RESEARCH ARTICLE

Effect of bacterial intra-species community interactions on the production and activity of volatile organic compounds

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ABSTRACT

Microorganisms experience intra- and inter-species interactions in the soil, and how these interactions affect the production of microbial volatile organic compounds (VOCs) is still not well-known. Here we evaluated the production and activity of microbial VOCs as driven by bacterial intra-species community interactions. We set up bacterial communities of increasing biodiversity out of 1–4 strains each of the Gram-positive *Bacillus* and Gram-negative *Pseudomonas* genera. We evaluated the ability of each community to provide two VOC-mediated services, pathogen suppression and plant-growth promotion and then correlated these services to the production of VOCs by each community. The results showed that an increase in community richness from 1 to 4 strains of both genera increased VOC-mediated pathogen suppression and plant-growth promotion on agar medium and in the soil, which was positively correlated with the production of pathogen suppressing and plant growth-promoting VOCs. *Pseudomonas* strains maintained while *Bacillus* strains reduced community productivity with an increase in community richness and produced eight novel VOCs compared with the monocultures. These results revealed that intra-species interactions may vary between Gram-negative and Gram-positive species but improved VOC-mediated functioning with respect to pathogen suppression and plant-growth promotion by affecting the amount and diversity of produced VOCs potentially affecting plant disease outcomes.

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1 Introduction

Plant roots are associated with a dense multispecies community that provides multiple benefits to plants, including plant growth stimulation and pathogen suppression (Mendes et al., 2015). The rhizosphere microbiome is often approached as the community of microbes in direct contact with plant host, mediated by diffusible molecules and contact-mediated

interactions (Westhoff et al., 2017). Recent studies have demonstrated that volatile organic compounds (VOCs) produced by microorganisms can have a deep impact on a range of organisms, including plants and pathogens (Schmidt et al., 2015). The VOCs are low molecular weight and high vapor pressure lipophilic compounds, and are generally produced by microbes through catabolic pathways (Schulz and Dickschat, 2007). Microbial VOCs have been reported for their antibacterial, antifungal, nematocidal activities, and cell-to-cell communication (Gu et al., 2007; Raza et al., 2016). In addition, VOCs not only improve plant growth but also induce plant defense against abiotic and biotic stresses (Kanchiswamy et al., 2015). Compared to other microbial compounds, VOCs can show an effect on longer distances far from the

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production point through the atmosphere and porous soils because of their diffusibility (Leff and Fierer, 2008). This implies that plant growth and health may be under the direct influence of a much bigger microbial community than originally expected (Tahir et al., 2017).

In the rhizosphere, the functionality and effects of the microbiome on plant fitness are directly determined by the species composition and biodiversity. Different species interact, sometimes, leading to death, generating new metabolic pathways or expressing silent secondary metabolites gene clusters (Jurburg and Salles, 2015; Hu et al., 2017). Such effects may also occur without contact via the production of VOCs (Garbeva et al., 2011). Most studies involving VOC-mediated interactions have been conducted using a single microorganism. Recently, many studies regarding communication among different soil bacterial species have been conducted, but such reports about the production and activity of VOCs in a mixed community are scarce. Schulz-Bohm et al. (2015) reported that a mixture of five phylogenetically different bacterial strains produced a different blend of VOCs compared to the monoculture. Alternatively, Tyc et al. (2015) did not find novel or new VOCs in a pairwise comparison of phylogenetically different bacterial species, but only the combination of VOCs produced as a monoculture. Recently, the effects of inter-species interactions on the production and activity of plant growth-promoting and pathogen inhibiting VOCs were explored (Raza et al., 2020). Different microbial species display different ecological characteristics in response to the presence of other similar or different species, so the cumulative outcome may change compared to individual species (Loreau and Hector, 2001). In this context, how intra-species interactions mediate the production and functioning of VOCs is still unknown. Here we tried further extending the knowledge of VOC-mediated functioning driven by bacterial intra-species community interactions. In our study system, we separately set up experimental bacterial communities of increasing biodiversity out of 1–4 strains each well-described Gram-positive and Gram-negative soil bacterial species *Pseudomonas fluorescence* and *Bacillus amyloliquefaciens*, respectively. We assessed the ability of each community to provide two microbiome services, pathogen suppression (*Ralstonia solanacearum*) and plant-growth promotion (*Arabidopsis thaliana*), without direct contact. We then related these two services to the amount and diversity of the VOC cocktails produced by each community.

