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Raza, Waseem, Wang, Jianing, Jousset, Alexandre et al. (5 more authors) (2020) Bacterial community richness shifts the balance between volatile organic compound-mediated microbe-pathogen and microbe-plant interactions. PROCEEDINGS OF THE ROYAL SOCIETY B-BIOLOGICAL SCIENCES. pp. 1-10. ISSN 1471-2954

https://doi.org/10.1098/rspb.2020.0403

PROCEEDINGS OF
THE ROYAL SOCIETY B
BIOLOGICAL SCIENCES

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Journal:	*Proceedings B*
Manuscript ID	RSPB-2020-0403.R1
Article Type:	Research
Date Submitted by the Author:	19-Mar-2020
Complete List of Authors:	Waseem, Raza; Nanjing Agricultural University, College of Resources and Environmental Sciences ; College of Resources and Environmental Sciences, Nanjing Agricultural University Wang, Jianing; Nanjing Agricultural University, College of Resources and Environmental Sciences Jousset, Alexandre; University of Goettingen, J.F. Blumenbach Institute of Zoology and Anthropology Friman, Ville-Petri; University of York, Biology Xinlan, Mei; Nanjing Agricultural University, College of Resources and Environmental Sciences Shimei, Wang; Nanjing Agricultural University, College of Resources and Environmental Sciences Wei, Zhong; Nanjing Agricultural University, Shen, Qi-rong; Nanjing Agricultural University, College of Resources and Environmental Sciences;
Subject:	Ecology < BIOLOGY, Microbiology < BIOLOGY, Plant science < BIOLOGY
Keywords:	Bacterial diversity, Community richness, Pathogen suppression, Plant growth promotion, Plant-microbe interactions
Proceedings B category:	Ecology

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Ethics

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Statement (if applicable):
CUST_IF_YES_ETHICS :No data available.

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Yes

Statement (if applicable):
All data generated or analyzed during this study are included in this article and its supplementary information files.

Conflict of interest

I/We declare we have no competing interests

Statement (if applicable):
CUST_STATE_CONFLICT :No data available.

Authors' contributions

This paper has multiple authors and our individual contributions were as below

Statement (if applicable):
WR, JW, AJ, VF, WZ, MX, WS and SQ developed the ideas; WR, AJ, VF and WZ designed the study; WR, JW, MX, WS and WZ set up the experiment; WZ, JW and WR collected data; WR and WZ analyzed the data and wrote the manuscript; AJ, VF, WZ and SQ provided comments on the manuscript.

Bacterial community richness shifts the balance between volatile organic compound-mediated microbe-pathogen and microbe-plant interactions

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Abstract

Even though bacteria are important in determining plant growth and health via volatile organic compounds (VOCs), it is unclear how these beneficial effects emerge in multi-species microbiomes. Here we studied this using a model plant-bacteria system, where we manipulated bacterial community richness and composition and determined the subsequent effects on VOC production and VOC-mediated pathogen suppression and plant growth-promotion. We assembled VOC-producing bacterial communities in different richness levels ranging from one to twelve strains using three soil-dwelling bacterial genera (*Bacillus, Paenibacillus* and *Pseudomonas*) and investigated how the composition and richness of bacterial community affect the production and functioning of VOCs. We found that VOC production correlated positively with pathogen suppression and plant growth-promotion and that all bacteria produced a diverse set of VOCs. However, while pathogen suppression was maximized at intermediate community richness levels when the relative amount and the number of VOCs were the highest, plant growth-promotion was maximized at low richness levels and was only affected by the relative amount of plant growth-promoting VOCs. The contrasting effects of richness could be explained by differences in the amount and number of produced VOCs and by opposing effects of community productivity and evenness on pathogen suppression and plant-growth promotion along the richness gradient. Together, these results suggest that the number of interacting bacterial species and the structure of the rhizosphere microbiome drive the balance between VOC-mediated microbe-pathogen and microbe-plant interactions potentially affecting plant disease outcomes in natural and agricultural ecosystems.

Keywords: Bacterial diversity, Community richness, Pathogen suppression, Plant growth promotion, Plant-microbe interactions

1. Introduction

Soil microbiome research has focused mainly on the beneficial effects of root-associated microbes that reside in the near vicinity of the plants. However, microbes also interact with each other and plants over long distances by producing volatile organic compounds (VOCs) that are a broad group of lipophilic compounds with low molecular weight (100–500 Da), high vapor pressure and low boiling point [2]. These properties facilitate evaporation and diffusion of VOCs over long distances through the atmosphere or porous soils from the point of production [3]. The VOCs have been reported for distinct bioactive functions, which are as diverse as the chemical structures of VOCs shaping a wide range of bacteria-bacteria and bacteria-plant interactions, including cell-to-cell communication, plant growth, flowering and photosynthesis stimulation, inhibition of parasites and pathogens and activation of systematic plant resistance against biotic and abiotic stresses [4, 5, 6, 7]. The composition of the emitted VOCs can also vary depending on the environmental conditions such as the substrate composition of the growth media [8]. While several VOCs have been shown to change pairwise interactions with plants and microorganisms [9, 10], it is less clear how the presence of other microbes in multi-species communities affects the production and functioning of VOCs. Here we studied this directly by manipulating bacterial community richness and composition and determining subsequent effects on VOC production and VOC-mediated pathogen suppression and plant growth-promotion.

Biodiversity is a key driver of several ecosystem functions [11] and the underlying bacterial interactions have been shown to affect the number, type and composition of produced antifungal VOCs [12, 13]. Bacterial community diversity could affect VOC production in many ways. First, multispecies communities could produce higher amounts and a greater number of VOCs by reaching higher cell densities compared to species grown in isolation due to complementary [14]

or facilitative [15] effects. Alternatively, it is possible that high bacterial community diversity could lead to increased antagonism within the bacterial community, which could then offset the VOC production by having a negative effect on the growth and overall metabolism of the community [16]. Increasing community diversity could thus either promote or constrain VOC production depending on the species interactions between the interacting community members that could be driven by competition for shared resources, cooperation, cheating or antibiosis [1, 3]. Second, increasing the number of species in a community could increase the number of unique VOCs that are produced if each species produces a different subset of compounds [17]. High community diversity could thus increase the range of VOC-mediated functions. Third, intra- and interspecific bacterial interactions could lead to the expression of certain 'emergent' VOCs that are not produced in monocultures. One potential mechanism for this could be interference competition which is often stronger in diverse bacterial communities due to the production of a high variety of antimicrobial compounds [18]. While co-culturing two to five bacteria together has been shown to induce the production of novel antifungal VOCs [3, 19], the effects of diversity on bacteria-specific VOCs have not yet been explored.

