Plant growth promotion mechanisms of Bacillus sp. NYG5

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Abstract

Background

Bacterial strains in the rhizosphere may secrete an array of volatile organic compounds (VOCs), many of which are vital for inter- and intra-kingdom signaling, modulating microbe-microbe and microbe-plant interactions. In this study, we tested the plant growth promoting effect of VOCs emitted by *Bacillus* sp. NYG5 on several plants, focusing on VOC-induced changes in plant metabolic pathways, soil chemical properties and rhizo-microbiome.

Results

NYG5 emitted VOCs were found to promote growth of *Arabidopsis thaliana*, *Nicotiana tabacum* and *Cucumis sativus*, while also altering the community composition of the rhizosphere microbiome. Furthermore, fluorescent spectral analysis of the soil showed a significant decrease in concentration of humic substances following exposure to NYG5-emitted VOCs. Utilizing SPME-GC-MS analysis, several novel VOCs were identified, some of which were shown to promote plant growth. Transcriptomic analysis of *N. tabacum* plants exposed to NYG5-emitted VOCs revealed the induction of several plant growth related pathways such as phenylpropanoid biosynthesis, metabolism of sugars, and signal transduction of plant hormones.

Conclusions

Exposure to NYG5-emitted VOCs significantly impacted several plant growth related pathways, increasing plant biomass. Additionally, these VOCs altered the community composition of the rhizosphere microbiome, and decreased the concentration of humic substances in the soil. These findings may suggest that bacterial VOCs significantly impact plant growth via various mechanisms.

Introduction

Plant growth promoting rhizobacteria (PGPR) are responsible for a wide range of beneficial effects on plants, often directly enhancing growth and crop yield. These benefits include, among others, nitrogen fixation (Vessey, 2003), phosphorus and potassium solubilization (Arora et al., 2013; Mehnaz, 2016), increasing metal availability (Ahmed and Holmström, 2014), as well as production of enzymes, plant hormones, and secondary metabolites (Mendes et al., 2013).

Volatile organic compounds (VOCs) are characterized by low boiling points and molecular weights (less than 300 Da), high vapor pressures and lipophilicity. These characteristics facilitate cellular membrane penetration, and evaporation and distribution in the atmosphere as well as in air or water-filled underground pores (Kanchiswamy et al., 2015). As a result, VOCs play a crucial role in chemical
communication between plants and rhizosphere organisms. Some bacterial VOCs were also found to
antagonize an array of known phytopathogens (Kai et al., 2007; Ossowicki et al., 2017; Liu et al., 2018;
Mannaa and Kim, 2018; Hazarika et al., 2019; Syed-Ab-Rahman et al., 2019). The ability of bacterial VOCs
to promote plant growth and resistance to abiotic stress, include increased leaf surface area and salinity
tolerance (Ryu et al., 2003; Park et al., 2015b; Tahir et al., 2017; Esmaeel et al., 2018).

In the current study, we demonstrate that VOCs emitted by Bacillus sp. NYG5 significantly increase
growth of different plants while also altering the rhizo-microbiome and affect soil chemical properties.

**Materials and methods**

**Bacterial strains and culture conditions**

Isolate NYG5 (closely related to Bacillus halotolerans) was isolated in our laboratory (Rana et al., 2024).
It was routinely grown on LB agar plates, and cultures were incubated in LB broth for overnight, shaking
at 170 rpm in 30°C. Bacillus halotolerans BH4 was received from DSMZ (DSM 8802). For two-
compartment plate assays, 50 µL of overnight culture, diluted to OD$_{600}$ = 0.5, was spread on one
compartment of the plate containing minimal agar media (2.5 g/L K$_2$HPO$_4$, 2.5 g/L KH$_2$PO$_4$, 1 g/L
(NH$_4$)$_2$HPO$_4$, 0.2 g/L MgSO$_4$·7H$_2$O, 0.01 g/L FeSO$_4$·7H$_2$O, 0.007 g/L MnSO$_4$·7H$_2$O, 15 g/L Bacto agar and
10 g/L of either galactose, arabinose, glucose, sucrose or mannitol). The plates were incubated at 30°C
for 48 hours before seedlings were added to the second compartment contained 50% MS media (see
details below).

**Plant material**

*A. thaliana* seeds were surface-sterilized (70% ethanol for 2 minutes followed by 10 minutes in 1%
sodium hypochlorite), rinsed three times in sterile, distilled water and placed on petri dishes containing
50% Murashige and Skoog salt (MS) medium (Duchefa Biochemie, Haarlem, Netherlands) containing
0.3% Gelrite and 1% sucrose, adjusted to pH 5.7, and seeds were than stratified for 2 days at 4°C in the
dark. Next, seeded plates were placed in 23°C growth room under 16 hours light/8 hours dark cycle at
100 µE fluorescent light intensity, and a relative humidity of 50–60% for 7 days. *N. tabacum* (cv. Samsun)
seeds were treated as described for the *A. thaliana*, excluding the stratification steps. *C. sativus* seeds
were sterilized as described above and directly applied on the 50% MS agar media in the two-
compartment petri plate assays (see below).