2 Materials and methods

2.1 Bacterial strains and assemblage of communities

Four strains each, two bacterial genera *Bacillus* and *Pseudomonas* isolated from the rhizosphere of different plants were selected (Table S1). We performed preliminary experiments to ensure that the pathogen suppression and plant-growth promotion by the selected bacterial strains were mediated only by VOCs (Table S1). For the assemblage of

bacterial communities, the strains were grown overnight in a nutrient broth at 30°C, washed twice with 0.85% NaCl and adjusted to the cell counts of 1×10^7 colony forming units (CFU) mL⁻¹. The strains of each genus were mixed separately in equal proportion (500 µL) to obtain 15 combinations (Table S2) with richness levels ranging from monocultures to the mixture of 4 strains considering that at each richness level, each genotype is replicated three times according to Bell et al. (2009). At each richness level, the total cell concentration of the monoculture or mixed cultures was 1×10^7 CFU mL⁻¹. The target pathogen used was *Ralstonia solanacearum* QLRs-1115 [RS; China General Microbiology Culture Collection Center accession No. 9487; Wei et al., 2011]. During the experiments, RS was grown overnight in nutrient medium at 30°C, washed twice and adjusted to the cell counts of 1×10^7 CFU mL⁻¹ with 0.85% NaCl. The bacterial strains and pathogenic strain were stored at -80°C in nutrient medium containing 70% glycerol.

2.2 Growth assay of bacterial monocultures and assembled communities

To determine the effect of intra-species interactions on the growth of bacterial communities, each well of the microtiter plates was filled with 195 µL minimal salt medium (Harwood and Cutting, 1990) modified with 0.4% tryptone soy broth (w/v) and 1.5% sucrose, and inoculated separately with 5 µL cell suspension of each bacterial community of both genera, prepared as described above (Table S2). The growth of *Pseudomonas* and *Bacillus* strains was determined by serial plating on nutrient agar medium after 36 h at 30°C and 170 r min⁻¹ and was represented as community productivity at different bacterial richness levels. The experiment was repeated twice in triplicate.

2.3 VOC-mediated pathogen suppression and plant-growth promotion assays

For VOC-mediated pathogen suppression by bacterial communities on agar medium, the cell suspension of bacterial strains was prepared separately as described above, at each richness level, was spotted at 2 points 5 cm apart (5 µL each) onto one compartment of the divided Petri plates (85-mm diameter) containing modified minimal salt agar medium prepared as described above except with 1.5% agar. While the cell suspension of RS was spot-inoculated 5 cm apart at 2 points onto the other compartment of the divided Petri plates containing nutrient agar medium. The plates were placed overnight at 30°C to initiate bacterial growth and then sealed with Parafilm and incubated at 30°C for 3 d further. The Petri plates only inoculated with RS were used as a control. After 3 d, the RS colonies were removed along with the agar medium using a sterilized scalpel, suspended (1 mL) and diluted (500 times) using sterilized water and spread on nutrient agar plates. The CFU mL⁻¹ of RS was determined after incubation for 2 d at 30°C. The experiment was repeated twice in triplicate. As evaluated in a previous experiment, the

growth of used *Bacillus* and *Pseudomonas* strains was not affected by the VOCs of RS (Raza et al., 2020).

For VOC-mediated pathogen-suppression assay in soil, the healthy soil was obtained from Yixing, China [for soil properties, see our previous report (Raza et al., 2015)]. The soil was sterilized at 121°C for 60 min and after cooling to room temperature, 7.0 g (dry weight) soil was first mixed with the cell suspensions of bacterial communities (1 mL), prepared separately as described above, for each richness level and then added to one compartment of the divided Petri plates except the controls where the soil was added without bacterial inoculation. The other compartment of the Petri plate was spot-inoculated with pathogen RS onto nutrient agar medium as described above. The plates were placed overnight at 30°C and then sealed with Parafilm and incubated again for 3 d. The CFU mL⁻¹ of RS was determined as described above and the VOC effects were presented as the percent increase or decrease in pathogen suppression relative to the control treatment. The experiment was repeated twice in triplicate.