Theory and experiments suggest that increasing community diversity and richness could predictably affect the production of VOCs by bacterial communities. However, it is still largely unknown how these changes affect the type and strength of VOC-mediated functioning with bacterial pathogens and plants. To address this shortcoming, we used a model plant-bacteria system to causally test how the microbial community richness affects the VOC-mediated functioning in terms of *Arabidopsis thaliana* plant growth-promotion and the suppression of a wide-spread bacterial pathogen, *Ralstonia solanacearum*, capable of infecting many plant species [20]. To achieve this, we assembled VOC-producing model bacterial communities in different richness levels ranging from one to twelve strains using three ubiquitous, soil-dwelling bacterial genera:

Bacillus, Paenibacillus and *Pseudomonas.* We then determined and classified the emitted VOCs by all bacterial communities and explored how this variation affected plant growth-promotion and pathogen suppression as a function of bacterial community richness.

2. Methods

(a) Bacterial strains

We used a total of twelve common soil bacterial strains belonging to *Bacillus, Paenibacillus* and *Pseudomonas* genera, which were isolated from the rhizosphere of different plant species (four strains from each genus; for more detail, see Table S1). The bacterial strains were selected based on the preliminary experiments, where we tested that pathogen suppression and plant growth-promotion were solely mediated by VOCs (Table S1). The bacterial strains were stored at -80°C in nutrient broth (BD Difco™, Becton, Dickinson and Company, USA) containing 70% glycerol and routinely grown on nutrient agar medium (Bacto® agar, Cat. No. 214030, Becton, Dickinson and Company, USA). We used the *Ralstonia solanacearum* QL-Rs1115 strain isolated in China [21] as our target pathogen, which was stored at -80°C in casamino acid-peptone-glucose (CPG) medium [1 g casamino acid (BD Bacto™, Becton, Dickinson and Company, USA), 10 g peptone (Sigma-Aldrich), 5 g glucose (Sigma-Aldrich) and pH 7.0] containing 70% glycerol [22].,

During the experiments, *R. solanacearum* was grown on CPG agar medium.

(b) Assembly of model rhizosphere bacterial communities

Single colonies of twelve bacterial strains (Table S1) were grown separately in nutrient broth as monocultures for 24 hours at 30°C before washing twice and adjusting to the final concentrations of 1×10^7 colony forming units (CFU)/ml with 0.85% NaCl. The monoculture cell suspensions of bacterial strains were mixed in equal proportions (500 µl) to assemble 43 model communities with

varying diversity (strain richness) levels and composition ranging from monocultures to 2, 3, 4, 6 and 12 species communities (Table S2) using broken stick design [23]. The final cell concentrations of monocultures and mixed co-culture communities were set to the same (1×10^7 CFU/ml). Each bacterial strain was replicated two times at each richness level except for richness levels 1 and 12. The assays for each model community were conducted in triplicate.

In order to verify whether all three bacterial genera could co-exist, we grew all the assembled bacterial communities in microtiter plates. Each well was filled with 195 µl of modified minimal salt medium amended with 1.5% sucrose, and 0.4% tryptone soy broth (w/v) and inoculated with 5 µl of bacterial communities, thereby mimicking the conditions used for VOC measurements later in the experiment. After 36 hours at 30°C, total bacterial, *Pseudomonas*, and *Paenibacillus* cell densities were determined by serial plating on nutrient agar medium, *Pseudomonas* selective agar (CFC) medium, and *Paenibacillus* selective nutrient agar medium supplemented with 10 µg/ml polymyxin B sulfate, respectively [24, 25]. *Bacillus* densities were determined by subtracting the *Pseudomonas* and *Paenibacillus* densities from the total bacterial densities. Plating method was chosen over the qPCR method to include only living cells to our analysis. Potential negative effects of selective plates on target bacteria were also confirmed: *Paenibacillus* and *Pseudomonas* genera were not negatively affected by the selective media as similar colony numbers were observed when the same samples were grown on nutrient agar medium (Figure S1). The bacterial cell densities were represented as community productivity at different bacterial richness levels.

(c) Measuring VOC-mediated pathogen suppression and plant growth-promotion by monocultures and communities

We assessed the VOC-mediated inhibitory potential of each bacterial monoculture and constructed community on *R. solanacearum* pathogen using divided Petri dish and soil systems. Briefly, a

single colony of *R. solanacearum* was grown in CPG medium for 24 hours at 30°C before washing twice with 0.85% NaCl and adjusting to a final concentration of 1×10^7 CFU/ml. Later, one half of the divided Petri dish (85 mm diameter) was filled with 15 ml of CPG agar medium and spot-inoculated with the cell suspension of *R. solanacearum* at five cm apart two locations (5 µl in each; Figure S2). The cell suspensions for 43 model communities (1×10^7 CFU/ml) were prepared as described above and spot-inoculated at five cm apart two locations (5 µl in each) on the other side of the Petri dish containing minimal salt agar medium (same as above but with 15 g agar/L; Figure S2). Petri dishes were incubated at 30°C for 12 hours to initiate bacterial growth and then sealed with Parafilm and incubated for further three days at 30°C. Three replicates were set up for each community, including negative control treatment with *R. solanacearum* growing in the absence of VOC-producing communities. Later, *R. solanacearum* colonies were removed along with agar medium using a sterilized scalpel, suspended in 1 ml of sterilized water, diluted by 500 times and spread on CPG agar plates to count the CFU/ml (cell densities) after incubation at 30°C for 2 days. The VOC effects were presented as the percentage increase or decrease in the pathogen suppression relative to the control treatment. Moreover, in a separate experiment, the effect of VOCs produced by *R. solanacearum* on the growth of monocultures of *Bacillus*, *Paenibacillus* and *Pseudomonas* bacterial strains was also evaluated in triplicate using the same method as described above including negative control treatments with bacterial monocultures growing separately in the absence of VOC-producing *R. solanacearum*. These results showed that the VOCs of *R. solanacearum* were not able to inhibit the cell densities of any of the bacterial strains from *Bacillus*, *Paenibacillus* and *Pseudomonas* genera (Figure S3).