**Two-compartment plate assay for growth promotion**

This assay was conducted in two-compartment Petri plates (Miniplast, Ein Shemer, Israel) containing a
plastic partition, preventing direct contact between plant and bacteria. 50 µL of each bacterial strain
(normalized to OD$_{600}$ = 0.5) were streaked on the minimal agar (described in bacterial strains and culture
conditions section) side of the two-compartment plates and incubated for 48 hours at 30°C. Next, three
to four, evenly germinated, 7 days old *A. thaliana* or *N. tabacum* seedlings were placed on the 50% MS
agar side of the two-compartment petri plates. The plates were double sealed with parafilm and incubated vertically at 23°C in 16-hour light/8-hour dark cycle for 14 days. For root length measurements, the plates were scanned using an office scanner (HP Scanjet G4010) and analyzed by ImageJ (version 1.52s). Plant biomass (fresh weight) was measured using an analytical balance (AS 220.R2, Radwag, Radom, Poland). Screening of the biological activity of individual VOCs was performed as described by Rana et al., (2024).

**Two-compartment container assay**

A (7.5 x 7.5 x 10 cm) polycarbonate container (TA 020080, Tarsons, Kolkata, India) with ten (2 mm) holes in the bottom was filled with 30 g of potting mix and placed on top of another, identical container, containing two 60 mm petri plates inoculated with the tested bacterial strains (NYG5, DH5α) or un-inoculated control plate. To examine the effect of VOCs on plants, three *A. thaliana* Col-0 seeds were added to each container (Fig. 1A, B). The containers were incubated at 23°C with a 16-hour light/8-hour dark cycle for 45 days. To examine the effect of VOCs on soil chemical properties, the same procedure was followed without addition of *A. thaliana* seeds.

**Rhizosphere sampling for microbiome analysis**

Rhizosphere samples were collected as follows, the plant roots were carefully separated from the soil by hand, the soil directly attached to the roots was collected using a soft brush for the microbiome analysis. The samples were stored at -80°C.

**DNA extraction**

0.2 g of each sample was used for total genomic DNA extraction using the NucleoSpin Soil, DNA isolation kit (Macherey Nagel, MN, USA) following the manufacturer’s instructions. DNA samples were stored at -80°C until further analysis. The quantification and quality of the DNA were determined using a Qubit 2.0 fluorometer (Thermo Fisher Scientific, MA, USA).

**Amplification of bacterial 16S rRNA genes**

DNA samples were amplified targeting the V3-V4 variable region of the microbial small subunit ribosomal RNA (16S rRNA) gene. A two-step PCR protocol was conducted using the CS1_515F (ACACTGACGACATGGTTCTACAGTGCCAGCMGCCGCGGTAA) and CS2_806R (TACGGTAGCAGAGACTTGGTCTGGACTACHVGGGTWTCTAAT) primer set (Green et al., 2015). Primers contained 5’ common sequence tags (CS1 and CS2). Each primer set also contained the 5’ common sequence tags and Fluidigm adaptor primers CS1F and CS2R as previously described by Green et al., (2015). PCR was conducted in a 50µL volume with the following PCR conditions: 1 cycle of 95°C for 5 min, followed by 30 cycles of 95°C for 30 s, 60°C for 30 s and 72°C for 60 s with final elongation of 72°C for 5 min. PCR products were purified using MinElute PCR Purification Kit (Qiagen, Hilden, Germany).

**Library preparation**
To study the microbial community composition, library preparation and sequencing of the rhizosphere samples were performed at the DNA Services Facility (University of Illinois, Chicago, IL, USA), using the Illumina NovaSeq platform for the sequencing, and producing 150-bp paired-end reads.

**Microbiome data analysis**

Data analysis was conducted as described in Sudakov et al., (2024). In short, FASTQ files were imported and processed using QIIME2 tools (version 2019.7) (Bolyen et al., 2019), and sequence quality was visualized using the `demux summarize` plugin. Subsequently, the `DADA2` plugin (Callahan et al., 2016) was employed for denoising, and the resulting 16S amplicon sequence variants (ASVs) were used for taxonomy classification with the Silva database (version 132) (Quast et al., 2013). Next, the `q2-diversity` plugin (Bolyen et al., 2019), DESeq2 (v1.38.2) (Love et al., 2014), ANCOMBC (v2.0.2) (Lin and Peddada, 2020) and phyloseq (v1.42) (McMurdie and Holmes, 2013) R packages were used to analyze microbiome composition. The effect of treatment on the bacterial community composition (Beta-diversity) was determined using the Bray–Curtis dissimilarity index. The Pairwise Permutational multivariate analysis of variance (PERMANOVA) test was employed to determine statistical significance between treatments.