For VOC-mediated plant-growth promotion assay by bacterial communities, the same methodology was used, except for the spot inoculation of RS, three seedlings of *A. thaliana* (Col-0) were transferred to one compartment of the divided Petri plates containing half-strength Murashige and Skoog (MS) agar medium (0.8% agar, pH 5.7). Before transfer, the surface-sterilized seeds were first vernalized for 2 d at 4°C and then placed in a growth chamber for 4 d (22°C temperatures, 12 h light, 12 h dark, 40 W fluorescent light). On the other compartment of the Petri plates, two spot inoculations of bacterial strains at different richness levels on the modified minimal salt agar medium and in the soil were performed as described above. The Petri plates without bacterial inoculation were used as controls. Two weeks later, the whole plant was removed and the fresh weight (mg plant⁻¹) was determined after washing plant with sterilized water and blot-drying. The VOC effects were presented as the percentage increase or decrease in plant growth relative to the control treatment and the experiment was repeated twice in triplicate.

2.4 VOC analysis using gas chromatography-mass spectroscopy (GC-MS)

To analyze the VOC profiles produced by all bacterial strains and communities, cell suspensions (1×10^7 CFU mL⁻¹) were prepared as described above and 2 spots (5 µL each) inoculated on minimal salt agar medium (same as above) in a 100 mL vial was prepared in triplicate and placed at a temperature of 30°C. After overnight growth, the vials were sealed and incubated at 30°C for 3 d. The vials without the inoculation of bacteria were used as controls. After incubation, 5 µL of (Z)-3-hexenyl acetate (1 mM) as an internal standard was added to the vials. Then, the extraction of VOCs using solid-phase microextraction (SPME) fiber and GC-MS analysis was performed as reported in our previous reports (Raza et al., 2015, 2016, 2020). The VOC was considered as identified if it had a match factor greater than 800, contained all main

peaks of the listed compound, and its retention index difference was not larger than five with the listed compound (for a semi-standard non-polar column). Except for 12 unidentified and 2 commercially unavailable VOCs, the production of identified VOCs was further confirmed by comparing them with standard compounds purchased from Sigma; Tokyo Chemical Industry Co., Ltd. Japan and Aladdin Reagent Database, Inc., Shanghai, China. The standards were analyzed similarly as those using SPME fiber. As an indirect approach to estimate the relative amount (concentration) of each VOC, the number of VOCs (number of peaks) produced in each treatment were recorded and the chromatographic peak area was expressed in arbitrary units (a.u.) relative to the peak area of (Z)-3-hexenyl acetate (internal standard).

2.5 Classification of VOCs into pathogen suppressing and plant growth-promoting compounds

Except for the 12 unidentified and 2 commercially unavailable VOCs, all VOCs were assessed for their pathogen suppression and plant growth-promoting activity. For pathogen-suppression assay, the cell suspension of RS prepared as described above was spot-inoculated at two points onto one compartment of the divided Petri plates containing nutrient agar medium and incubated overnight at 30°C. Later, the other half of the Petri plates were separately inoculated with 30 µL of solutions of pure VOCs (10, 50, 250, 1, and 5 µg mL⁻¹) in methanol on an approximately 10 mm (diameter) sterile filter paper disk (Whatman filter paper, 6 µm pore size) to give a final amount of 0.3, 1.5, 7.5, 30, and 150 µg final amount of each pure VOC, respectively. Petri plates were sealed with Parafilm and incubated at 30°C for 3 d. Later, the CFU mL⁻¹ of RS was determined and expressed as described above.

For the plant-growth promotion assay, the same methodology was used, except for the spot inoculation of RS, three *Arabidopsis* seedlings were placed onto the other half of the Petri plates containing half-strength MS agar medium. The plates were sealed with Parafilm and after two weeks, the fresh weight of the plant was determined and expressed as described above. For pathogen suppression and plant-growth promotion, the sterile filter paper disks inoculated with nothing or with methanol were used as control treatments (both control treatments did not show any difference). The experiment was repeated twice in triplicate.