The Petri dish assays were validated using a sterilized soil system as follows [26]. The soil (pH 6.5, organic matter 11.65 g/kg, and available N, P, and K contents 41.3, 238.7, and 177.5 mg/kg, respectively) was collected from Yixing, China, and sterilized 121°C for 60 min. One ml of

each bacterial monoculture and community (1×10^7 cells/ml) was mixed with 7.5 g of soil (dry weight) and inoculated to one half of the divided Petri dish. The other half of the dish was filled with CPG agar and spot-inoculated with *R. solanacearum* as described above (Figure S2). Three replicates were set up for each treatment including negative control with *R. solanacearum* in the absence of VOC-communities. Dishes were incubated at 30°C for 12 hours to initiate bacterial growth and then sealed with Parafilm and incubated for three days at 30°C. The VOC-effects on the cell densities of *R. solanacearum* was quantified similarly as described above.

We used the *A. thaliana* plant model system to assess whether changes in microbial community richness and composition affected plant growth via changes in VOC composition. The Petri dish system was used in a similar way as described above in triplicate, including a negative control treatment where *A. thaliana* grew in the absence of VOC-producing bacteria. The cell suspensions of 43 model communities (1×10^7 CFU/ml) were spot-inoculated on one side of the Petri dish as described above and incubated at 30°C for 12 hours to initiate bacterial growth (Figure S2). Later, three *Arabidopsis* Col-1 seedlings were placed onto the other half of the Petri dish containing half-strength Murashige and Skoog agar medium (0.8% agar and pH 5.7). Before that, *Arabidopsis* seeds were surface sterilized, vernalized for 2 days at 4°C in the dark on half-strength Murashige and Skoog agar medium with 1.5% sucrose and then placed in a growth chamber (22°C temperature, 12h light, 12h dark, 40W fluorescent light) for three days. The Petri plates were sealed with parafilm and placed in a growth chamber. After two weeks, plants were gently removed from the medium, roots washed with sterilized water and the whole plant was blot dried and weighted to determine the plant fresh weight (mg/plant). To determine VOC-mediated plant growth-promotion in the soil, a similar system was used as when evaluating VOC-mediated pathogen suppression in the soil except that the pathogen was replaced with three *Arabidopsis* seedlings inoculated onto half-strength Murashige and Skoog agar medium. After two weeks, plant fresh

180 weight (mg/plant) was determined as described above. The VOC effects were presented as the
181 percentage increase or decrease in plant growth relative to control treatment.

182 **(d) Analysis of VOC profiles produced by bacterial strains and assembled communities**

183 To analyze the VOC profiles produced by all bacterial monocultures and communities, cell
184 suspensions (1×10^7 CFU/ml) were prepared as described above and two spots (5 µl each)
185 inoculated on minimal salt agar medium (15 g agar/L) in a 100-ml vial and placed at 30°C. After
186 12 hours of growth, vials were sealed and incubated for further 72 hours at 30°C. Three replicates
187 were set up for each treatment and vials without the inoculation of bacteria were used as controls.
188 After incubation, 10 µl of (Z)-3-hexenyl acetate (5 mM) as an internal standard was added into the
189 vial. Later, a solid-phase microextraction (SPME) fiber [Supelco (Bellefonte, PA) stable flex
190 divinylbenzene/carboxen/polydimethylsiloxane (DCP, 50/30 µm)] was inserted into the vial and
191 incubated further 30 min at 30°C and another 30 min at 50°C. The SPME fiber was then inserted
192 into the injector of gas chromatography-mass spectrometry (GC-MS) (Finnigan Trace DSQ, Austin,
193 TX, USA) and desorbed at 220°C (1 min) with an RTX-5MS column (30 m, 0.25-mm inside
194 diameter, 0.25 µm). The following oven temperature protocol was used: 33°C (3 min), 180°C
195 (10°C/min), and 240°C (30°C/min) and finally for 5 min at 240°C. The mass spectrometer was
196 operated at 70eV and 220°C in the electron ionization mode with a scan from 50 to 500 m/z.
197 Chromatographs were obtained and analyzed by AMDIS 2.73 (National Institute of Standards and
198 Technology, Gaithersburg, USA). The mass spectra of deconvoluted VOC peaks were compared
199 with those in the NIST/EPA/NIH Mass Spectrometry Library with respect to the spectra in the
200 Mainlib and/or Replib databases (Agilent Technologies, Santa Clara, CA, USA). Later, the Kovats
201 retention indexes were calculated for each compound using an alkane calibration mix and
202 compared with those found in NIST/EPA/NIH Mass Spectrometry Library. The compound was
203 considered identified if its mass spectra matched well with a listed compound, had match

factor >800 and the difference between the retention index calculated for the detected compound and the listed compound (for a semi-standard non-polar column) was not larger than five. Except for 14 unidentified and four commercially unavailable VOCs (Data-set S1), the production of 67 identified VOCs was further confirmed by comparing with standard compounds [Sigma, Tokyo Chemical Industry Co., Ltd. (TCI, Tokyo, Japan) and Aladdin Reagent Database, Inc. (Shanghai, China)]. The standards were mixed and measured using SPME fibers as described above. The peaks similar to the control treatment (without bacterial inoculation) were not considered for the identification of VOCs. The number of VOCs produced in each treatment were recorded and the chromatographic peak area was expressed as the relative peak area to (Z)-3-hexenyl acetate (internal standard) in arbitrary units (a.u.) as an indirect approach to estimate the relative amount (concentration) of each VOC.