**Extraction and Identification of bacterial volatiles using SPME-GC-MS**

**a. Bacterial sample preparation for volatile profile sampling**

100 µL of NYG5 bacterial overnight culture diluted to OD$_{600}$ = 0.5, was added to a sterile GC vial containing 15 mL minimal agar media (as described above) with galactose. The serum bottles were sealed with sterile crimp aluminum seals with Butyl/PTFE septa and incubated at 30°C for 48 hours. Serum bottles filled only with agar (without bacteria) were used as control.

**b. VOC collection**

Volatile collection was conducted using 50/30 µm divinylbenzene (DVB)–carboxen (CAR)–PDMS SPME fiber (Supelco, Bellefonte, PA, USA). Vials containing samples were kept at room temperature with the SPME fiber inserted into the headspace above the bacterial sample for 18 hours (absorption time). VOCs absorbed to the fiber were desorbed and analyzed using GC-MS (Agilent 7890B, 5977A MSD), Inlet: 260°C; Split 4:1 for 0.5 min; then split ~ 35:1, using HP-5ms Ul; 30 m x 0.25 mm ID x 0.25 um film Column. Carrier gas was: He; 1.2 ml/min; constant flow, GC Oven: 30°C (1min) 10°C/min to 150°C (0min) 20°C/min to 320 (2min), with Transfer Line: 250°C and MS: scan 40–500 amu; 1562 u/sec. The Total Ion Count (TIC) chromatograms of NYG5 were compared with control samples to identify bacterial VOCs. All compounds were identified using the NIST/EPA/NIH mass spectral library (version 2.0, 2012).

**Soil chemical properties**

Soil samples were dried in a 40°C oven for 48 hours. For analysis of available N-NO$_3$ and N-NH$_4$, soils were extracted with 1 mol/L KCl, and for P-PO$_4$, soils were extracted with 0.5 mol/L NaHCO$_3$ using the ‘Olsen P’ test (Black et al., 1983). Recovered N-NO$_3$, N-NH$_4$, and P-PO$_4$ were colorimetrically quantified using a Gallery Plus Discrete Analyzer (Thermo-Fisher, Waltham, MA, USA). For quantification of chlorine
(Cl), potassium (K), sodium (Na), soil solutions were mixed with double-deionized water (1:5 soil to water ratio), shaken for 1 hour, centrifuged for 5 minutes at 4000 rpm, and the supernatants were filtered using 1 µM filter disks (Sartorius, Gottingen, Germany). Cl concentrations in the soil solution extract were quantified using the Gallery Plus Discrete Analyzer. Na and K levels were measured using a M410 flame spectrophotometer (Sherwood Scientific, Cambridge, UK).

**Fluorescence excitation-emission spectra**

Soil extracts were prepared as described above for Cl K and Na. Fluorescence of aqueous soil extracts was measured in 10 mm-quartz cuvettes at a 25 ± 1°C using FluoroMax+ (Horiba, Kyoto, Japan), with an excitation spectral range of 240–450 with 5 nm increments and emission range between 300 and 600 nm with 5 nm increments and 0.1 s integration time. The entrance and exit slits were 6 nm. The excitation-emission spectra were further processed by blank (DDW) subtraction, Rayleigh scatter removal and subsequent missing data interpolation using drEEM toolbox (version 0.6.3) for MATLAB 2021a (The Mathworks Inc., MA, USA) (Murphy et al., 2013).

**Transcriptomic analysis**

**Sample preparation**

Evenly germinated tobacco seedlings were added to one side of two-compartment plates and exposed to one of three treatments: NYG5-emitted VOCs, BH4-emitted VOCs and a control plate without bacteria. Plant samples were collected at three different time points: 24 hours, 72 hours and one week following the start of the experiment. Five replicates per treatment were collected, each containing of at least nine tobacco seedlings. The seedlings were flash frozen in liquid nitrogen. Next, samples were ground to powder in a pre-chilled pestle and mortar filled with liquid nitrogen. 75 mg of each replicate was added to a RNase/DNase free sterile Eppendorf tube and flash frozen in liquid nitrogen. Total RNA was extracted using the InviTrap® Spin Plant RNA Mini Kit 50 (Invitek diagnostics, Berlin, Germany), according to manufacturer's instructions. DNA was removed from the samples using Turbo DNase (AM2238, Thermo Fisher Scientific, MA, USA), and RNA concentration was measured using a Qubit 2.0 fluorometer (Thermo Fisher Scientific, MA, USA) with broad spectrum RNA reagents and gel electrophoresis.