2.6 Statistical analysis

The differences in bacterial communities, strains, and genera were analyzed using ANOVA and Tukey's tests. Regression analysis was used to analyze the relationship between bacterial community richness and VOC-mediated pathogen suppression and plant-growth promotion, the relative amount of VOCs, number of VOCs, and total community growth (cell counts mL⁻¹). The identity effect of bacterial strains was analyzed by performing a separate generalized linear model

(GLM) expressing pathogen suppression and plant-growth promotion as a function of the presence of each bacterial strain (based on binary predictors). Statistical analyses were conducted with SPSS version 19.0 statistical software (SPSS, Inc., Chicago, IL, USA).

3 Results and discussion

3.1 Effect of monocultures and community richness on VOC-mediated pathogen suppression and plant-growth promotion

In this study, all eight tested bacterial strains showed VOC-mediated pathogen suppression and plant-growth promotion. Among different *Pseudomonas* strains, Q8r1-96 showed the highest VOC-mediated pathogen suppression both on agar medium ($F_{3,8} = 52.73$, $P < 0.0001$) and in the soil ($F_{3,8} = 155.7$, $P < 0.0001$; Fig. S1A). Among the *Bacillus* strains, NJN-6 showed the highest VOC-mediated pathogen suppression on agar medium ($F_{3,8} = 187.4$, $P < 0.0001$), and strain SQR-9 was the most effective in soil ($F_{3,8} = 207.3$, $P < 0.0001$; Fig. S1B). For VOC-mediated plant-growth promotion, among the *Pseudomonas* strains, Q2-87 showed the highest plant-growth promotion both on agar medium ($F_{3,8} = 69.53$, $P < 0.0001$) and in soil ($F_{3,8} = 158.5$, $P < 0.0001$; Fig. S1C). Among the *Bacillus* strains, strain NJN-6 exhibited highest plant-growth promotion both on agar medium ($F_{3,8} = 162.9$, $P < 0.0001$) and in soil ($F_{3,8} = 298.2$, $P < 0.0001$; Fig. S1D). Except for VOC-mediated pathogen-suppression activity of *Bacillus* strains, the most effective strain of both *Pseudomonas* and *Bacillus* genera on agar medium was also the most effective in the soil. However, some strains showed differences in their VOC-mediated effects on agar medium and in soil (Fig. S1), which might be related to the differences in nutrient composition of agar medium and soil as VOC production can vary depending on the substrate composition (Lazazzara et al., 2017).

The results of the bacterial community richness and VOC-mediated activity revealed that the increase in community richness of *Pseudomonas* strains increased pathogen suppression, which on agar medium was 26% at richness level 1 and 33% at richness level 4 ($F_{3,41} = 19.9$, $P < 0.0001$; Fig. 1A). Similar results were obtained in soil, where 24% pathogen suppression at richness level 1 rose to 31% at richness level 4 ($F_{3,41} = 32.7$, $P < 0.0001$; Fig. S2A). For the *Bacillus* strains, an increase in community richness levels increased the pathogen suppression from 26% at richness level 1 to 35% at richness level 4 on agar medium ($F_{3,41} = 27.1$, $P < 0.0001$; Fig. 1B), while in soil, 24% pathogen suppression at richness level 1 rose to 31% at richness level 4 ($F_{3,41} = 86.9$, $P < 0.0001$; Fig. S2B).

For VOC-mediated plant-growth promotion, the increase in community richness of *Pseudomonas* strains increased the plant-growth promotion from 65% at richness level 1 to 109% at richness level 4 on agar medium ($F_{3,41} = 16.1$, $P < 0.0001$; Fig. 1C). In soil, similar results were obtained and an increase

in plant-growth promotion by 55% at richness 1 rose to 90% at richness level 4 ($F_{3,41} = 13.7$, $P = 0.001$; Figure S2C). For *Bacillus* strains, plant-growth promotion of 43% at richness level 1 rose to 114% at richness level 4 on agar medium ($F_{3,41} = 13.8$, $P < 0.0001$; Fig. 1D), while in soil, the increase in plant-growth promotion by 48% at richness level 1 rose to 88% at richness level 4 ($F_{3,41} = 23.9$, $P < 0.0001$; Fig. S2D). The VOC-mediated effects observed on the agar medium and in soil showed some differences; however, in general, both showed a significant positive correlation (Fig. S3), which recommends that VOCs produced on agar medium provides a realistic estimate of VOC-mediated activity in the soil.