(e) Classification of emitted compounds into pathogen-suppressing and plant growth-promoting VOCs

To evaluate the effect of different concentrations of identified VOCs (GC-MS analysis) on pathogen suppression, the Petri dish system was used in a similar way as described above. The cell suspension of *R. solanacearum* (1×10^7 CFU/ml) was spot-inoculated at two locations (5 µl in each) on one side of the Petri dish and incubated at 30°C for 12 hours to initiate bacterial growth. Later, stock solutions (20 µg/ml, 100 µg/ml, 500 µg/ml, 2 mg/ml and 10 mg/ml) of 67 commercially available pure VOCs (Dataset S1) were prepared separately in methanol by serial dilutions and the other side of Petri dish was inoculated with 15 µl of stock solutions to give 0.3 µg, 1.5 µg, 7.5 µg, 30 µg and 150 µg final amount of each VOC on a ~10 mm diameter sterile filter paper disc (Whatman™ filter paper, 6 µm pore size), respectively. Petri dishes were sealed with Parafilm and incubated for three days at 30°C. The sterile filter paper discs inoculated with nothing or with methanol were used as control treatments (no difference found between these control treatments).

The VOC-effects on the cell densities *R. solanacearum* was quantified similarly as described above. [7, 25].

To evaluate the effect of VOCs on plant growth, the same methodology described above was used, with one exception: instead of the pathogen, three *Arabidopsis* Col-1 seedlings were placed onto the other half of the Petri dish containing half-strength Murashige and Skoog salt agar medium. After two weeks, plant fresh weight (mg/plant) was determined as described above. The VOC effects were presented as the percentage increase or decrease in plant growth relative to control treatment [7, 25].

(f) Statistical analysis

The statistical differences between bacterial strains and genera were analyzed using ANOVA and Tukey's tests. Linear regression analysis was used to analyze separately the VOC-mediated pathogen suppression and plant growth-promotion, relative amount of VOCs (sum of relative peak area to (Z)-3-hexenyl acetate of detected GC-MS peaks), number of VOCs (number of peaks) and VOC composition (first axis of the principal component analysis on non-transformed data), and total community abundance, genera abundances and community evenness (at genera level) as the function of bacterial community richness (factor with 6 levels); significance at P=0.05. Similarly, to link VOCs production with VOC-mediated activity, we separately analyzed the VOC-mediated pathogen suppression and plant growth-promotion as the function of the relative amount of produced VOCs, number of VOCs and VOC composition; significance at P=0.05. To further link VOC profiles and community properties to functioning, we used separate models to explain plant growth-promotion and pathogen suppression with bacterial genera, community abundances and community evenness, community richness and strain identity effects and relative amount, number and composition of VOCs. To uncover the most parsimonious GLMs with the best explanatory power, and to avoid potential correlations between different explanatory variables, sequential

252 analyses were performed using stepwise model selection based on Akaike information criteria
253 (AIC). Statistical analyses were conducted with SPSS version 19.0 statistical software (SPSS, Inc.,
254 Chicago, IL, USA).
255

256 ## 3. Results

257 **(a) Production, classification and activity of pathogen-suppressing and plant growth-**
258 **promoting VOCs by bacterial species and genera**

259 All twelve bacterial strains were effective at VOC-mediated pathogen suppression and plant
260 growth-promotion, though some bacterial strains were more effective than the others on agar
261 medium and/or in soil (Figure S4a-b). Overall, these effects were similar regardless if they were
262 measured on agar media or in the soil ($F_{1, 70}$=0.02, P=0.891 for pathogen suppression and $F_{1,}$
263 $_{70}$=2.20, P=0.143 for plant growth-promotion). As a result, VOC-mediated pathogen suppression
264 and plant growth-promotion observed on agar media and in the soil were highly positively
265 correlated (R^2=0.20; P<0.0001 and R^2=0.61; P<0.0001, respectively; Figure S5), which suggests
266 that VOCs activity on agar media provided a realistic estimate of VOC activity in the natural soil.
267 At the genera level, *Paenibacillus* showed relatively lower pathogen suppression ($F_{2, 33}$=14.73,
268 P<0.0001) and *Bacillus* genera relatively lower plant growth-promotion on agar medium ($F_{2,}$
269 $_{33}$=28.01, P=0.001; Figure S6a-b), while no between-genera differences were observed in the soil
270 (Figure S6a-b).

271 We next compared the relative amount and number of VOCs produced by different bacterial
272 genera and strains. We found that *Paenibacillus* genera produced higher relative amount ($F_{2, 33}$=
273 263.3, P<0.0001) and number ($F_{2, 33}$= 61.8, P<0.0001) of total VOCs compared to *Pseudomonas*
274 and *Bacillus* genera, which did not differ from each other (Figure S6c-d). However, bacterial strains

showed significant variation in the relative amount ($F_{11, 24}$= 357.2, P<0.0001) and number ($F_{11, 24}$= 54.6, P<0.0001) of produced VOCs within each genus (Figure S7a-f).

When VOC effects were tested as pure compounds, most of the produced VOCs had pathogen-suppressing activity (52%; Figure S6a-b) and only 7% had plant growth-promoting activity (Figure S6c), while both pathogen-suppressing and plant growth-promoting activities were increased with the increase in the concentration of VOCs (Figure S8). At the genera level, we found that in total 49 VOCs produced by *Paenibacillus* genera showed pathogen suppression, while *Pseudomonas* and *Bacillus* genera produced 33 and 40 pathogen-suppressing VOCs, respectively (Figure S6c-d; Data-set S1). As a result, the relative amount ($F_{2, 33}$= 46.9, P<0.0001) and the number of pathogen-suppressing VOCs ($F_{2, 33}$= 34.6, P=0.001) were the highest with *Paenibacillus* genera (Figure S6c-d). In contrast, only eight *Paenibacillus*, eight *Pseudomonas* and five *Bacillus* VOCs showed plant growth-promotion (Figure S6c-d; Data-set S1). While the highest relative amount of plant growth-promoting VOCs was produced by *Bacillus* genera ($F_{2, 33}$= 42.6, P<0.0001; Figure S6c), *Paenibacillus* and *Pseudomonas* genera both produced the most diverse selection of plant growth-promoting VOCs ($F_{2, 33}$=10.5, P=0.011; Figure S6d). These results suggest that while all bacteria from each genus produced both types of VOCs, most of the produced VOCs had pathogen-suppressing effect and that the *Paenibacillus* genera showed the highest relative VOC production in general.

