1 Rhizosphere-enriched microbes as a pool to design synthetic 2 communities for reproducible beneficial outputs

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17

18 **Abstract**

19 Composts represent a sustainable way to suppress diseases and improve plant growth.
20 Identification of compost-derived microbial communities enriched in the rhizosphere
21 of plants and characterization of their traits, could facilitate the design of microbial
22 synthetic communities (SynComs) that upon soil inoculation could yield consistent
23 beneficial effects towards plants. Here, we characterized a collection of compost-
24 derived bacteria, previously isolated from tomato rhizosphere, for *in vitro* antifungal
25 activity against soil-borne fungal pathogens and for their potential to change growth
26 parameters in *Arabidopsis*. We further assessed root-competitive traits in the
27 dominant rhizospheric genus *Bacillus*. Certain isolated rhizobacteria displayed
28 antifungal activity against the tested pathogens and affected growth of *Arabidopsis*,
29 and Bacilli members possessed several enzymatic activities. Subsequently, we
30 designed two SynComs with different composition and tested their effect on
31 *Arabidopsis* and tomato growth and health. SynCom1, consisting of different bacterial
32 genera, displayed negative effect on *Arabidopsis in vitro*, but promoted tomato
33 growth in pots. SynCom2, consisting of Bacilli, didn't affect *Arabidopsis* growth,
34 enhanced tomato growth and suppressed Fusarium wilt symptoms. Overall, we found
35 selection of compost-derived microbes with beneficial properties in the rhizosphere of
36 tomato plants, and observed that application of SynComs on poor substrates can yield
37 reproducible plant phenotypes.

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Introduction

The soil layer surrounding plant roots, the rhizosphere, hosts a mesmerizing diversity of microorganisms that are highly affected by plant activity and represents one of the most complex terrestrial ecosystems^{1,2}. The microbial communities in the rhizosphere, collectively known as the microbiome, are highly diverse and contain members having neutral, deleterious or beneficial effects on plants^{2,3}. Generally, the structure of the microbiome is determined by the chemical environment of the rhizospheric soil, with main drivers being the root exudates^{1,3}, the soil organic matter⁴ and exogenously-applied organic amendments⁵. Increasing evidence suggests that plants actively recruit beneficial bacteria that can either promote plant growth and health or can actively repress proliferation of soil-borne pathogens^{6,7}. Thus, the root-associated microbiome is a subset of the more diverse microbiome of the bulk soil⁸. Perturbations in the composition of the root-associated microbiome can affect plant phenotypes, thus understanding the processes determining the composition of the rhizospheric community but also how specific members of the microbiome can help the plant are of high importance.

A considerable amount of research has been devoted to the identification of individual beneficial microbes responsible for plant growth and/or health. Plant growth-promoting rhizobacteria (PGPR) are widely studied beneficial microorganisms because of their ability to enhance plant growth and yield of several economically important crops. PGPRs can stimulate plant growth via the production of phytohormones (e.g. auxins, cytokinins and ethylene)⁹, the enzymatic activity they display (e.g. aminocyclopropane-1-carboxylate (ACC) deaminase)¹⁰ or by facilitating nutrition, e.g. phosphate solubilization¹¹. PGPRs can also protect plants against soil-borne plant pathogens by producing antibiotics, hydrogen cyanide (HCN), and

65 siderophores^{12,13}, or through the activity of fungal cell wall-degrading enzymes^{14,15}.
 66 Root colonization by some rhizobacteria can also induce systemic defense responses
 67 (induced systemic resistance; ISR) in the plant, that are effective against various plant
 68 pathogens in the leaves¹⁶.

69 The beneficial role of the root microbiome on plant health becomes more
 70 evident in disease-suppressive soils and organic amendments, in which disease will
 71 not develop even in the presence of a pathogen and a susceptible host¹⁷. Composts
 72 from different sources are the most widely-studied organic amendments that display
 73 suppressive properties against soil-borne pathogens and promote plant growth¹⁸. The
 74 phenomenon of suppressiveness is attributed to the microbial activity since it is
 75 abolished after sterilization or pasteurization^{18,19}. A substantial number of bacterial,
 76 oomycete and fungal pathogens, including *Fusarium* and *Verticillium* spp., are targets
 77 of disease suppression¹⁸. Despite the extensive literature demonstrating the
 78 suppressiveness of composts, practical application is still limited due to the
 79 inconsistency of results and the unpredictable outcome in different pathosystems^{20,21}.