These results revealed that intra-species interactions within a community improved the VOC-mediated activity with respect to pathogen suppression and plant-growth promotion. Our outcomes are in agreement with several studies that reported positive relationships between the microbial community and plant performance. For example, Wagg et al. (2011) and Hu et al. (2017) reported that the microbial diversity of communities containing four and eight microbes, respectively, showed positive relationships with plant performance. Alternatively, Jousset et al. (2011) reported a hump-shaped association between toxin production and bacterial community richness. In another report, while the VOC-mediated pathogen suppression increased, VOC-mediated plant-growth promotion decreased with an increase in bacterial community richness up to richness level 4 (Raza et al., 2020). These differences in results may be related to the use of different bacterial strains compared with those in our study.

3.2 Bacterial strains identity effect on VOC-mediated pathogen suppression and plant-growth promotion

The results of the strain identity effect revealed that among the *Pseudomonas* strains, Q8r1-96 was the most effective strain and played a significant role in VOC-mediated pathogen suppression and plant-growth promotion both on agar medium and in soil (Fig. S4A). The strain Q2-87 showed a significant contribution only on VOC-mediated plant-growth promotion and strain Phl1c2 showed a significant contribution only on VOC-mediated pathogen suppression on agar medium. Further confirmation was obtained from the GLM analysis showing that except for strain Q2-87 for pathogen suppression and strain Phl1c2 for plant-growth promotion, all other strains showed significant contribution (Table S3). Among the *Bacillus* strains, SQR-9 was the most effective strain, which displayed a significant role in VOC-mediated pathogen suppression and plant-growth promotion both on agar medium and in soil (Fig. S4B). The strain NJN-6 showed significant contribution only in VOC-mediated plant-growth promotion and strain T-5 showed a significant contribution only in VOC-mediated pathogen suppression. GLM analysis further revealed that except for strains NJN-6 and FZB42 for pathogen suppression and strain T-5 for plant-growth promotion, all other strains showed a significant contribution.

Fig. 1 Volatile organic compounds (VOCs)-mediated pathogen suppression and plant-growth promotion at different bacterial community richness levels on agar medium. Left panels show the pathogen suppression (A) and plant-growth promotion (B) by the VOCs produced by *Pseudomonas* strains and right panels show pathogen suppression (C) and plant-growth promotion (D) by the VOCs produced by *Bacillus* strains at different community richness levels. The pathogen suppression was determined as cell counts mL^{-1} after exposure to the VOCs produced by each bacterial community and expressed as a percentage decrease compared to the control (no VOC exposure). The plant-growth promotion was determined as plant fresh weight (mg) after exposure to the VOCs produced by each bacterial community and expressed as a percentage increase compared to the control. Each data point represents each replicate.

Moreover, the strain identity effect appeared to be more prominent for both the *Pseudomonas* and *Bacillus* genera compared to the community richness effect (Table S3). Similar results were reported by Jousset et al. (2014), who showed a significant strain identity effect of *P. fluorescence* PF-5 as its presence strongly increased the concentration of antibiotics at each level of diversity. Similarly, Hazard et al. (2017) reported that the intraspecific identity of Ectomycorrhiza fungi becomes more important than richness in modulating plant and fungal performance. Alternatively, Hu et al. (2017) revealed that the identity effect of *Pseudomonas* strains was significant to siderophore production and phosphate solubilization; however, generally, the diversity effects were stronger than the strain identity effects. Bacteria from complex communities of competing and cooperating organisms differ in functionality with both the identity of the present species and their interactions (Becker et al., 2012). In this study, while all strains were playing their role, some strains were dominated the community for VOC production and functioning.

3.3 Effect of monocultures and community richness on the production of pathogen suppressing and plant growth-promoting VOCs

Among different *Pseudomonas* strains, PF-5 showed the highest relative amount ($F_{3,8} = 359.4$, $P < 0.0001$) and number ($F_{3,8} = 235.7.2$, $P < 0.0001$) of all VOCs (Fig. S5A). While the highest relative amount ($F_{3,8} = 343.4$, $P < 0.0001$) and numbers ($F_{3,8} = 13.33$, $P = 0.015$) of pathogen-suppressing VOCs were produced by strains PF-5 and Q8r1-96. For plant growth-promoting VOCs, while the highest relative amount of VOCs ($F_{3,8} = 57.26$, $P = 0.001$) was produced by strains Ph11C2 and PF5, the highest number ($F_{3,8} = 298.2$, $P < 0.0001$) of VOCs was produced by strain PF-5 (Fig. S5B). Among the *Bacillus* strains, SQR-9 showed the highest relative amount ($F_{3,8} = 655.5$, $P < 0.0001$ for all VOCs, $F_{3,8} = 581.6$, $P < 0.0001$ for pathogen-suppressing VOCs) and numbers ($F_{3,8} = 64.5$, $P = 0.001$ for all VOCs, $F_{3,8} = 64.6$, $P = 0.001$ for pathogen-suppressing VOCs) of all VOCs and