(b) Effect of bacterial community richness on the VOC-mediated pathogen suppression and plant growth-promotion

We next explored how bacterial community richness affected the VOC-mediated pathogen suppression and plant growth-promotion using agar media assays (quantitatively similar results obtained in the soil; Figure S9a-b). We found that bacterial community richness and pathogen suppression showed a hump-shaped relationship ($F_{2, 126}$=90.4, P<0.0001) where pathogen

suppression peaked at the intermediate community richness (4 species) reaching 40% suppression efficiency and then decreasing to 8% efficiency at richness level 12 compared to non-VOC control (Figure 1a). This pattern could be explained well with the relative amount ($F_{2, 127}$=58.18, P<0.0001; Figure 1b), number ($F_{2, 126}$=67.7, P<0.0001; Figure 1C) and composition ($F_{2, 126}$=13.68, P<0.0001; Figure S10a) of produced pathogen-suppressing VOCs, which all showed a similar hump-shaped relationship peaking at richness level 4 and then decreasing at richness levels 6 and 12. Together, pathogen suppression showed highly significant and positive relationships with the relative amount, number and composition of pathogen-suppressing VOCs (Figure 2a-b; Figure S10b; Table S3).

In contrast, the highest plant growth-promotion was observed at low community richness levels ($F_{1, 127}$=13.8, P<0.0001). Specifically, a 67% increase in plant growth-promotion observed at the richness level 1 decreased to 17% increase at richness level 4, and at richness level 12, an average of 33% decrease in plant growth-promotion was observed compared to control treatment (Figure 1d). Reduction in the plant growth-promotion correlated clearly with a decrease in the relative amount of plant growth-promoting VOCs ($F_{1, 127}$= 39.9, P<0.0001; Figure 1e) resulting in 90% decrease between richness levels 1 and 12. However, similar to pathogen-inhibiting VOCs, the number of plant growth-promoting VOCs peaked at intermediate richness levels reaching up to 139% increase at the richness level 4 and then decreasing down to 19% increase at the richness level 12 compared to the richness level 1 ($F_{2, 126}$ =56.1, P<0.0001; Figure 1f). The composition of plant growth-promoting VOCs did not show any relationship with plant growth-promotion (Figure S10c). As a result, plant growth showed a highly significant and positive relationship only with the relative amount of plant growth-promoting VOCs (Figure 2c-d; Figure S10D; Table S3).

(c) Linking pathogen suppression and plant growth-promotion with the production of VOCs

We next investigated if VOC-mediated functioning could be explained by the emission of certain VOCs. A total of 85 different VOCs were produced by all bacterial communities. Except for three

VOCs (1, 2-ethanediol 1, 2-diphenyl; 9-decen-i-ol and 5-octadecene), the relative amount of VOCs varied significantly between communities with different richness levels (Dataset S1). Interestingly, 15 VOCs were produced only in communities. Similarly, 49 VOCs produced at richness levels 1-4 were absent from the VOC profiles of 6 and 12 species communities (Figure S11A; Data-set S1). Out of 85 VOCs in total, 41 VOCs showed pathogen-suppressing activity. Of these, 4 pathogen-suppressing VOCs were not produced at the community richness level 1, and 26 pathogen-suppressing VOCs produced at richness levels 1-4 were absent from the VOC profiles of 6 and 12 species communities (Figure S11b; Data-set S1). When chemical groups of VOCs were evaluated, 80% (61) of the identified VOCs produced by twelve bacterial strains belonged to alkane, alcohol, aldehyde, benzene, ketone and fatty acid groups. Almost all alcohol, aldehyde, benzene and ketone group VOCs showed pathogen-suppressing activity. Other VOC groups related to pathogen suppression included naphthalene, phenol, sulfur and nitrogen containing compounds (Figure S12).

Only six out of 85 VOCs were found to show plant growth-promoting activity (Figure S8C). Of these compounds, four VOCs were not produced at richness level 12, while tetradecane was only produced at richness levels 6 and 12 albeit in low relative amount (Figure S11c; Data-set S1). Interestingly, two of the plant growth-promoting VOCs (indole, heptadecane) also showed antibacterial activity against *R. solanacearum* (Figure S8a-b). When chemical groups of VOCs were evaluated, plant growth-promoting VOCs mainly belonged to the alkane (4) group; while one VOC belonged to the diol and one to the nitrogen-containing compounds group (Figure S12). These results suggest that bacterial interactions within communities can trigger and abolish the production of certain pathogen-suppressing and plant growth-promoting VOCs.

(d) Linking bacterial community properties with pathogen suppression and plant growth-promotion

Lastly, we explored if richness-mediated VOC effects could be explained by certain underlying community properties such as community productivity, evenness, genera abundances or strain identity effects. While the community productivity increased with bacterial richness ($F_{1, 127}$=36.8, P=0.004; Figure 4a), the relative abundance of all three genera showed a parabolic relationship with the richness reaching the lowest abundances at the intermediate richness levels and the highest abundances when grown in the low or high richness level communities (Figure 4b). Moreover, while the community evenness of bacterial genera did not differ at the lower richness levels (in 2-4 species communities), it considerably decreased at the higher richness levels ($F_{4, 88}$=41.00, P<0.0001; Figure 4c). As a result, bacterial community properties showed contrasting effects on VOCs functioning; while total community productivity was positively linked with pathogen suppression, it showed a negative effect on the plant growth-promotion (Table S4). In contrast, while community evenness had no effect on the pathogen suppression, it was positively linked with the plant growth-promotion (Table S4). Furthermore, while the densities of *Pseudomonas* and *Paenibacillus* genera showed a negative relationship with pathogen suppression, the densities of all three genera showed positive effects on the plant growth-promotion (Table S4). Finally, some strains had strong and often opposing identity effects on both the pathogen suppression and plant growth-promotion (Table S4). These results suggest that bacterial community properties had contrasting effects on VOC-mediated functioning, which likely constrained simultaneous expression of pathogen suppressing and plant growth-promoting VOCs.

4. Discussion

While the role of individual VOC on plant physiology and antimicrobial activity has been well described [3, 13], their production and effects in complex microbial communities are poorly understood. Especially, VOC-mediated effects on bacterial pathogens and plants remain unclear.

