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

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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.

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

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15 **Abstract**

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

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

38 # 1. Introduction

39 Soil microbiome research has focused mainly on the beneficial effects of root-associated microbes
40 that reside in the near vicinity of the plants. However, microbes also interact with each other and
41 plants over long distances by producing volatile organic compounds (VOCs) that are a broad group
42 of lipophilic compounds with low molecular weight (100–500 Da), high vapor pressure and low
43 boiling point [2]. These properties facilitate evaporation and diffusion of VOCs over long distances
44 through the atmosphere or porous soils from the point of production [3]. The VOCs have been
45 reported for distinct bioactive functions, which are as diverse as the chemical structures of VOCs
46 shaping a wide range of bacteria-bacteria and bacteria-plant interactions, including cell-to-cell
47 communication, plant growth, flowering and photosynthesis stimulation, inhibition of parasites and
48 pathogens and activation of systematic plant resistance against biotic and abiotic stresses [4, 5, 6,
49 7]. The composition of the emitted VOCs can also vary depending on the environmental conditions
50 such as the substrate composition of the growth media [8]. While several VOCs have been shown
51 to change pairwise interactions with plants and microorganisms [9, 10], it is less clear how the
52 presence of other microbes in multi-species communities affects the production and functioning of
53 VOCs. Here we studied this directly by manipulating bacterial community richness and
54 composition and determining subsequent effects on VOC production and VOC-mediated pathogen
55 suppression and plant growth-promotion.

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

61 or facilitative [15] effects. Alternatively, it is possible that high bacterial community diversity could
62 lead to increased antagonism within the bacterial community, which could then offset the VOC
63 production by having a negative effect on the growth and overall metabolism of the community
64 [16]. Increasing community diversity could thus either promote or constrain VOC production
65 depending on the species interactions between the interacting community members that could be
66 driven by competition for shared resources, cooperation, cheating or antibiosis [1, 3]. Second,
67 increasing the number of species in a community could increase the number of unique VOCs that
68 are produced if each species produces a different subset of compounds [17]. High community
69 diversity could thus increase the range of VOC-mediated functions. Third, intra- and interspecific
70 bacterial interactions could lead to the expression of certain 'emergent' VOCs that are not produced
71 in monocultures. One potential mechanism for this could be interference competition which is often
72 stronger in diverse bacterial communities due to the production of a high variety of antimicrobial
73 compounds [18]. While co-culturing two to five bacteria together has been shown to induce the
74 production of novel antifungal VOCs [3, 19], the effects of diversity on bacteria-specific VOCs
75 have not yet been explored.
76 Theory and experiments suggest that increasing community diversity and richness could
77 predictably affect the production of VOCs by bacterial communities. However, it is still largely
78 unknown how these changes affect the type and strength of VOC-mediated functioning with
79 bacterial pathogens and plants. To address this shortcoming, we used a model plant-bacteria system
80 to causally test how the microbial community richness affects the VOC-mediated functioning in
81 terms of *Arabidopsis thaliana* plant growth-promotion and the suppression of a wide-spread
82 bacterial pathogen, *Ralstonia solanacearum*, capable of infecting many plant species [20]. To
83 achieve this, we assembled VOC-producing model bacterial communities in different richness
84 levels ranging from one to twelve strains using three ubiquitous, soil-dwelling bacterial genera:

85 *Bacillus, Paenibacillus* and *Pseudomonas*. We then determined and classified the emitted VOCs
86 by all bacterial communities and explored how this variation affected plant growth-promotion and
87 pathogen suppression as a function of bacterial community richness.
88

89 ## 2. Methods

90 ### (a) Bacterial strains

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

103 ### (b) Assembly of model rhizosphere bacterial communities

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

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

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

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

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

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

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

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

163 We used the *A. thaliana* plant model system to assess whether changes in microbial
164 community richness and composition affected plant growth via changes in VOC composition. The
165 Petri dish system was used in a similar way as described above in triplicate, including a negative
166 control treatment where *A. thaliana* grew in the absence of VOC-producing bacteria. The cell
167 suspensions of 43 model communities (1×10^7 CFU/ml) were spot-inoculated on one side of the
168 Petri dish as described above and incubated at 30°C for 12 hours to initiate bacterial growth (Figure
169 S2). Later, three *Arabidopsis* Col-1 seedlings were placed onto the other half of the Petri dish
170 containing half-strength Murashige and Skoog agar medium (0.8% agar and pH 5.7). Before that,
171 *Arabidopsis* seeds were surface sterilized, vernalized for 2 days at 4°C in the dark on half-strength
172 Murashige and Skoog agar medium with 1.5% sucrose and then placed in a growth chamber (22°C
173 temperature, 12h light, 12h dark, 40W fluorescent light) for three days. The Petri plates were sealed
174 with parafilm and placed in a growth chamber. After two weeks, plants were gently removed from
175 the medium, roots washed with sterilized water and the whole plant was blot dried and weighted
176 to determine the plant fresh weight (mg/plant). To determine VOC-mediated plant growth-
177 promotion in the soil, a similar system was used as when evaluating VOC-mediated pathogen
178 suppression in the soil except that the pathogen was replaced with three *Arabidopsis* seedlings
179 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