80 The improvement of microbial isolation techniques allowed the identification
 81 of several microbial genera that are beneficial for plants^{22,23}. Species belonging to
 82 *Bacillus*, *Enterobacter*, *Pseudomonas*, *Streptomyces* and *Trichoderma* are really
 83 active in the root environment and have the potential for disease control and plant-
 84 growth promotion¹⁸. However, to determine the contribution of individual strains
 85 from complex microbial communities to plant health is a daunting task. Various *in*
 86 *vitro* screening techniques are employed to identify microbes that could potentially
 87 improve plant growth and health; yet none of these screening methods guarantee
 88 plant-associated phenotypes²⁴. Also, binary mutualistic interactions between plants
 89 and microbes are commonly applied for the identification of macroscopically visible

beneficial outcomes but only a limited number have been applied successfully in practice²⁵. The aforementioned suggest that these *in vitro* assays fail to predict *in vivo* effects as they cannot capture all variables of nature's complexity.

In this study, we build on our previous work where we demonstrated that the activity of the microorganisms contained in a compost promoted plant growth and enhanced the protection of tomato plants against *Verticillium dahliae* (Vd) and *Fusarium oxysporum* f. sp *lycopersici* (Fox). Community-level analysis of the culturable microbiome revealed differential distribution of the microbial communities between the compost and the rhizosphere, suggesting that plants recruit beneficial soil microorganisms for their own benefit²⁶. The aims of the present study are: (a) to assess if bacterial genera enriched in the rhizosphere of tomato plants have antifungal potential and can improve plant fitness; (b) to test whether the composition of synthetic communities (SynComs) can affect growth and defense outputs; and (c) to use simplified SynComs in sterile peat-based growth substrate and reproduce the suppressive and growth promotion properties of the compost on tomato plants. Using this approach, we provide with evidence that rhizosphere-enriched microbes can be pools for the construction of beneficial SynComs. Inoculation of an organic amendment lacking phytobeneficial properties with such SynComs can produce a growth substrate with predictable and consistent growth-promoting and disease suppressive properties.

110

111 **Results**

112 **Production of antifungal and growth-modulating secondary metabolites is**
113 **widespread among rhizosphere-enriched bacteria**

114 To understand whether the previously observed disease suppressiveness of soil-borne
 115 pathogens²⁶ is attributed to specific microbes selected in tomato rhizosphere, we
 116 tested in dual-culture assays the antagonistic activity of the rhizobacterial strains
 117 against *Vd* and *Fox* (Fig. 1). Initially, all 143 isolated strains were evaluated for direct
 118 antagonism through the production of diffusible secondary metabolites (DSM). A
 119 high percentage of these (32.9%, 47 strains) suppressed *Vd* growth whereas only 9
 120 strains (6.3%) led to significant inhibition of *Fox* growth (Fig. 1a). Suppression of
 121 *Fox* ranged from 45.3% to 56.7%, while inhibition rates against *Vd* ranged from 3.3%
 122 to 51.3%. All the *Enterobacter cloacae* strains and strains *Bacillus licheniformis*
 123 (3Ba9, 3Ba17), *Bacillus subtilis* (1Ba19), *Chryseobacterium* sp. (2Ba19),
 124 *Ochrobactrum* sp. (2Ba57) and *Stenotrophomonas maltophilia* (2Ba31) were found to
 125 inhibit the growth of both *Vd* and *Fox*. From all the *Bacillus* strains tested, a relatively
 126 high percentage (42.6%, 20 isolates) could suppress mycelial growth of *Vd*. Next, we
 127 selected the 47 strains out of the 143 total isolates, causing significant *Vd* and *Fox*
 128 growth inhibition and tested their ability to suppress fungal growth through the
 129 production of VOCs (Fig. 1b). We observed a marked suppression of *Vd* by
 130 *Acidovorax* sp., that inhibited fungal growth by 55.3%, while 16 more isolates
 131 inhibited *Vd* growth more than 10%, with inhibition ranging from 10.3 to 28.4%.
 132 Twenty-eight isolates exhibited moderate inhibition, ranging from 0 to 9.6%, whereas
 133 strains *Microbacterium foliorum* (2Ba37) and *B. licheniformis* (3Ba44) slightly
 134 promoted *Vd* fungal growth by 2.5 and 1.5%, respectively. Regarding *Fox*, 18 strains
 135 led to pathogen inhibition ranging from 10.2 to 23%, whereas 21 strains showed less
 136 than 10% suppression. Seven *Bacillus* (1Ba2, 1Ba18, 1Ba43, 3Ba3, 3Ba30, 3Ba44,
 137 3Ba45) and one *Microbacterium* (3Ba33) strains promoted *Fox* growth but to a small
 138 extent (0.5 to 4.4%). Interestingly, only three *S. maltophilia* (1Ba16, 2Ba27, and

