min to 160 °C (held for 2 min), and then increased by 5 °C/min to 230 °C, where it was held for 1 min. The MS was operated in electron-ionization mode with helium as the carrier gas at 1.0 mL/min. The ion source, transfer line, and injector were kept at 230 °C, 240 °C, and 255 °C, respectively, and spectra were recorded in the 50-400 amu range. Total VOC production was estimated by adding all GC peak areas and expressing each compound as a percentage of this total. The VOCs were identified by matching retention indices and mass spectra with the FFNSC and NIST-2020 libraries. The relative abundance of each compound was adjusted by subtracting the peak areas of the uninoculated control.

Statistical analysis

The data was statistically analysed using Operational Statistics (OPSTAT) software. The metabolome data were analysed using MetaboAnalyst 6.0.[22] Partial least squares–discriminant analysis (PLS-DA) was performed using MetaboAnalyst (https://www.metaboanalyst.ca), a comprehensive web-based platform, to differentiate the overall volatile profile patterns among bacterial isolates from cowpea nodules (CPH62, CPH64, CPK42, CPM11) and their control group, as well as bacterial isolates from garden pea nodules (GP44, GP71, GP84, GP102) and their respective control group. Prior to analysis, the dataset was formatted to include volatile names and sample names. Additionally, heatmaps were created using normalized data in MetaboAnalyst to visualize the relative abundance of key volatiles. These heatmaps display the major volatiles emitted by each bacterial isolate in a matrix format, highlighting patterns and group-specific differences. A Venn diagram was prepared to represent the common VOC's between the root nodule associated bacterial groups using the "Venny 2.1.0" tool.[23]

RESULTS

Partial Least Squares-Discriminant Analysis of the VOCs produced by the bacterial isolates

A total of one hundred and fifty nine VOCs were detected from the uninoculated control and the eight bacterial strains by HS-SPME-GC-MS. Among the VOCs produced by the bacterial isolates, esters (14.46%), ketones (13.83%), alcohols (12.57%) and hydrocarbons (9.4%) were the most prevalent, while other classes of VOCs were detected at lower frequencies. While 33.13% of the compounds were exclusive to cowpea nodule isolates, 38.50% of the compounds were exclusive to garden pea nodule isolates. The compounds that were common between both groups accounted for 28.40% of the total compounds detected. The VOCs with potential plant growth promotion activity are listed in Table. The data matrix of the VOCs produced by the cowpea and garden pea isolates was analysed separately by PLS-DA to identify the variation and grouping trends of the VOCs with respect to the uninoculated control.

This distinguished the overall differences in metabolic profiles between the groups.

The score plots illustrated clear differences in the volatile compounds produced by the different bacterial groups and the control. The volatile compounds from bacterial isolates of cowpea (Figure 1a) and garden pea (Figure 1b) nodules exhibited distinct separation from each other and from the control, indicating differential expression patterns across the isolates

Table. Volatile organic compounds with potential plant growth promoting effects produced by the root nodule-associated bacterial strains

Volatile Organic Compound	Relative Abundance (%)	Produced by group	Functional	References
3-methyl-1-butanol	19.59	*Enterobacter* sp. CPH64,	Alcohol	24-28
	11.43	*Enterobacter* sp. GP44,		
	2.83	*Chryseobacterium* sp. CPM11,		
	15.03	*Enterobacter* sp.CPK42,		
	23.91	*Enterobacter* sp. GP84		
2,3,5-Trimethyl pyrazine	1.28	*Bacillus* sp. GP102	Pyrazine	17,29-31
	0.58	*Enterobacter* sp. GP71,		
	0.8	*Enterobacter* sp. CPH64		
Dimethyl silanediol	5.23	*Bacillus* sp. GP102,	Alcohol	32
	1.23	*Chryseobacterium* sp. CPM11,		
	0.41	*Enterobacter* sp. CPH64,		
	2.04	*Stenotrophomonas* sp. CPH62		
	0.74	*Enterobacter* sp.CPK42,		
	0.9	*Enterobacter* sp. GP44,		
	0.77	*Enterobacter* sp. GP84		
	0.98	*Enterobacter* sp. GP71		
2-Tridecanone	6.74	*Enterobacter* sp. CPH64,	Ketone	33-39
	2.02	*Enterobacter* sp. GP44,		
	3.17	*Enterobacter* sp.CPK42,		
	1.76	*Enterobacter* sp. GP84		
	0.39	*Chryseobacterium* sp. CPM11		
Butane 1-methoxy 3-methyl	23.88	*Enterobacter* sp. GP44,	Methoxy	5,12,40
	31.94	*Enterobacter* sp. GP84		
Hexanol 2-ethyl-	12.24	*Enterobacter* sp. GP71,	Alcohol	41,42
	3.23	*Bacillus* sp. GP102,		
	1.71	*Enterobacter* sp. GP44,		
	1.18	*Chryseobacterium* sp. CPM11,		
	6.02	*Stenotrophomonas* sp. CPH62,		
	2.97	*Enterobacter* sp. CPK42		
Palmitic acid	2.22	*Bacillus* sp. GP102	Fatty acids	43
	0.39	*Enterobacter* sp. CPH64,		
	0.46	*Enterobacter* sp. CPK42		
	0.49	*Stenotrophomonas* sp. CPH62		
	0.92	*Enterobacter* sp. GP71,		
	0.34	*Enterobacter* sp. GP84,		
2-methyl-1-butanol	7.08	*Enterobacter* sp. CPH64,	Alcohol	5,44-47
	4.76	*Enterobacter* sp. GP44,		
	3.24	*Enterobacter* sp.CPK42		
	0.89	*Chryseobacterium* sp. CPM11		