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

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

217 To evaluate the effect of different concentrations of identified VOCs (GC-MS analysis) on
218 pathogen suppression, the Petri dish system was used in a similar way as described above. The cell
219 suspension of *R. solanacearum* (1×10^7 CFU/ml) was spot-inoculated at two locations (5 µl in each)
220 on one side of the Petri dish and incubated at 30°C for 12 hours to initiate bacterial growth. Later,
221 stock solutions (20 µg/ml, 100 µg/ml, 500 µg/ml, 2 mg/ml and 10 mg/ml) of 67 commercially
222 available pure VOCs (Dataset S1) were prepared separately in methanol by serial dilutions and the
223 other side of Petri dish was inoculated with 15 µl of stock solutions to give 0.3 µg, 1.5 µg, 7.5 µg,
224 30 µg and 150 µg final amount of each VOC on a ~10 mm diameter sterile filter paper disc
225 (Whatman™ filter paper, 6 µm pore size), respectively. Petri dishes were sealed with Parafilm and
226 incubated for three days at 30°C. The sterile filter paper discs inoculated with nothing or with
227 methanol were used as control treatments (no difference found between these control treatments).

228 The VOC-effects on the cell densities *R. solanacearum* was quantified similarly as described above.
229 [7, 25].

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

236 **(f) Statistical analysis**

237 The statistical differences between bacterial strains and genera were analyzed using ANOVA and
238 Tukey's tests. Linear regression analysis was used to analyze separately the VOC-mediated
239 pathogen suppression and plant growth-promotion, relative amount of VOCs (sum of relative peak
240 area to (Z)-3-hexenyl acetate of detected GC-MS peaks), number of VOCs (number of peaks) and
241 VOC composition (first axis of the principal component analysis on non-transformed data), and
242 total community abundance, genera abundances and community evenness (at genera level) as the
243 function of bacterial community richness (factor with 6 levels); significance at P=0.05. Similarly,
244 to link VOCs production with VOC-mediated activity, we separately analyzed the VOC-mediated
245 pathogen suppression and plant growth-promotion as the function of the relative amount of
246 produced VOCs, number of VOCs and VOC composition; significance at P=0.05. To further link
247 VOC profiles and community properties to functioning, we used separate models to explain plant
248 growth-promotion and pathogen suppression with bacterial genera, community abundances and
249 community evenness, community richness and strain identity effects and relative amount, number
250 and composition of VOCs. To uncover the most parsimonious GLMs with the best explanatory
251 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

275 showed significant variation in the relative amount ($F_{11, 24}$= 357.2, P<0.0001) and number ($F_{11, 24}$=
276 54.6, P<0.0001) of produced VOCs within each genus (Figure S7a-f).

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

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

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

299 suppression peaked at the intermediate community richness (4 species) reaching 40% suppression
300 efficiency and then decreasing to 8% efficiency at richness level 12 compared to non-VOC control
301 (Figure 1a). This pattern could be explained well with the relative amount ($F_{2, 127}$=58.18, P<0.0001;
302 Figure 1b), number ($F_{2, 126}$=67.7, P<0.0001; Figure 1C) and composition ($F_{2, 126}$=13.68, P<0.0001;
303 Figure S10a) of produced pathogen-suppressing VOCs, which all showed a similar hump-shaped
304 relationship peaking at richness level 4 and then decreasing at richness levels 6 and 12. Together,
305 pathogen suppression showed highly significant and positive relationships with the relative amount,
306 number and composition of pathogen-suppressing VOCs (Figure 2a-b; Figure S10b; Table S3).
307 In contrast, the highest plant growth-promotion was observed at low community richness
308 levels ($F_{1, 127}$=13.8, P<0.0001). Specifically, a 67% increase in plant growth-promotion observed
309 at the richness level 1 decreased to 17% increase at richness level 4, and at richness level 12, an
310 average of 33% decrease in plant growth-promotion was observed compared to control treatment
311 (Figure 1d). Reduction in the plant growth-promotion correlated clearly with a decrease in the
312 relative amount of plant growth-promoting VOCs ($F_{1, 127}$= 39.9, P<0.0001; Figure 1e) resulting in
313 90% decrease between richness levels 1 and 12. However, similar to pathogen-inhibiting VOCs,
314 the number of plant growth-promoting VOCs peaked at intermediate richness levels reaching up
315 to 139% increase at the richness level 4 and then decreasing down to 19% increase at the richness
316 level 12 compared to the richness level 1 ($F_{2, 126}$ =56.1, P<0.0001; Figure 1f). The composition of
317 plant growth-promoting VOCs did not show any relationship with plant growth-promotion (Figure
318 S10c). As a result, plant growth showed a highly significant and positive relationship only with the
319 relative amount of plant growth-promoting VOCs (Figure 2c-d; Figure S10D; Table S3).