139 3Ba7), one *Ochrobactrum* (2Ba57), one *E. cloacae* (2Ba47), one *Acidovorax* (1Ba38)
 140 and one *B. megaterium* (1Ba20) strains could suppress fungal growth of both
 141 pathogens more than 10% (Fig. 1b). This first experiment suggests that compost-
 142 derived bacteria isolated from tomato rhizosphere have the potential to inhibit fungal
 143 growth, mostly through production of DSM and in a lesser extent through VOCs.

144 Previously, the compost-derived and rhizosphere-enriched isolates were not
 145 only associated with disease suppressiveness, but also with plant growth promotion²⁶.
 146 Therefore, the 47 strains displaying an inhibitory effect on *Vd* and *Fox* mycelial
 147 growth *in vitro* (Fig. 1) were tested for their ability to promote plant growth *in vitro*.
 148 Seven-d-old *Arabidopsis* seedlings were exposed to each bacterial strain and shoot
 149 biomass, primary root length and number of lateral roots were determined after 8 d of
 150 co-cultivation. Eleven out of 47 isolates significantly increased shoot fresh weight, 6
 151 increased primary root length and 7 increased the number of lateral roots compared to
 152 the control (Fig. 2; green-colored boxes). A number of *S. maltophilia*, *E. cloacae*,
 153 *Delftia* sp., *Agrobacterium tumefaciens*, *Bacillus humi* and *B. licheniformis* strains led
 154 to significant increase in plant shoot fresh weight, whereas the remaining strains did
 155 not increase shoot biomass (Fig. 2a). Three *S. maltophilia* strains, *Delftia* sp., one
 156 *Chryseobacterium* strain and *Lysinibacillus sphaericus* significantly increased
 157 primary root length (Fig. 2b; green boxes), whereas *E. cloacae* 2Ba47 and 17 *Bacillus*
 158 strains showed negative effects on primary root length of seedlings (Fig. 2b; red
 159 boxes). Two *S. maltophilia* strains, two *Ochrobactrum* sp., one *E. cloacae*, one
 160 *Chryseobacterium* and *Advenella* sp. significantly increased the number of lateral
 161 roots (Fig. 2c; green boxes) whereas 12 *Bacillus* strains led to decreased number of
 162 lateral roots compared to the control (Fig. 2c; red boxes). The *B. megaterium* 1Ba20
 163 induced negative effects on all plant growth parameters determined. These results

164 indicate that the bacterial isolates when tested individually *in vitro* can have
165 differential effects on plant growth, despite their isolation from the rhizosphere of
166 plants showing growth promotion.

167 Another way to test if bacterial strains have the potential to affect plant
168 physiology, is by evaluating their ability to produce/modify hormonal signals⁴⁸. Auxin
169 is one of the hormones closely related to plant growth and development⁴⁹. Production
170 of the auxin analog IAA was possible by 28 out of 47 strains (Fig. 3a). Four
171 *Chryseobacterium*, 2 *Ochrobactrum*, one *S. maltophilia*, both *A. tumefaciens*, *Delftia*
172 sp. and *Acidovorax* sp. strains produced IAA. A high percentage of the *Bacillus*
173 strains assayed (85%, 17 strains out of 20) produced IAA, however at lower levels.
174 Ethylene (ET) is a plant hormone that depending on its levels in plants can have either
175 positive or negative effects on growth⁵⁰. ET depletion is achieved by many PGPR
176 strains through ACC deaminase enzymatic activity¹⁰. ACC deaminase activity was
177 measured by determining the production of a-ketobutyrate from ACC. *Delftia* sp.
178 2Ba46b was the most efficient strain, among the 25 strains that exhibited ACC
179 deaminase activity (Fig. 3b). All the *Delftia* sp., *Acidovorax* sp. *Advenella* sp.,
180 *Pseudomonas stutzeri*, *A. tumefaciens*, *Chryseobacterium* sp., *E. cloacae*,
181 *Ochrobactrum* sp., *S. maltophilia* strains and 3 *Microbacterium* sp. strains were found
182 positive to ACC deaminase activity. These assays show that a big proportion of the
183 tested isolates have the potential to either synthesize hormonal analogs (IAA) or
184 modify hormonal precursors (ACC), affecting as such plant growth.