and the control. In the score plot of cowpea nodule isolates, component 1 and component 2 accounted for 35.3% and 52.8%, respectively, of the variation, while in the score plot of garden pea nodules, component 1 and component 2 accounted for 30% and 14.4%, respectively of the variation.

Venn diagram analysis and hierarchical clustering of VOCs produced by the bacterial isolates

The Venn diagram analysis (Figure 2) was employed to examine the uniqueness and commonalities among the VOCs produced by the cowpea and garden pea isolates. Among the cowpea isolates, *Chryseobacterium* sp.

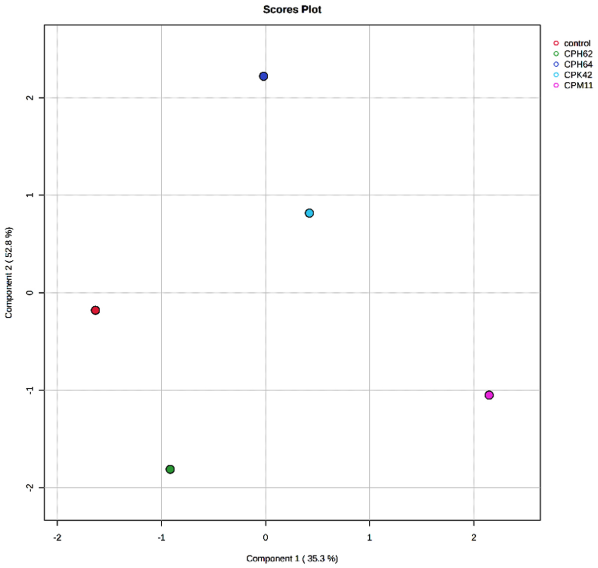

Figure 1a. Partial Least Squares-Discriminant Analysis (PLS-DA) of the VOCs detected in the uninoculated control and cowpea nodule isolates

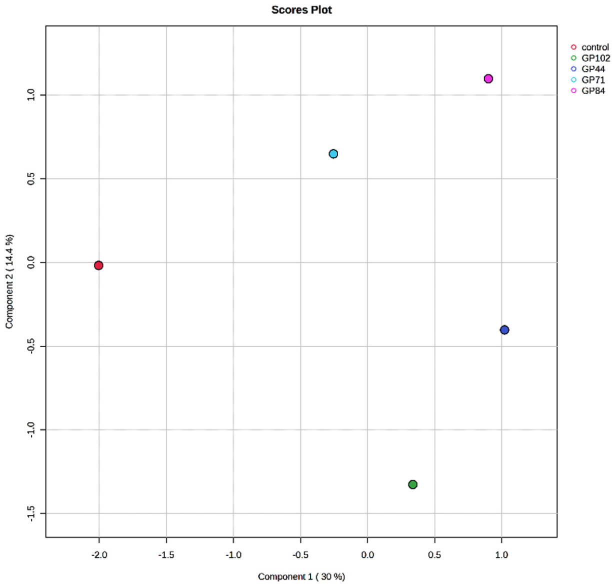

Figure 1b. Partial Least Squares-Discriminant Analysis (PLS-DA) of the VOCs detected in the uninoculated control and garden pea root nodule isolates