**Library preparation and sequencing**

RNA libraries were prepared from total RNA extracted from tobacco plants using the NEXTFLEX Rapid Directional RNA-Seq Kit 2.0 (PerkinElmer, MA, USA) after poly(A) capture using NEXTFLEX poly(A) bead capture. Poly(A) capture, reverse transcription, and library preparation were performed on a SciClone NGSx workbench (PerkinElmer, MA, USA), according to the manufacturer’s instructions. Input RNA levels were 150 ng, cDNA was generated and 15 cycles of PCR were performed. Libraries were initially evaluated by sequencing on an Illumina MiniSeq mid-output flow cell (300 cycles), followed by deep sequencing on an Illumina NovaSeq6000 S4 lane. Libraries were prepared by the RUSH University Genomics and Microbiome Core Facility (GMCF), and NovaSeq sequencing was performed at the DNA Services facility at the University of Illinois at Urbana-Champaign.
Data analysis of Nicotiana tabacum RNAseq

Raw-reads were subjected to a filtering and cleaning procedure, the Trimmomatic tool (Bolger et al., 2014) was used to remove Illumina adapters from the reads. Next, the FASTX Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/index.html, version 0.0.13.2) was used to trim read-end nucleotides with quality scores < 30, using the FASTQ Quality Trimmer, and the FASTQ Quality Filter was used to remove reads with less than 70% base pairs with a quality score ≤ 30. Clean reads were mapped to the reference genome of Nicotiana tabacum TN90 cultivar (GCF_000715135.1; https://ftp.ncbi.nlm.nih.gov/genomes/all/GCF/000/715/135/GCF_000715135.1_Ntab-TN90/GCF_000715135.1_Ntab-TN90_genomic.fna.gz) using STAR software (v. 2.7.1a) (Dobin et al., 2013). Gene abundance estimation was performed using Cufflinks (v. 2.2) (Trapnell et al., 2010), combined with gene annotations from NCBI database (https://ftp.ncbi.nlm.nih.gov/genomes/all/GCF/000/715/135/GCF_000715135.1_Ntab-TN90/GCF_000715135.1_Ntab-TN90_genomic.gff.gz). PCA analysis and heatmap visualization were performed using R Bioconductor (Gentleman et al., 2004). Gene expression values were computed as Fragments Per Kilobase of transcript per Million mapped reads (FPKM). Differential expression analysis was completed using the DESeq2 R package (Love et al., 2014). Genes that varied from the control more than twofold, with an adjusted p-value of no more than 0.05, were considered differentially expressed (Benjamini and Hochberg, 1995). Venn diagrams were produced using the Venny 2.0.2 online tool (https://bioinfogp.cnb.csic.es/tools/venny/index2.0.2.html). KOBAS 3.0 tool (Xie et al., 2011; http://kobas.cbi.pku.edu.cn/kobas3/?t=1) was used to detect the statistical enrichment of differential expression genes in KEGG pathways. Plots were produced using the SRplot online tool (Tang et al., 2023).

Results

The effect of NYG5-emitted VOCs on A. thaliana and its rhizo-microbiome

To determine the role of NYG5-emitted VOCs in plant growth promotion, we utilized a two-compartment container assay (Fig. 1A), seedlings were grown for 30 days in the presence of bacteria-emitted VOCs (Fig. 1B). Plant parameters and alterations in the rhizo-microbiome of A. thaliana were evaluated across three treatments: (1) Exposure to VOCs emitted by NYG5, (2) Exposure to VOCs emitted by DH5α E. coli, and (3) control without bacteria. A significant increase in several parameters (plant height, number of leaves and biomass) was observed in A. thaliana plants exposed to NYG5-emitted VOCs compared to DH5α and control treatments (Fig. 1C). Significant differences were observed in alpha-diversity (Shannon’s index) between microbiomes of plants exposed to NYG5-emitted VOCs compared to DH5α (p = 0.012) VOCs, and the bacteria-free control (p = 0.013) (Fig. 1D). Significant differences in bacterial community composition were observed in plants exposed to NYG5-emitted VOCs vs. DH5α and control (p < 0.002) treatments (Fig. 1E). The dominant classes in all treatments were Alpha-, Gamma- and Deltaproteobacteria, Bacteroidia, Verrucomicrobiae, and Actinobacteria. Taxonomic and linear discriminant analysis Effect Size (LEfSe, (Segata et al., 2011)) indicate significant differences between
features of the communities in NYG5-emitted VOCs treatments vs. DH5α and controls, with the main differences being an increased relative abundance of Gammaproteobacteria and decrease in Deltaproteobacteria (Fig. 2A, B). LEfSe was employed to quantify the differentially abundant genera. A significant increase in relative abundance of sequences related to Micropepsis, Nitrosospira and Rhodanobacter genera was detected in the NYG5-emitted VOCs treatments. Relative abundance of sequences related to Devosia, Optitutus and Lacunisphaera were significantly reduced (LEfSe p < 0.05) (Fig. 2C, D). These findings indicate that VOCs produced by NYG5 have a significant impact on both the A. thaliana plant and its rhizo-microbiome.