pathogen-suppressing VOCs (Fig. S5C), while strain FZB42 showed the highest number ($F_{3,8} = 298.2$, $P < 0.0001$) and strain SQR-9 showed the highest relative amount ($F_{3,8} = 307.4$, $P < 0.0001$) of plant growth-promoting VOCs (Fig. S5D).

We next explored how bacterial community richness affected the production of pathogen suppressing and plant growth-promoting VOCs on agar medium. The results showed that community richness of strains of both genera had a positive effect on the production of pathogen suppressing and plant growth-promoting VOCs. For *Pseudomonas* strains, an increase in community richness up to level 4 increased the relative amount of pathogen-suppressing VOCs by 2.8 folds ($F_{3,41} = 44.3$, $P < 0.0001$) and the numbers of pathogens suppressing VOCs by 2.1 folds ($F_{3,41} = 92.8$, $P < 0.0001$) compared with richness level 1 (Fig. 2A–B). Similarly, an increase in community richness up to level 4 increased the relative amount of plant growth-promoting VOCs by 2.6 folds ($F_{3,41} = 117.2$, $P < 0.0001$) and number of plant growth-promoting VOCs by 2.0 folds ($F_{3,41} = 45.5$, $P < 0.0001$) compared to richness level 1 (Fig. 2C–D).

For the *Bacillus* strains, an increase in community richness up to level 4 increased the relative amount of pathogen-suppressing VOCs by 2.4 folds ($F_{3,41} = 29.4$, $P < 0.0001$) and numbers of pathogen-suppressing VOCs by 1.6 folds ($F_{3,41} = 20.7$, $P < 0.0001$) compared to richness level 1 (Fig. 2E–F).

Similarly, an increase in community richness up to level 4 increased the relative amount of plant growth-promoting VOCs by 3.6 folds ($F_{3,41} = 141.6$, $P < 0.0001$) and number of plant growth-promoting VOCs by 2.3 folds ($F_{3,41} = 112.1$, $P < 0.0001$) compared to richness level 1 (Fig. 2G–H). While all used strains could variably able to produce both pathogen suppressing and plant growth-promoting VOCs in monocultures; the majority of produced VOCs were pathogen-suppressing and the relative amount and numbers of pathogen suppressing and plant growth-promoting VOCs were significantly positively correlated with pathogen suppression and plant-growth promotion, respectively (Figs. S6, S7). These results revealed that intra-species interactions within a community improved the VOC-mediated functioning in terms of pathogen suppression and plant-growth promotion by affecting the amount and diversity of produced VOCs. Our results are in agreement with several studies that reported positive relationships between the microbial community and plant performance (Wagg et al., 2011; Hu et al., 2017). However, Jousset et al. (2011) reported a hump-shaped pattern between toxin production and bacterial community richness. Similarly, in another report, while the production of pathogen-suppressing VOCs increased up to richness level 4, the production of plant growth-promoting VOCs decreased with an increase in bacterial community richness (Raza et al., 2020).

Fig. 2 Volatile organic compounds (VOCs) produced by *Pseudomonas fluorescens* and *Bacillus amyloliquefaciens* strains at different community richness levels. The top panel shows the relative amount (A) and number (B) of pathogen-suppressing VOCs and the relative amount (C) and number (D) of plant growth-promoting VOCs produced by *Pseudomonas fluorescens* strains. The bottom panel shows the relative amount (E) and number (F) of pathogen suppressing VOCs and the relative amount (G) and number (H) of plant growth-promoting VOCs by *Bacillus amyloliquefaciens* strains. Each data point shows the effect of each replicate. The number of VOCs represents the relative peak area to (Z)-3-hexenyl acetate (internal standard) in arbitrary units (a.u.), as detected by GC-MS analysis.