CPM11 produced seventeen distinct compounds and *Enterobacter* sp. CPH64 produced sixteen distinct compounds, *Stenotrophomonas* sp. CPH62 produced ten distinct compounds and *Enterobacter* sp. CPK42 produced eleven unique compounds. *Chryseobacterium* sp. CPM11 and *Stenotrophomonas* sp. CPH62 produced five VOCs that were common amongst them. Two common VOCs were produced by *Enterobacter* sp. CPH64 and *Stenotrophomonas* sp. CPH62, while five common VOC's were detected in *Stenotrophomonas* sp. CPH62 and *Enterobacter* sp. CPK42. The collective analysis of the cowpea isolates revealed a shared set of 10 compounds among them. This suggests shared metabolic pathways among these bacterial isolates. The investigation of volatile organic compounds produced by garden pea nodule isolates revealed distinctive chemical profiles for each isolate. *Bacillus* sp. GP102 featured an exclusive repertoire of 18 compounds, *Enterobacter* sp. GP84 produced 14, *Enterobacter* sp. GP71 produced twenty-one unique compounds, and *Enterobacter* sp. GP44 produced 11 distinctive elements. Commonalities were observed between the *Enterobacter* sp. GP71 and *Bacillus* sp. GP102 isolates, which share six common compounds. Intriguingly, no common compounds were identified between *Bacillus* sp. GP102 and *Enterobacter* sp. GP44, but one common compound was found between *Enterobacter* sp. GP71 and *Enterobacter* sp. GP44. A substantial overlap of eleven common compounds was observed among the four isolates, emphasizing shared metabolic pathways across the isolates.

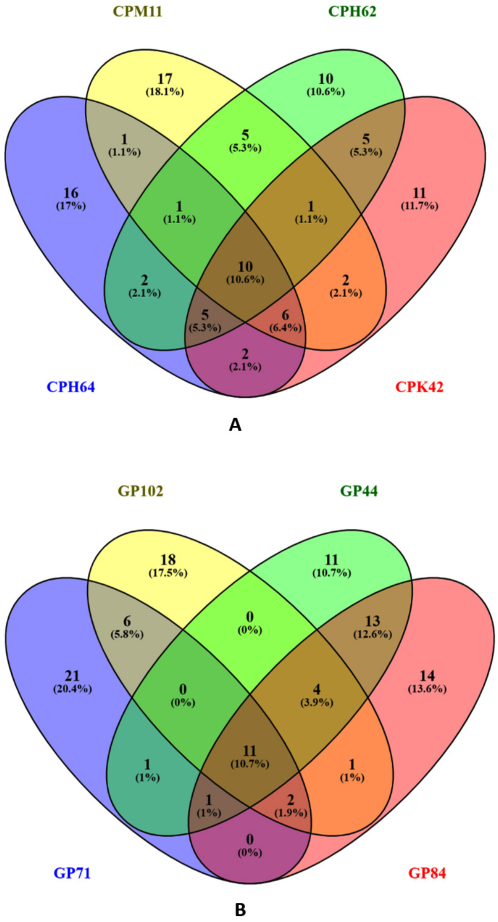

Figure 2. Venn diagram displaying the number of volatile organic compounds coincident between the bacterial strains isolated from (A) cowpea and (B) garden pea root nodules

The differential expression of bacterial volatile compounds with plant growth promotion abilities was visualized via hierarchical clustering with heatmaps. The volatile compounds were differentially accumulated across isolates obtained from cowpea, garden pea nodule isolates and the control (Figures 3 and 4). Higher expression levels of volatiles are denoted by red on the heatmap, whereas lower expression levels are represented by green. In summary, the detailed examination of volatile compounds provided valuable insights into the distinctive characteristics and potential functional roles of each bacterial isolate, shedding light on their unique chemical signatures.

DISCUSSION

Root nodules harbour several non-rhizobial bacteria. Twenty strains were isolated from the root nodules of *Phaseolus vulgaris* L., which include six different genera, viz. *Enterobacter*, *Stenotrophomonas*, *Chryseobacterium*, *Arthrobacter*, *Pseudomonas* and *Rhizobium*.[48] Members of the family Enterobacteriaceae were the most abundant among the nonrhizobial genera, in cowpea nodules along with *Stenotrophomonas* and *Chryseobacterium*.[49]