Here we investigated this by addressing how the composition and richness of bacterial communities affect the production of different VOCs and VOC-mediated functioning in terms of pathogen suppression and plant growth-promotion. We found that the majority of produced VOCs were pathogen-suppressing and that bacterial strains from all genera produced both types of VOCs in monocultures. However, VOC production was dramatically changed when the strains were grown together in communities. Specifically, we found that pathogen suppression was maximized at intermediate community richness levels when the relative amount and number of produced pathogen-suppressing VOCs were the highest. In contrast, plant growth-promotion was unaffected by the number of VOCs and maximized at low community richness levels when the relative amount of produced plant growth-promoting VOCs was the highest. Interestingly, community productivity and evenness had contrasting effects on the VOC functioning in this study: productivity promoted the pathogen suppression but constrained the plant growth-promotion, while evenness promoted the plant growth-promotion but constrained the pathogen suppression. Together these results suggest that species interactions within communities can change VOC-mediated functioning by affecting the amount and diversity of produced VOCs. VOC-mediated microbe-microbe and microbe-plant functions are thus likely to be optimized with contrasting community structures due to non-linear and contrasting relationships with community diversity, productivity and evenness.

Of all the detected VOCs, 41 VOCs (52%) showed pathogen suppression and their relative amount and numbers peaked at the intermediate community richness levels, which was highly correlated with VOC-mediated pathogen suppression. Moreover, compared to monocultures, 14 unique VOCs, including four pathogen-suppressing VOCs, were produced in more diverse bacterial communities including two to four strains. These results suggest that the addition of new species likely increased the metabolic potential of the community by stimulating the production of antimicrobial compounds with greater chemical diversity and activity [16, 27]. However, the

393 relative amount and number of pathogen-suppressing VOCs decreased at higher richness levels
394 and 26 VOCs including 10 pathogen-suppressing VOCs were not observed at 12 strain bacterial
395 community. These results are in line with a previous study, which found a similar hump-shaped
396 pattern between toxin production and bacterial community richness [28]. Bacteria often sense and
397 respond to the presence of competitors by turning more antagonistic by upregulating secondary
398 metabolism and by producing antimicrobial compounds like antibiotics [29, 30]. The secondary
399 metabolism is also the main driver of antimicrobial VOC production that has been shown to change
400 in the presence of competitors [18, 31]. It is thus possible that the presence of other bacterial strains
401 promoted the production of pathogen-suppressing VOCs because they were also used in
402 interference competition between VOC-producing species [18]. Some previous studies have also
403 reported a relationship between increased VOC-mediated suppression of fungal pathogens and
404 increasing microbial diversity [12, 32]. However, in this study, increasing community diversity
405 beyond four strains could have intensified interference competition to the extent that it led to a
406 decrease in the production of pathogen-suppressing VOCs. In addition, quorum sensing, cross-talk
407 between species, chemical cues from competitors (antibiotics), silence gene clustering or cross-
408 feeding generating new metabolic pathways at community levels, etc. might also affect the
409 production of VOCs [16, 28, 30, 33]. While linking community effects on certain species is difficult,
410 we found that community evenness decreased with richness and that *Paenibacillus* genera
411 dominated at the 12-strain community (Figure 3b-c). Interestingly, *Paenibacillus polymyxa* WR-2
412 strain had a strong negative effect on pathogen suppression in general, which suggests that it might
413 have played an important role in reducing VOC-mediated pathogen suppression at high richness
414 levels (Table S4). We also found that community productivity had a positive relationship with
415 pathogen suppression, indicative of a positive link between bacterial metabolic activity and VOC-
416 mediated pathogen suppression. However, most pathogen-suppressing VOCs were produced at

417 intermediate richness levels when all genera were found to be at very similar abundances. As a
418 result, intra- and inter-bacterial species interactions might be more important for the expression of
419 pathogen-suppressing VOCs instead of bacterial growth and metabolic activity.

420 Of all detected 85 VOCs, only six showed plant growth-promoting activity (7% of all
421 VOCs). Moreover, and in contrast to pathogen-suppressing VOCs, plant growth-promotion was
422 the highest in bacterial monocultures and steadily decreased with increasing community richness
423 turning into plant growth-inhibition at 12-strain community. While a clear positive correlation was
424 found with the relative amount of VOCs and plant growth-promotion, the numbers or composition
425 of plant growth-promoting VOCs had no effect. This is likely explained by the low number of plant
426 growth-promoting VOCs produced in general and by the fact that all genera tended to emit them
427 similarly. Moreover, some of the plant growth-promoting VOCs were not detected at higher
428 richness levels, which could also partly explain the reduction in VOC-mediated plant growth-
429 promotion along the richness gradient. One potential explanation for this pattern is that the presence
430 of other bacteria triggered a switch from the expression of plant growth-promoting to pathogen-
431 suppressing VOCs due to bacterial competition, which has previously shown to upregulate
432 antibacterial activity including VOC production [19, 30, 31]. Moreover, we found that the
433 community evenness and the abundance of all genera promoted, while community productivity
434 constrained the VOC-mediated plant growth-promotion.

435 These results clearly show that bacterial interactions within multi-species communities can
436 affect the VOC production, which in turn can change VOC-mediated functioning in terms of
437 pathogen suppression and plant growth-promotion. Furthermore, VOC-mediated microbe-
438 pathogen and microbe-plant interactions were optimized with different community structures due
439 to non-linear and contrasting relationships with community diversity, productivity and evenness.
440 These results suggest that VOC-mediated interactions in communities cannot be predicted based