**Effect of NYG5-emitted VOCs on soil chemical properties**

To evaluate whether bacteria-emitted VOCs alter soil chemical properties, we utilized a two-compartment container assay (Fig. 3A) similar to the one used for microbiome analysis. In this experiment a container with perforated bottom was filled with soil and placed on top of a second container with a plate with a single bacterial strain. We applied NYG5, DH5α and BH4 (non-growth promoting B. halotolerans), and an empty plate as a negative control. Fluorescence spectra excitation-emission matrix revealed a single peak in the soil samples of all treatments (Fig. 3B), that was representative of humic acid substances (Gao et al., 2017). The peak magnitude (excitation at 350 nm and emission at 450 nm) of soils exposed to NYG5-emitted VOCs, was significantly lower (p < 0.0001) than the other treatments (Fig. 3C). In addition, the pH of soil samples treated with NYG5-emitted VOCs was significantly higher (p = 0.001) than the other treatments (Fig. 3D). Difference in soil macronutrients between treatments were insignificant (Supplementary Fig. 1). These results may suggest that NYG5-emitted VOCs lead to reduction in humic substances either directly or through alterations of the rhizomicrobiome consequently affecting soil composition.

**Bacterial growth conditions for optimal VOC production**

A two-compartment petri plate assay was used to screen for bacterial growth conditions for the optimal production of plant growth promoting VOCs (Fig. 4A). NYG5 was incubated on agar media with different carbon sources on one side of the plate, and A. thaliana (Col-0) seedlings were grown on the other. When incubated on galactose (as a single carbon source), NYG5 was found to emit VOCs that significantly increased A. thaliana plant biomass and root length compared to other tested carbon sources (glucose, arabinose, sucrose and mannitol) (Fig. 4A, B). Additional model plants (N. tabacum and Cucumis sativus) tested using this setup also displayed promising plant growth promotion effects (Fig. 4C-F). These results indicate that NYG5's volatile profile is dictated by available carbon sources. This assay can be utilized for screening of the effect of different media components on VOCs production.

**Growth promotion effect of NYG5-emitted specific VOCs**

Following the calibration of optimal growth condition for the production of plant growth promoting VOCs, Solid-phase microextraction (SPME) combined with chromatography-mass spectrometry (GC-MS) was
utilized to identify NYG5-emitted VOCs profile. From these, several identified, commercially available VOCs and their derivatives (Supplementary Fig. 2) were tested in vitro assessing their growth promoting effect on *N. tabacum*. Several VOCs consistently increased either plant biomass or root length compared to control (Supplementary Fig. 2). Furthermore, we examined the growth-promoting potential of 2,3-hexanedione, a VOC previously identified by our laboratory (Rana et al., 2024). The compound exhibited a significant impact on plant biomass and root length at concentrations of 0.01 and 0.05 ppm (Fig. 5A, B). These findings suggest that a single NYG5-emitted VOC may be sufficient to efficiently increase plant growth.