In the present study, HS-SPME-GC-MS analysis revealed the production of volatile compounds, such as 3-methyl-1-butanol, 2-methyl-1-butanol, 2,3,5-trimethyl pyrazine, dimethyl silanediol, 2- tridecanone, butane 1-methoxy 3-methyl, hexanol 2-ethyl- and palmitic acid, which were previously shown to have plant growth-promoting activities, plant defence, improved chlorophyll content, fruit ripening and stress mitigation activities.[50,51] To analyse these compounds, the Partial Least Squares Discriminant Analysis (PLS-DA) which is a multivariate statistical technique in metabolomics is increasingly being used to analyse volatile profiles of Plant Growth-Promoting Bacteria (PGPB)[52-54] was employed. By integrating VOC peak intensities with supervised classification, PLS-DA effectively differentiates bacterial strains based on their characteristic volatile signatures.[55] Recent studies have profiled VOC blends from several PGPR, such as *Pseudomonas koreensis*, *P. fluorescens*, *Lysinibacillus sphaericus*, and *Paenibacillus alvei*, using HS-SPME-GC-MS coupled with PLS-DA.[52] This approach has revealed species-specific and strain-specific VOC patterns, identified novel compounds and certain VOCs as potential biomarkers for PGPR classification and functional prediction.[52] The PLS-DA has also proven to be effective in distinguishing bacterial VOCs from control samples.[55]

Alcoholic VOCs such as 3-methyl-1-butanol have been widely recognized for their plant growth-promoting (PGP) properties. The VOC 3-methyl-1-butanol has been shown to enhance the growth of *Medicago sativa* and *Arabidopsis thaliana* under iron-limited conditions.[24] It has

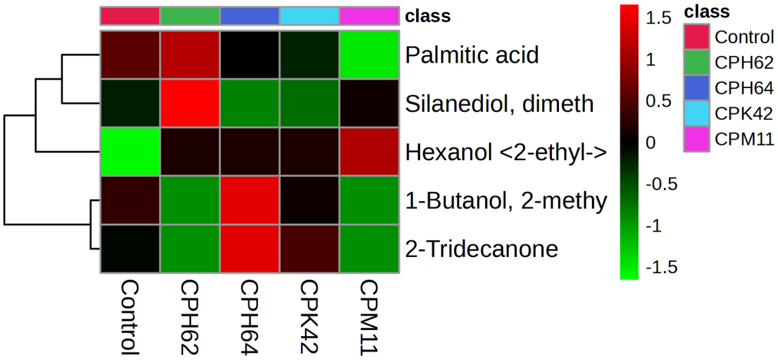

Figure 3. Heatmap of the VOCs with putative plant growth promotion abilities produced by bacterial isolates obtained from cowpea nodules. The columns represent the bacterial isolates, and the rows represent the different metabolites identified. The colour codes indicate the abundance of each VOC

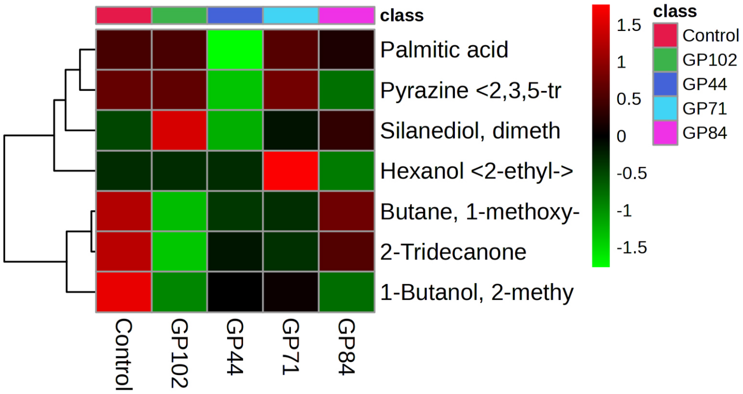

Figure 4. Heatmap of the VOCs with putative plant growth promotion abilities produced by bacterial isolates obtained from garden pea nodules. The columns represent the bacterial isolates, and the rows represent the VOC's identified. The colour codes indicate the abundance of each VOC

been reported that bacterial strains isolated from agave and cactus roots emitted 3-methyl-1-butanol, which positively influenced the growth and maturation of several Agave species.[25] The 3-methyl-1-butanol produced by rhizospheric bacteria was reported to increase superoxide dismutase (SOD) activity, elevate chlorophyll levels and promote the growth of maize and wheat.[26] It has also been identified as a strong inducer of plant growth and systemic resistance,[5] regulate lateral root formation via jasmonic acid signalling without activating auxin-responsive pathways, thereby enhancing avocado growth and productivity.[27] Further confirmation of the PGP potential of 3-methyl-1-butanol by Ayuso Calles et al.[28] showed that the VOCs emitted by *Rhizobium* sp. GPTR29 increased plant development under controlled, salt-stress, and greenhouse conditions.