185

186 ***Bacillus* rhizobacteria possess multifaceted beneficial characteristics**

187 Enrichment of selected members of the microbiome in the rhizosphere can usually
188 explain the beneficial effects conferred on plant fitness^{16,51}. In our case, *Bacillus*
189 isolates had increased representation among the rhizosphere strains (47 strains out of
190 143) accounting for 33% of the rhizospheric isolates (Supplementary Information
191 Table S1). Hence Bacilli were selected to be characterized for an array of enzymatic
192 activities and genetic markers associated with biological control activities. Microbial
193 versatility in the rhizosphere and microbial biocontrol potential is usually linked to the
194 production of hydrolytic enzymes (chitinases, glucanases, proteases, cellulases)^{52,53},
195 while cyclic lipopeptides (CLPs) produced by microbes can inhibit growth of
196 phytopathogens, facilitate root colonization and even stimulate host defense
197 mechanisms⁵⁴. Fifteen strains (71%) showed pectinase activity including all *B.*
198 *licheniformis* and *B. subtilis* isolates; 8 strains (38%) exhibited cellulase activity; 6
199 strains (29%) showed proteolytic activity including all *B. subtilis* isolates; 10 strains
200 (48%) showed chitinase activity including all *B. licheniformis* strains and eight strains
201 (38%) showed phosphate solubilization potential including all *B. subtilis* strains (Fig.
202 4a). From these data, it becomes apparent that the isolated Bacilli constitute a pool of
203 enzymatic activities.

204 Except for general biocontrol activities, the 21 *Bacillus* strains were examined
205 for the presence of CLP biosynthetic genes, since production of CLPs is linked not
206 only to antimicrobial activity but also to biofilm formation⁵⁵. Ten out of 21 strains had
207 between one and three CLP biosynthetic genes. The most frequent gene marker was
208 *srfAA* (9 positive strains), followed by *bacA* (4 positive strains). The gene markers
209 *srfAB* and *bmyB* were found in two *B. licheniformis* strains (3Ba17, 3Ba28 and
210 3Ba16, 3Ba17, respectively), *fend* was found in *B. subtilis* CCS strain and *spaS* was
211 detected in *B. chandigarhensis* (3Ba3) strain. The most frequent genotypes were

212 *srfAA-bacA* found in all *B. subtilis* and *B. megaterium* strains and *srfAA-bmyB* found
 213 in two *B. licheniformis* (3Ba16, 3Ba17) strains. We further tested the *in vitro* ability
 214 of Bacilli strains to form biofilm (Fig. 4b). Eleven out of 21 strains (52%) showed
 215 significant biofilm formation with 4 *B. licheniformis* strains forming more biofilm
 216 than the remaining strains. Moreover, all *B. subtilis* strains were positive in biofilm
 217 formation and the same was observed for *B. chandigarhensis*, *B. megaterium* and *B.*
 218 *humi* 1Ba28 strains. Generally, the abovementioned data show that among Bacilli
 219 there are isolates with the potential to efficiently proliferate in the rhizosphere.

220

221 **Design of bacterial synthetic communities of different composition yields** 222 **different outputs in *Arabidopsis* and tomato**