**Effect of NYG5 VOCs on *N. tabacum* gene expression and metabolic pathways**

To elucidate growth promotion mechanisms of NYG5-emitted VOCs, RNA-seq analysis was performed on *N. tabacum* seedlings exposed to VOCs emitted by either NYG5 or BH4 (a non-growth-promoting *Bacillus halotolerans* strain), as well as on a bacterial-free control group utilizing two-compartment plate assays described above (Fig. 4A). Seedling samples were collected at three time points: 24 hours, 72 hours and 7 days following the beginning of the exposure. Approximately 20 million reads per sample were generated with 96% mapping to the *N. tabacum* reference genome. Transcriptional profiles of the different treatment groups were combined from all three time points. Genes that were differentially expressed (Log Fold Change (LFC) > 2, *p* adj < 0.05) in the NYG5 treatment compared to both BH4 and control were considered significant (Fig. 6A). This analysis revealed 1761 up-regulated and 1199 down-regulated genes. Pathway enrichment analysis revealed the upregulation of various plant growth associated functions, such as phenylpropanoid biosynthesis, amino sugar and nucleotide sugar metabolism, starch and sucrose metabolism, and plant hormone signal transduction. Down-regulated pathways included various RNA related functions such as mRNA surveillance, RNA transport RNA polymerase, ribosome biogenesis and spliceosome, and different structural components 2-oxalocarbonic acid and peroxisome. Notably, several components within the phenylpropanoid biosynthesis pathway, involved in synthesis of lignin monomers, were significantly upregulated (Fig. 6C and supplementary Fig. 3). One of these is caffeic acid 3-O-methyltransferase (COMT) (LFC = 6.9, *p* adj = 1.16e-14), which has been known to play a role in the lignin (Ma and Xu, 2008) and suberin (Graça, 2015) monomer synthesis. Staining of lignin and suberin did not reveal significant differences in the abundance of suberin between the different treatments (data not shown). Furthermore, genes associated with the synthesis of lignin or suberin polymers were not up-regulated in this experiment, this may be due to the young age of the tested *N. tabacum* seedlings. However, these results suggest that NYG5-emitted VOCs may potentially to enhance the synthesis of lignin and suberin, influencing the structural and protective properties of the plant.

**Discussion**

The rhizosphere is a dynamic environment characterized by active interactions between microorganisms and plants, influencing various aspects of plant growth and health. One type of such interaction involves PGPR-secreted VOCs, which were found to play a crucial role in facilitating microbe-microbe and
microbe-plant interactions in the rhizosphere (Tahir et al., 2017; Fincheira and Quiroz, 2018; Vaishnavi and Osborne, 2021). VOCs diffuse through the soil, reaching the plant root system and affecting plant growth (Park et al., 2015b; Camarena-Pozos et al., 2019). VOCs were found to affect plant signaling pathways, ultimately leading to changes in its gene expression and overall physiology (Zhang et al., 2007; Hao et al., 2016; Gao et al., 2022). Furthermore, VOCs may extensively influence the composition of the plant microbiome, attracting beneficial microorganisms to the rhizosphere (Liu and Brettell, 2019), and decreasing phytopathogens proliferation, thus promoting plant growth and health. PGPR-emitted VOCs may also act as signaling molecules between different types of microorganisms in the rhizosphere (Garbeva et al., 2014; Schulz-Bohm et al., 2015), leading to the formation of beneficial microbial communities.

The current study provides clear insights on the mechanisms by which NYG5 promotes plant growth through the secretion of VOCs. These include reshaping of the plant rhizo-microbiome, altering soil chemical properties and modification of specific metabolic pathways in the plant. To the best of our knowledge, this is the first study to combine those three aspects of bacterial plant growth promotion.

**NYG5-emitted VOCs affect growth and rhizo-microbiome in A. thaliana:**

The capacity of NYG5-emitted VOCs to stimulate plant growth discovered in this study are consistent with previous research on plants exposed to bacterial VOCs (Tahir et al., 2017). In the current study, we demonstrated that NYG5-emitted VOCs stimulated biomass and root length of several plants including cucumber and tobacco, which have significant agricultural value. We further investigated this phenomenon by assessing the differences in the rhizo-microbiomes of the VOC treated plants, due to the fact that changes in the abundance or composition of the plant microbiome may be one of the mechanisms leading to increased plant growth (Qu et al., 2020). We report that NYG5-emitted VOCs significantly alter the relative abundance of several taxa in the rhizo-microbiome of A. thaliana, including an increase in *Gammaproteobacteria* and a decrease in *Deltaproteobacteria*. Interestingly, some members of these taxa are associated with a healthy plant microbiome and were shown to have beneficial interactions with plants (Edwards et al., 2015; Köberl et al., 2017). Genus level analysis of the data revealed that exposure to NYG5-emitted VOCs significantly increased the relative abundance of several bacterial genera. These include the root-colonizing *Rhodanobacter* (Vannier et al., 2023) and ammonia-oxidizing *Nitrosospira* (Liang and Bowatte, 2022). Members of these genera have been previously found to be associated with a healthy plant microbiome (Liu and Zhang, 2021). VOCs secreted by NYG5 may act directly on the microbiome, altering the abundance of potentially beneficial bacteria. Alternatively, the abundance of these taxa may also be indirectly influenced by VOCs altering the composition of secreted root exudates or modifying soil chemical properties. Additional research is required for the elucidation of the mechanism of action of NYG5-emitted VOCs on the rhizo-microbiome.