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

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

323 VOCs (1, 2-ethanediol 1, 2-diphenyl; 9-decen-i-ol and 5-octadecene), the relative amount of VOCs
324 varied significantly between communities with different richness levels (Dataset S1). Interestingly,
325 15 VOCs were produced only in communities. Similarly, 49 VOCs produced at richness levels 1-
326 4 were absent from the VOC profiles of 6 and 12 species communities (Figure S11A; Data-set S1).
327 Out of 85 VOCs in total, 41 VOCs showed pathogen-suppressing activity. Of these, 4 pathogen-
328 suppressing VOCs were not produced at the community richness level 1, and 26 pathogen-
329 suppressing VOCs produced at richness levels 1-4 were absent from the VOC profiles of 6 and 12
330 species communities (Figure S11b; Data-set S1). When chemical groups of VOCs were evaluated,
331 80% (61) of the identified VOCs produced by twelve bacterial strains belonged to alkane, alcohol,
332 aldehyde, benzene, ketone and fatty acid groups. Almost all alcohol, aldehyde, benzene and ketone
333 group VOCs showed pathogen-suppressing activity. Other VOC groups related to pathogen
334 suppression included naphthalene, phenol, sulfur and nitrogen containing compounds (Figure S12).
335 Only six out of 85 VOCs were found to show plant growth-promoting activity (Figure S8C).
336 Of these compounds, four VOCs were not produced at richness level 12, while tetradecane was
337 only produced at richness levels 6 and 12 albeit in low relative amount (Figure S11c; Data-set S1).
338 Interestingly, two of the plant growth-promoting VOCs (indole, heptadecane) also showed
339 antibacterial activity against *R. solanacearum* (Figure S8a-b). When chemical groups of VOCs
340 were evaluated, plant growth-promoting VOCs mainly belonged to the alkane (4) group; while one
341 VOC belonged to the diol and one to the nitrogen-containing compounds group (Figure S12). These
342 results suggest that bacterial interactions within communities can trigger and abolish the production
343 of certain pathogen-suppressing and plant growth-promoting VOCs.

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

346 Lastly, we explored if richness-mediated VOC effects could be explained by certain underlying
347 community properties such as community productivity, evenness, genera abundances or strain
348 identity effects. While the community productivity increased with bacterial richness ($F_{1, 127}$=36.8,
349 *P*=0.004; Figure 4a), the relative abundance of all three genera showed a parabolic relationship
350 with the richness reaching the lowest abundances at the intermediate richness levels and the highest
351 abundances when grown in the low or high richness level communities (Figure 4b). Moreover,
352 while the community evenness of bacterial genera did not differ at the lower richness levels (in 2-
353 4 species communities), it considerably decreased at the higher richness levels ($F_{4, 88}$=41.00,
354 *P*<0.0001; Figure 4c). As a result, bacterial community properties showed contrasting effects on
355 VOCs functioning; while total community productivity was positively linked with pathogen
356 suppression, it showed a negative effect on the plant growth-promotion (Table S4). In contrast,
357 while community evenness had no effect on the pathogen suppression, it was positively linked with
358 the plant growth-promotion (Table S4). Furthermore, while the densities of *Pseudomonas* and
359 *Paenibacillus* genera showed a negative relationship with pathogen suppression, the densities of
360 all three genera showed positive effects on the plant growth-promotion (Table S4). Finally, some
361 strains had strong and often opposing identity effects on both the pathogen suppression and plant
362 growth-promotion (Table S4). These results suggest that bacterial community properties had
363 contrasting effects on VOC-mediated functioning, which likely constrained simultaneous
364 expression of pathogen suppressing and plant growth-promoting VOCs.