3.4 Relationship between pathogen suppression and plant-growth promotion on the production of VOCs

We next investigated whether the production of certain VOCs by bacterial communities could explain the VOC-mediated functioning. For *Pseudomonas* strains, 45 VOCs were produced and except 3, all showed significant differences in their production at different bacterial richness levels (Data-Set 1). When VOCs were screened for their activity, 25 (54%) pathogen-suppressing VOCs were found (Fig. S8A–B). The results of the relative amounts of pathogen-suppressing VOCs at different community richness levels varied. There were 5 VOCs produced at different bacterial richness levels but not at richness level 4, while one VOC was produced only at richness level 4. The production of 14 VOCs was highest at richness level 4, and among those, only 2 VOCs showed significant differences with other richness levels (Data-Set 1). Compared to the monocultures, three new VOCs were produced by *Pseudomonas* strains at different community richness levels (Fig. S9A) and one of those VOCs showed pathogen suppression activity (Fig. S9B), although it was produced in a relatively small amount (Data-Set 1). Except for the two VOCs produced only at richness level 4, one new VOC was produced in all richness levels. There were only three (6%) plant growth-promoting VOCs produced at different bacterial community richness levels of *Pseudomonas* strains (Fig. S8C). Among those, hexadecane was equally produced at all richness levels while heptadecane and tetracosane showed the highest relative amount at richness levels 4 and 2, respectively (Data-Set 1). For *Bacillus* strains, 36 VOCs were produced and except 4, all showed significant differences in their production at different bacterial richness levels (Data-Set 2). When VOCs were screened for their activity, 23 (63%) VOCs showed pathogen-suppressing activity (Fig. S8A–B). The results of the relative amount of pathogen-suppressing VOCs at different community richness levels varied. There were 5 VOCs produced at different bacterial richness levels but not at richness level 4, while one VOC was produced only at richness level 4. The production of 10 VOCs was found highest at richness level 4, and among those, 5 VOCs showed significant differences with other richness levels (Data-Set 2). Compared with monocultures, 8 new VOCs were produced by *Bacillus* strains at different community richness levels and 5 of those VOCs showed pathogen-suppressing activity (Fig. S9C–D). Except 2 VOCs, produced only at richness levels 2 and 4, respectively, other new VOCs were produced at all richness levels (Data-Set 2). There were only three (9%) plant growth-promoting VOCs produced at different community richness levels of *Bacillus* strains (Fig. S8C). Among those, the relative amount of 1,3-propanediol was highest at richness level 3, while the highest production of heptadecane was found at richness level 2. Hexadecane also showed highest production at richness levels 1 and 2.

Most of the VOCs produced by both *Pseudomonas* and *Bacillus* strains showed that pathogen-suppression activity

and only three plants growth-promoting VOCs were produced by both genera. The increase in relative amount and number of pathogen suppressing and mainly plant growth-promoting VOCs with the increase in community richness was due to the mixture of VOCs produced by all four bacterial isolates of both genera. Tyc et al. (2015) reported the production of more numbers of VOCs in pairwise combinations, which were the mixture of VOCs of two bacterial isolates; no novel or different VOCs were found. Conversely, Schulz-Bohm et al. (2015) reported that the mixture of five phylogenetically different bacterial strains produced a different blend of VOCs compared to the monocultures. In another study, the emergence of nine new VOCs was found with the increase in community richness as the result of inter-species interactions, including three pathogen-suppressing VOCs (Raza et al., 2020). In our study, the underlying mechanisms behind VOC-mediated pathogen suppression and plant-growth promotion seemed to be different as most of the VOCs showed pathogen-suppression activity and only one VOC, heptadecane, produced by *Pseudomonas* strains showed both pathogen suppression and plant growth-promoting activity (Fig. S8). Bacteria often upregulate levels of secondary metabolism and turn more antagonistic as a result of interactions between species or by sensing chemical cues from the competitors (Haas and Keel, 2003; Dubuis and Haas, 2007; Garbeva et al., 2011). It is thus possible that in this study, the presence of other bacterial strains promoted antagonism and thus new pathogen-suppressing VOCs were produced because they are also used in interference competition between VOC-producing species (Becker et al., 2012). This effect was found to be prominent in Gram-positive *Bacillus* strains as eight new VOCs, including five pathogen-suppressing VOCs, were produced at different community richness levels compared to the monocultures. Alternatively, Gram-negative *Pseudomonas* strains produced only three new VOCs, including one pathogen-suppressing VOC but in a relatively small amount.