441 on VOC expression patterns observed in bacterial monocultures [34]. Our results are in contrast
442 with several previous studies. For example, Wagg et al. [11] and Hu et al. [35] have reported
443 positive relationships between microbial diversity and plant performance in communities
444 containing four and eight microbes, respectively. It is thus possible that diversity-functioning
445 relationships between soil bacteria and plants are less predictable, especially when mediated
446 through VOCs. Moreover, soil is a complex and heterogenous environment, and in reality,
447 rhizosphere bacterial communities are composed of thousands of interacting bacterial strains.
448 Because analyzing this many interactions at the same time is practically impossible, we used small
449 model communities consisting of 12 bacterial strains belonging to three genera. Even though, our
450 model system does not reflect the natural soil conditions, it can help to understand how interspecies
451 bacterial interactions can change the production and activity of VOCs. In the future, it would be
452 interesting to study the underlying ultimate mechanisms like quorum sensing, cross-talk, chemical
453 cues (antibiotics), silence gene clustering or cross-feeding, etc. driving the VOC production within
454 the communities. Moreover, it would be interesting to explore how the VOCs produced in the soil
455 affect the microbiota residing in the aerial parts of the plant for example in leaves and flowers, that
456 could affect pollination [36]. Our results also show that bacterial communities can interact with
457 plants and plant pathogens over long distances through VOCs, and crucially, that bacterial
458 interactions within communities change their effects on plants or pathogens in the absence of direct
459 contact. Thus, it is important to move beyond plant rhizosphere microbiomes to explore microbe-
460 microbe-plant interactions over larger spatial scales that also include VOC-mediated long-distance
461 interactions in porous soils [37]. For example, plant root VOCs were reported to disperse over 12
462 cm distances mediating long-distance belowground interactions in the soil [2] indicative of
463 interactions between microbial metapopulations. From the applied perspective, our study suggests
464 that VOC-mediated functions could potentially be employed to manipulate rhizosphere

465 microbiome composition to simultaneously improve multiple ecosystem functions including
466 pathogen suppression and plant growth.

467 **Authors' contributions**

468 WR, JW, AJ, VF, WZ, MX, WS and SQ developed the ideas; WR, AJ, VF and WZ designed the
469 study; WR, JW, MX, WS and WZ set up the experiment; WZ, JW and WR collected data; WR and
470 WZ analyzed the data and wrote the manuscript; AJ, VF, WZ and SQ provided comments on the
471 manuscript.

472 **Data accessibility**

473 All data generated or analyzed during this study are included in this article and its supplementary
474 information files. The supplementary information data has also been submitted to Dryad
475 (https://doi.org/10.5061/dryad.dbrv15dxn)

476 **Competing interests**

477 The authors declare no competing interest.

478 **Funding**

479 The work was supported by the National Natural Science Foundation of China (grant numbers
480 31601835, 41671248, 41671256); National Key Basic Research Program of China (grant number
481 2015CB150503), the Fundamental Research Funds for the Central Universities (grant numbers
482 KYT201802, KJQN201745), 973 project (grant number 2015CB150500) and Jiangsu Science and
483 Technology Department (grant numbers BK20171373, BK20170085). Ville-Petri Friman is
484 supported by the Wellcome Trust (reference no. 105624) through the Centre for Chronic Diseases
485 and Disorders (C2D2) and Royal Society Research Grants (RSG\R1\180213 and CHL\R1\180031)
486 at the University of York, UK. Alexandre Jousset is supported by the Nederlandse Organisatie voor

Wetenschappelijk Onderzoek (ALW.870.15.050) and the Koninklijke Nederlandse Akademie van Wetenschappen (530 - 5CDP18).

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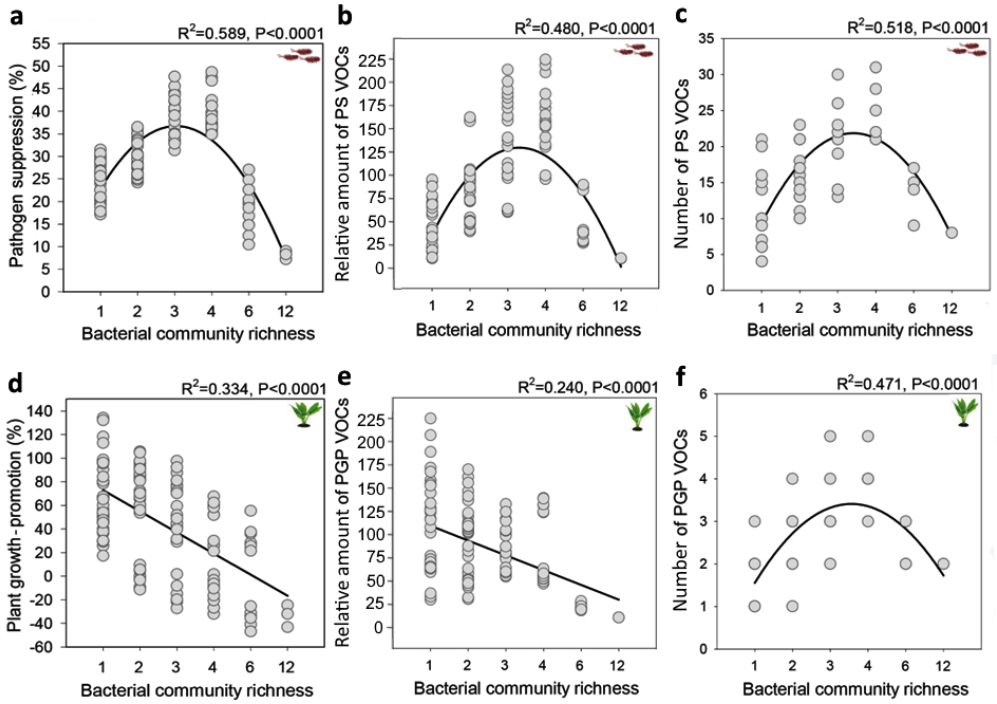
Figure 1: Effect of bacterial community richness on volatile organic compound (VOC)-mediated pathogen suppression (PS) and plant growth promotion (PGP) and on the relative amount and number of produced pathogen-suppressing and plant growth-promoting VOCs. Top panels show the effect of bacterial community richness on VOC-mediated pathogen suppression (A) and on the relative amount (B), and number (C) of pathogen-suppressing VOCs. Bottom panels show the effect of bacterial community richness on VOC-mediated plant growth-promotion (D) and on the relative amount (E) and number (F) of plant growth-promoting VOCs. The relative amount of VOCs shows the chromatographic peak area that was expressed relative to the peak area of (Z)-3-hexenyl acetate (internal standard) as an indirect approach to estimate the relative concentration of each VOC, while number of VOCs means the total number of VOCs produced at each community richness level. In all panels, each observation shows the effect of each replicate of each bacterial monoculture or community. The experiments were repeated twice in triplicate.