365 **4. Discussion**

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

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

386 Of all the detected VOCs, 41 VOCs (52%) showed pathogen suppression and their relative
387 amount and numbers peaked at the intermediate community richness levels, which was highly
388 correlated with VOC-mediated pathogen suppression. Moreover, compared to monocultures, 14
389 unique VOCs, including four pathogen-suppressing VOCs, were produced in more diverse
390 bacterial communities including two to four strains. These results suggest that the addition of new
391 species likely increased the metabolic potential of the community by stimulating the production of
392 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.

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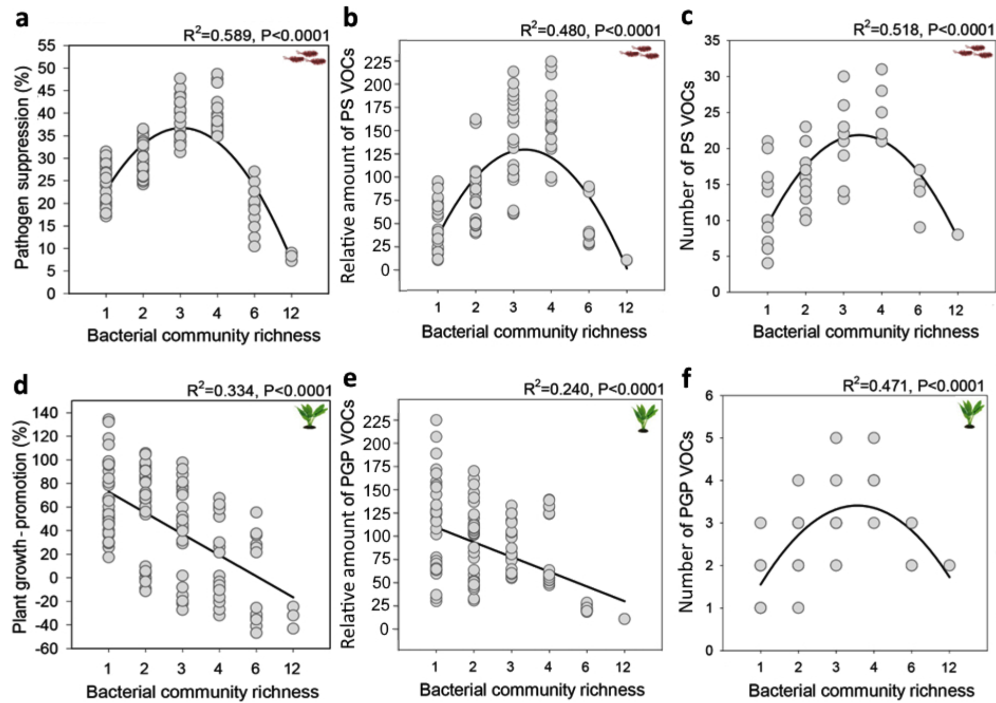
581 **Figure 1:** Effect of bacterial community richness on volatile organic compound (VOC)-mediated
582 pathogen suppression (PS) and plant growth promotion (PGP) and on the relative amount and
583 number of produced pathogen-suppressing and plant growth-promoting VOCs. Top panels show
584 the effect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