**NYG5-emitted VOCs affecting humic substances in soil**
A single peak representative of humic substances was identified in the soils used in this study using fluorescent excitation-emission spectra analysis. The significant decrease in the peak maxima observed following exposure to NYG5-emitted VOCs may indicate a decrease in the concentration of humic substances in the treated soil. These substances, which are mainly composed of humic acids, fulvic acids, and humins, are generally associated with healthy soil and enhanced plant growth (Fuentes et al., 2018; Canellas et al., 2020), mainly by contributing to soil structure and texture (Ampong et al., 2022) and plant hormone-like activity (Yang et al., 2021). Reduction in humic substances in soil may occur due to their degradation by recruited beneficial bacteria (Tikhonov et al., 2010) including *Gammaproteobacteria* (Rocker et al., 2012; Park et al., 2015a) which were found here to be significantly more abundant following treatment with NYG5-emitted VOCs. Humic substances are complex organic compounds in soil, they contain essential nutrients that are often in forms that are not directly accessible to most microorganisms and plants. Degradation of humic substances by bacteria and fungi transforms these complex molecules into simpler forms (Grinhut et al., 2007), enhancing nutrient cycling in the soil, making essential elements more accessible to plants (Jin et al., 2024). In our study, we observed a noticeable negative correlation between the levels of humic substances and the corresponding pH values within the soil samples. Specifically, as humic substances decreased, there was a tendency for pH levels to increase, indicating a potential relationship between the abundance of humic substances and soil acidity. This suggests that the degradation of humic substances may contribute to the reduction of soil acidity over time. These results highlight the importance of microbial activity in the soil ecosystem for optimizing nutrient availability and supporting plant growth.

NYG5-emitted VOCs affecting plant metabolic pathways

Transcriptomic analysis of *N. tabacum* seedlings exposed to NYG5-emitted VOCs revealed significant differences in the gene expression profiles of several plant metabolic pathways. Some upregulated pathways were strongly correlated with plant growth, including those encoding for phenylpropanoid and diterpenoid biosynthesis, metabolism of amino sugars, nucleotide sugars, starch and sucrose, and signal transduction of plant hormones. The phenylpropanoid biosynthesis pathway is mainly regarded as one of the most important pathways in the plant, responsible for biosynthesis of different metabolites, including lignins, flavonoids, and coumarins (Fraser and Chapple, 2011). In our study, expression of the COMT gene, a key component in the phenylpropanoid biosynthesis pathway, was significantly stimulated by NYG5 VOCs. COMT has been previously recognized as a crucial component in suberin biosynthesis, facilitating the catalysis of ferulic acid - the main aromatic monomer of suberin polyester (Graça, 2015). Moreover, COMT was found to be involved in the biosynthesis of lignin, catalyzing the methylation reactions of monomeric lignin precursors (Ma and Xu, 2008). However, an actual increase in suberin and lignin concentrations was not detected. We hypothesize that, at the tested age of the studied *N. tabacum* seedlings (14 days old), suberin and lignin may not have yet reached quantifiable levels in the plant. This assumption is based on the absence of enrichment in the lignin and suberin pathways in our analysis, and the inability to visualize both polymers through staining (data not shown). We speculate that extending *N. tabacum* growth period under VOCs exposure may result in an increased synthesis of both
lignin and suberin polymers. Taken together, these findings indicate that NYG5 may play an important role in promoting plant growth through the regulation of various plant metabolic pathways via the emission of VOCs.

**Study summary**

In the current study, we describe the effects of VOCs produced by *Bacillus* sp. NYG5 on the soil-microbiome-plant system. Our results indicate that these VOCs can modify the chemical properties of soil, altering the levels of humic substances. VOCs were also shown to impact the plant microbiome, fostering the growth of certain bacteria while hindering the growth of others. These microbiome alterations correlate with plant growth and development. Furthermore, we found that exposure to these VOCs induced changes in plant gene expression, altering expression of several growth-related metabolic pathways. These findings emphasize the importance of bacterial VOCs in plant-microbe interactions and the need for further research to understand the full extent of their impact and specific mechanisms of action. The results of this study may have broad implications for agriculture and ecosystem management and can promote the development of new strategies for cultivating healthy and productive plants.

**Declarations**

**Declaration of competing interest**

The authors declare that they have no known competing financial interests or personal relationships that could have influenced the work reported in this paper.

**Author Contribution**

K.S. Planning, methodology, analysis and interpretation of all experiments, writing of the original draft. A.R. Investigation and visualization of the rhizomicrobiome experiment, Review & editing of the original draft. A.F.D. Analysis of the RNAseq data. A.G. Analysis and interpretation of SPME-GC-MS VOC experiments S.C. Conceptualization VOC experiments and review & editing of the original draft. J.A.S. Conceptualization and planning of fluorescent spectral analysis, review & editing of the original draft. E.C. and D.M. Supervision, conceptualization, methodology, Resources, review & editing of the original draft, funding acquisition. All authors read and approved the final manuscript.