Figure 2: The relationship of volatile organic compound (VOC)-mediated pathogen suppression (PS) and plant growth promotion (PGP) with the relative amount and numbers of pathogen-suppressing and plant growth-promoting VOCs, respectively, produced by bacterial communities at different richness levels. Top panels show the relationship between VOC-mediated pathogen suppression and the relative amount (A) and number (B) of pathogen-suppressing VOCs. Bottom panels show the relationship between VOC-mediated plant growth-promotion and the relative amount (C) and number (D) of plant growth-promoting VOCs. The relative amount of VOCs shows the chromatographic peak area that was expressed relative to the peak area of (Z)-3-hexenyl acetate (internal standard) as an indirect approach to estimate the relative concentration of each VOC, while number of VOCs means the total number of VOCs produced at each community richness level. In all panels, each observation shows the effect of each replicate in each bacterial monoculture or community. The experiments were repeated twice in triplicate.

605 **Figure 3:** Effect of bacterial community richness on community productivity (total bacterial
606 abundance), genera abundances and genera evenness. The relationships between bacterial
607 community richness and total bacterial community productivity (A), genera abundances (B) and
608 community evenness based on bacterial genera abundances (C). In panels A and B, CFU denotes
609 for bacterial cell numbers per ml in terms of colony forming units. In panel B, black, dark grey and
610 light grey data points represent *Paenibacillus*, *Bacillus* and *Paenibacillus* genera, respectively. In
611 all panels, each observation shows the effect of each replicate in each bacterial monoculture or
612 community. The experiments were repeated twice in triplicate.



Effect of bacterial community richness on volatile organic compound (VOC)-mediated pathogen suppression and plant growth promotion and on the relative amount and number of produced pathogen-suppressing and plant growth-promoting VOCs.

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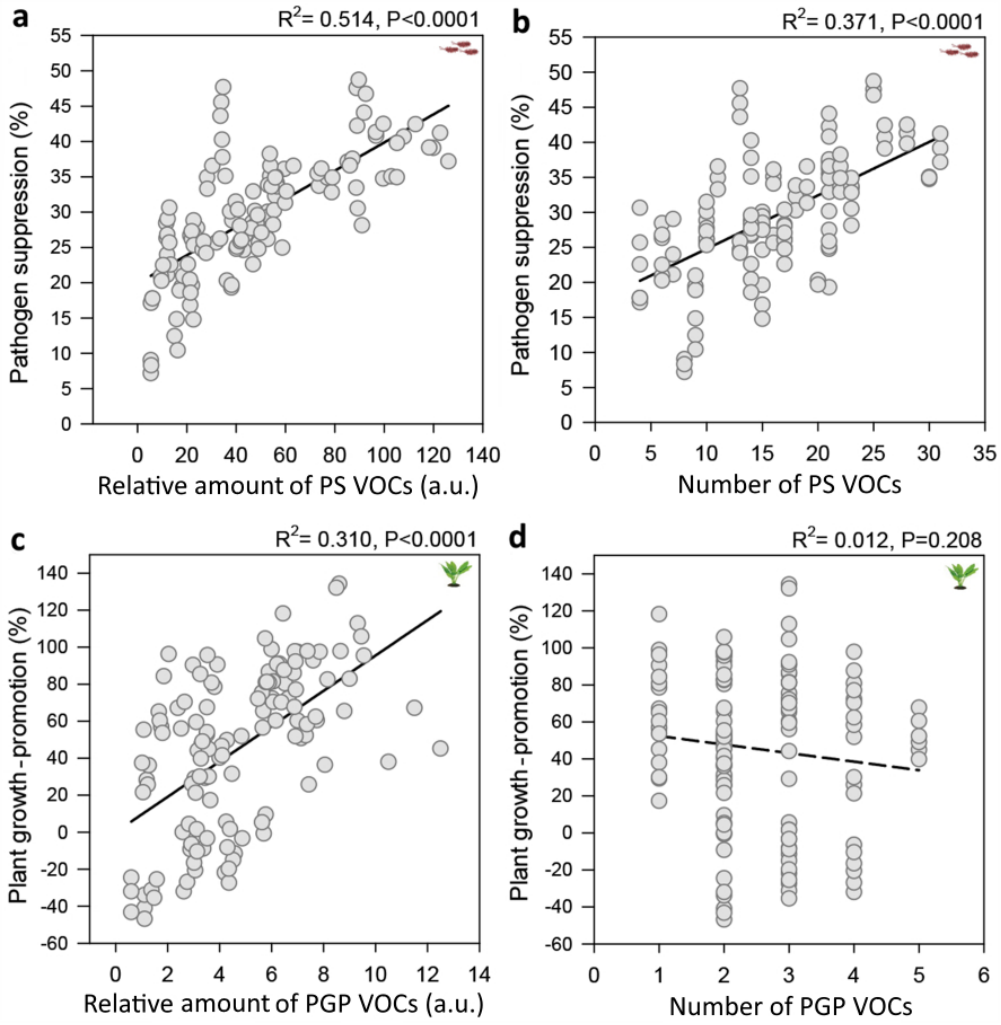

The relationship of volatile organic compound (VOC)-mediated pathogen suppression and plant growth promotion with the relative amount and numbers of pathogen-suppressing and plant growth-promoting VOCs, respectively, produced by bacterial communities at different richness levels.

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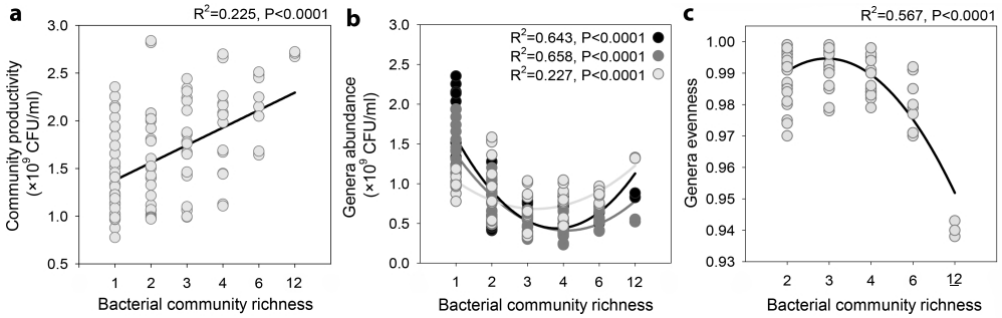

Effect of bacterial community richness on community productivity (total bacterial abundance), genera abundances and genera evenness.

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