The alcoholic VOC 2-methyl-1-butanol has been well documented for its plant growth-promoting and antimicrobial properties by Ayuso Calles et al.[28] who identified 2-methyl-1-butanol as one of the major compounds associated with growth promotion and the induction of systemic resistance in plants. Several 1-butanol derivatives, including 3-methyl-1-butanol, 2-methyl-1-butanol, and 1-butanol-3-methyl acetate, have been reported to inhibit the mycelial growth of *Pythium ultimum*, *Rhizoctonia solani*, and *Sclerotium sclerotiorum*[44,45] demonstrated that VOCs emitted by *Fusarium oxysporum*, including 3-methyl-1-butanol and 2-methyl-1-butanol, effectively suppressed mycelial growth and reduced the microsclerotia viability of fungal pathogens. In addition, the pathogenic fungus *Ceratocystis fimbriata* has been shown to be inhibited by *Pseudomonas chlororaphis* subsp. *aureofaciens*, which emits both 3-methyl-1-butanol and 2-methyl-1-butanol. It has been reported that VOCs such as 2-methyl-1-butanol, 1-decene, and 2-heptylfuran produced by *Trichoderma* significantly increased the fresh weight and chlorophyll content of *Arabidopsis thaliana*.[47]

The alcoholic VOC 2-ethyl-1-hexanol has been previously reported. The VOC from *Bacillus* sp. JC03, which emitted relatively high levels of 2-ethyl-1-hexanol, tetrahydrofuran-3-ol, and 2-heptanone, all of which contributed to enhanced plant growth.[41] Further demonstrated that 2-ethyl-1-hexanol exhibited strong antifungal activity against *Rhizoctonia solani*, indicating its potential as a biological control agent for managing rice sheath blight[42] confirmed that 2-ethyl-1-hexanol promoted growth in *Arabidopsis thaliana*.[41] VOCs such as dimethyl silanediol produced by *Pseudomonas pseudoalcaligenes* can alleviate drought stress in maize by modulating the plants defence system.[32]

The ketonic compound 2-tridecanone, widely regarded as a plant growth-promoting VOC, has been well characterized in earlier studies, several VOCs emitted by *Bacillus* sp. BCT9—namely 3-hydroxy-2-butanone, 2,3-butanediol, 2-nonanone, 2-undecanone, 2-tridecanone, and 2-pentadecanon which stimulated seed germination and significantly influenced the growth of *Lactuca sativa* during both germination and seedling stages. High concentrations of 2-tridecanone in leaf tissues have been associated with plant resistance, producing antibiosis and antixenosis effects against spider mites and other arthropods,[33,35-37] It has been reported that 2-tridecanone plays a key role in plant resistance mechanisms against the South American tomato pinworm.[39]

Pyrazine compounds, such as 2,3,5-trimethylpyrazine, were also detected in the present study. It has been reported that 2,3,5-trimethylpyrazine emitted by *Bacillus* strain HA and *Bacillus* strain A8a modulated root development, increased the fresh weight of *Arabidopsis thaliana*, and inhibited the growth of *Fusarium solani,* and soil-borne oomycetes. Antimicrobial VOCs produced by *Bacillus* spp., including 2-decanone, 2,3,5-trimethylpyrazine, and 2-methylbutanoic acid, have demonstrated antagonistic activity against major phytopathogens such as *Alternaria alternata*, *Fusarium solani*, *Fusarium kuroshium*, and *Phytophthora cinnamomi*.[17,30,31] Butane-1-methoxy-3-methyl produced by *B. subtilis* GB03 and *B. amyloliquefaciens* IN937a promoted growth and induced systemic acquired resistance in *A. thaliana*.[5] The plant growth ability of acidic compounds such as palmitic acid was proven by Xiong et al.,[43] who showed that root exudates from the halophyte *Limonium sinense* help in the colonization by the *Bacillus flexus* KLBMP 4941, which promotes host plant growth under salt stress due to the presence of organic acids such as palmitic acid, in the root exudates. Palmitic acid also had positive effects on the growth, motility, chemotaxis, and root colonization by the bacterium.

CONCLUSION

In the past, much emphasis has been greatly placed on bacterial phytohormones for their role in plant growth promotion, but recent trends have shown that other compounds of bacterial origin such as VOCs, also play a major role in plant growth promotion and disease resistance. Given the relatively limited knowledge on the VOCs produced by vegetable root nodule associated bacteria, there is a pressing need for extensive documentation and further research to decipher their roles. Such an exercise would pave the way for discovering novel aspects of nodule associated bacterial metabolism in relation to plant growth promotion and microbial interactions.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

AUTHORS' CONTRIBUTION

GS conceived the study. ASB executed the study. AVG, GS and DK supervised the study. ANL and KSS carried out the HS-SPME-GC–MS analysis. SK carried out the statistical analysis. ASB wrote the manuscript. GS revised the manuscript. All authors read and approved the final manuscript for publication.

FUNDING

None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

This article does not contain any studies on human participants or animals performed by any of the authors.

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