3.5 Linking bacterial community productivity with the production of pathogen suppressing and plant growth-promoting VOCs

We next investigated if and how the production of pathogen suppressing and plant growth-promoting VOCs were linked with bacterial community productivity (growth). The community productivity of *Pseudomonas* strains showed non-significantly increasing trend with the increase in community richness (Fig. 3A) and showed a hump-shaped relationship with both the relative amount and numbers of pathogen suppressing and plant growth-promoting VOCs (Fig. 4A–D). Alternatively, the community productivity of *Bacillus* strains decreased significantly with an increase in bacterial community richness (Fig. 3B) and showed a hump-shaped relationship with both the relative amount and numbers of pathogen suppressing and plant growth-promoting VOCs (Fig. 4E–H). These results revealed that the bacterial community interactions played an important role in the production of VOCs and

Fig. 3 Effect of bacterial community richness on community productivity (total bacterial abundance). Relationships between *Pseudomonas* (A) and *Bacillus* (B) on bacterial community richness and total bacterial community productivity. CFU denotes bacterial cell numbers per ml in terms of colony forming units. Each observation shows the effect of each replicate in each bacterial monoculture or community.

Fig. 4 Interaction of community productivity (growth) at different bacterial richness levels with the relative amount and numbers of VOCs produced by *Pseudomonas* and *Bacillus* strains. The top panel shows the interaction of community productivity at different richness levels with the relative amount (A) and numbers (B) of pathogen suppressing (PS) and relative amount (C) and numbers (D) of plant growth-promoting (PGP) VOCs produced by *Pseudomonas* strains. The bottom panel shows the interaction of community productivity at different richness levels with the relative amount (E) and numbers (F) of PS and relative amount (G) and numbers (H) of plant growth-promoting (PGP) VOCs produced by *Bacillus* strains.

the maximum production of VOCs was achieved at the intermediate growth of bacterial communities of both *Pseudomonas* and *Bacillus* genera. The high and low community productivity reduced the production of pathogen suppressing and plant growth-promoting VOCs. Schulz-Bohm et al. (2015) also reported that both the composition of microbial

communities and interactions among community members play an important role in the production of VOCs. Bacterial community productivity is shaped by natural selection in response to resource conditions over their ancestral history (Maitra and Dill, 2015). In this study, *Pseudomonas* strains might maintain community productivity because of their

versatile metabolism (Rojo, 2010) compared to *Bacillus* strains, which showed a decrease in community productivity with the increase in community richness. This decrease in community productivity might trigger the antagonistic interactions because of interference competition among *Bacillus* strains as is evident from the production of eight new VOCs at different community richness levels, including five pathogen-suppression VOCs, while this antagonism was unsubstantial in the case of *Pseudomonas* strains.

4 Conclusion

In heterogeneous environments like the soil, different bacterial strains have different ecological characteristics, and the bacterial cells are patterned by their interactions with neighboring cells and the abiotic environment (Liu et al., 2017). Here we evaluated how intra-species interactions of each Gram-positive and Gram-negative genus mediate the production and functioning of VOCs. The results clearly showed that the bacterial interactions within multispecies communities affect the amount and type of produced VOC. This effect not only includes the blend of VOCs from all strains in a community but also the production of new VOCs. In turn, this changes the VOC-mediated functioning in terms of pathogen suppression and plant-growth promotion. However, the emergence of new VOCs depends on the interactions within species. While both genera *Bacillus* and *Pseudomonas* showed similar results for VOC-mediated pathogen suppression and plant-growth promotion, the intra-species interactions revealed some differences. Gram-positive *Bacillus* strains showed a decrease in community productivity and triggered antagonism, which resulted in the emergence of new pathogen-suppressing VOCs. Together, this study suggests that both bacterial identity and diversity need to be considered for understanding the VOC-mediated activity of bacterial communities. From the applied perspective, our study suggests that VOC-mediated functions could be employed to manipulate the microbiome composition of the rhizosphere to simultaneously improve multiple ecosystem functions including pathogen suppression and plant growth.

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