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Data Availability

Data used in this study have been deposited in the NCBI Sequence Read Archive under BioProject accession number PRJNA1106390 (rhizo-microbiome amplicon sequencing) and PRJNA1106659 (transcriptome analysis).

References

Paraburkholderia phytofirmans PsJN-plants interaction: From perception to the induced 

14. Fincheira P, Quiroz A. Microbial volatiles as plant growth inducers. 

15. Fraser CM, Chapple C. The Phenylpropanoid Pathway in Arabidopsis. 
Arab B. 2011;9:e0152. 10.1199/tab.0152.

methodology to assess the quantity and quality of humic substances in 


the growth-promoting effect of volatile organic compounds produced by 
Microbacterium aurantiacum GX14001 on tobacco (Nicotiana benthamiana). BMC 

10.3389/fmicb.2014.00289.

Bioconductor: open software development for computational biology and 


22. Green SJ, Venkatramanan R, Naqib A. Deconstructing the polymerase chain 
reaction: Understanding and correcting bias associated with primer degeneracies 

23. Grinchut T, Hadar Y, Chen Y. Degradation and transformation of humic 
substances by saprotrophic fungi: processes and mechanisms. Fungal Biol Rev. 

Comparative digital gene expression analysis of the Arabidopsis response to 

Lipopeptide mediated biocontrol activity of endophytic Bacillus subtilis against 


Figures
Figure 1

Effect of bacterial VOCs on *A. thaliana* plant growth and diversity indices of bacterial communities. (A, B) Schematic description of the experimental setup. Plants were grown for 30 days in two-compartment containers. Four plant growth parameters (C) were measured. Statistical significance was assessed using Tukey HSD, with asterisks indicating significant differences ($p<0.005$), error bars indicating SEM (n=9). Alpha diversity (D) was calculated using Shannon's index, and significance of the differences was
assessed using the pairwise Wilcoxon test. Beta-diversity was evaluated using the Bray–Curtis similarity index (E), and visualized using principal coordinate analysis, and significance of the differences in community composition was calculated using pairwise PERMANOVA ($p<0.05$, n=5).

Figure 2

Effect of bacteria-emitted VOCs on the *A. thaliana* microbiome. Differences in differential abundance at class (A, B) and genus (C) levels between NYG5, DH5α-emitted VOCs, or controls were calculated using LEfSe analysis ($p<0.05$, n=5). Representative differences in abundance of specific genera between treatments (D).
Figure 3

Effect of bacteria-emitted VOCs on soil chemical properties. (A) Experiment design. (B) Fluorescence spectra emission-excitation matrix peak representative of humic acids, in all soil samples. (C) Magnitude of humic acid emission-excitation matrix peak; and (D) pH levels in bacterial VOC-exposed soils. Significance of the differences was assessed using Tukey HSD, with asterisks indicating significant difference \( (p<0.05) \), error bars indicating SEM (C) \( n=9 \) (D) \( n=15 \).
Figure 4

Effect of carbon source on NYG5 plant growth promoting VOCs. (A) Schematic description of the experiment, conducted in two-compartment petri plates without direct contact between bacteria and plants. *A. thaliana* growth promotion by NYG5-emitted VOCs using minimal media supplemented with different carbon sources (B-C). *N. tabacum cv. Samsun* (D-E) and (D-E) and *Cucumis sativus* (F-G) growth promotion by NYG5-emitted VOCs using minimal media supplemented with galactose. *Cucumis*
*sativus* was grown in two compartment and removed for analysis. Error bars indicate SEM, asterisks indicate significant difference following Tukey HSD (C) (p<0.05, n=9) and student t test (D, F) (p<0.05, n=6).

**Figure 5**

Growth promotion effect of 2,3-hexanedione. (A) Plant growth promotion effect of different concentrations of 2,3-hexanedione on *N. tabacum*. Error bars indicate SEM, asterisks indicate significant difference from control following Tukey HSD (p<0.05, n=9). (B) Concentration dependent effect of 2,3-hexanedione on growth of *N. tabacum* seedling.
Figure 6

Transcriptomic analysis of *N. tabacum* following exposure to NYG5-emitted VOCs. Venn diagrams showing (A) up and (B) down-regulated genes as a function of the three treatments. Significantly regulated genes of NYG5 compared to both BH4-emitted VOCs and control treatments (circled in red in A and B) were used to identify (C) up and (D) down regulated pathways. Venn diagrams were generated using Venny 2.0.2, pathway enrichment analyses were conducted using KOBAS 3.0. (n=5).

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.
• SupplementaryFig1macroelements.jpg
• SupplementaryFig2othertestedVOCs.jpg
• SupplementaryFig3KEGGpathway.jpg