593 **Figure 2:** The relationship of volatile organic compound (VOC)-mediated pathogen suppression
594 (PS) and plant growth promotion (PGP) with the relative amount and numbers of pathogen-
595 suppressing and plant growth-promoting VOCs, respectively, produced by bacterial communities
596 at different richness levels. Top panels show the relationship between VOC-mediated pathogen
597 suppression and the relative amount (A) and number (B) of pathogen-suppressing VOCs. Bottom
598 panels show the relationship between VOC-mediated plant growth-promotion and the relative
599 amount (C) and number (D) of plant growth-promoting VOCs. The relative amount of VOCs shows
600 the chromatographic peak area that was expressed relative to the peak area of (Z)-3-hexenyl acetate
601 (internal standard) as an indirect approach to estimate the relative concentration of each VOC,
602 while number of VOCs means the total number of VOCs produced at each community richness
603 level. In all panels, each observation shows the effect of each replicate in each bacterial
604 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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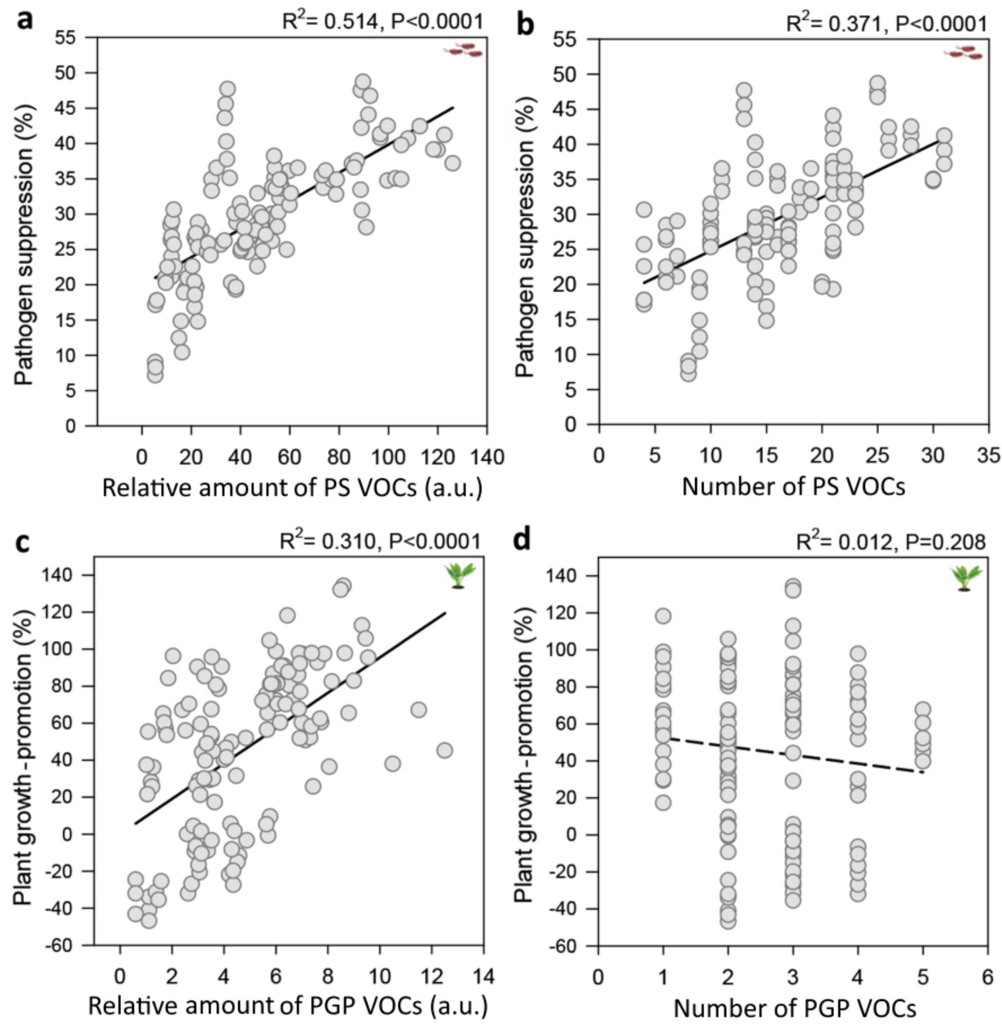

The relationship of volatile organic compound (VOC)-mediated pathogen suppression and plant growth promotion with the relative amount and numbers of pathogen-suppressing and plant growth-promoting VOCs, respectively, produced by bacterial communities at different richness levels.

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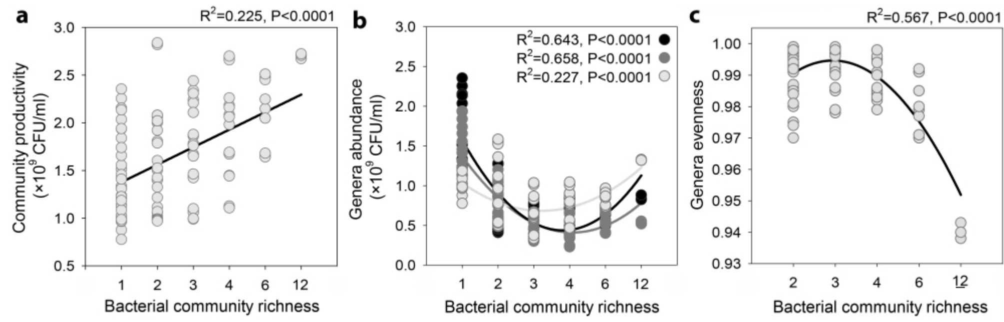

Effect of bacterial community richness on community productivity (total bacterial abundance), genera abundances and genera evenness.

84x26mm (300 x 300 DPI)