223 In an effort to assess how the rhizosphere enrichment of diverse bacterial isolates
 224 from a compost can benefit the host plant²⁶, we designed two simplified SynComs
 225 from the bacteria characterized in the current study. SynCom1 was composed of 25
 226 strains, aiming to reflect the representation of the most abundant phylogenetic groups
 227 detected in the rhizosphere (Supplementary Information Table S3). SynCom2 was
 228 composed of 25 *Bacillus* strains (Supplementary Information Table S3), in order to
 229 test if the dominance of the Phylum Firmicutes in the rhizosphere of tomato plants
 230 (33%, Supplementary Information Table S1) can explain by itself the beneficial
 231 effects on plant fitness²⁶. Initially, SynComs were tested *in vitro* for their effect on
 232 *Arabidopsis* growth, 20 d after root inoculation. Treatment of plants with SynCom1
 233 caused adverse effects in plant growth and resulted in reduction of shoot and root
 234 fresh weight and root length (Fig. 5). In contrast to SynCom1, treatment of plants with
 235 SynCom2 did not affect shoot and root fresh weight, however primary root length was

shorter compared to control plants (Fig. 5b). Root hairs' mean length of SynCom2-treated plants was significantly longer compared to the controls, however the number of mature root hairs was not altered (Supplementary Information Fig. S1). These data show that while the SynComs contain microbes enriched in the rhizosphere of tomatoes showing growth promotion, *in vitro* assays with *Arabidopsis* cannot capture the complexity of the interaction between a community and a host that can support it.

To tackle the limitations of the *in vitro* experimentation, we tested the effect of the two SynComs on tomato plants in more pragmatic conditions performing pot experiments. In contrast to the *Arabidopsis* findings, in both SynCom treatments of tomato, the plants displayed increased growth as assessed by total leaf area, plant height and total number of leaves compared to the control plants (grown in sterile peat-based substrate) (Fig. 6). The effects were more striking in SynCom1-treated tomato plants compared to plants treated with SynCom2, as they showed greater growth in all parameters assayed (Fig. 6).

The members of the tested SynComs derive from a compost not only promoting tomato growth but also suppressing disease caused by soil-borne fungal pathogens²⁶. Since both SynComs could benefit tomato growth, we then wondered if the tested SynComs could also reduce the symptoms caused by *Fox* after tomato infection. Interestingly, plants treated with SynCom2 showed less disease severity compared to the controls and SynCom1-treated plants (Fig. 7). The first *Fox* symptoms appeared in the form of wilting and yellowing especially on older leaves at 14 dpi and were recorder until 22 dpi (Fig. 7a). Disease symptoms progressed more rapidly in the control and SynCom1-treated plants, whereas SynCom2-treated plants showed less prominent symptoms and slower disease development (Fig. 7a, c). At 22 dpi, the disease severity in SynCom2-treated plants was 28.9% whereas in control and

261 SynCom1-treated plants reached 58.4% and 48.7% respectively (Fig. 7b). The relative
262 AUDPC values over 22 d of disease progress were higher in control (15.6%) and
263 SynCom1-treated plants (15.9%) compared to SynCom2-treated plants (7.3%) (Fig.
264 7b). These data show that substrate inoculation with SynCom2 could reproduce the
265 effect of the compost on disease suppressiveness. The ability of SynCom1 to promote
266 growth in tomato but not to suppress disease, in the tested conditions, highlights the
267 complexity behind naturally-observed growth promotion and further experimentation
268 is required for its elucidation.

269

270 **Discussion**

271 Modern agriculture relies on the extensive use of chemicals to combat plant diseases
272 and increase yields, which have nevertheless a negative impact on terrestrial
273 ecosystems and human health. Therefore, the development and application of
274 alternative and sustainable ways to deal with phytopathogens is a matter of urgency.
275 In this respect, composts with suppressive properties are used to control soil-borne
276 pathogens, that conventional methods, such as synthetic pesticides and resistant
277 cultivars fail to control^{18,21,56}. However practical application of composts in
278 agriculture is limited due to their unpredictable and inconsistent growth-promoting
279 and disease-suppressive properties¹⁸. Plants shape the structure of rhizosphere
280 microbial communities by altering the chemical environment of the rhizosphere
281 through root exudation¹. Exudates can stimulate the abundance of selected microbial
282 groups with beneficial properties that can largely contribute in the fitness and
283 longevity of plants^{6,7}. Several studies using cultivation-based techniques allowed the
284 identification of antagonistic bacteria and fungi that when proliferating in the

rhizosphere promoted plant growth and protected host plants from pathogens. In several cases, such isolated strains have been used to fortify composts and improve their consistency to control diseases^{57,58}. Herein, we extend on our previous work by evaluating the antifungal and growth promotion potential of the bacteria enriched in the rhizosphere of tomato plants²⁶, and by designing synthetic communities we aim to understand how the composition of microbial consortia can explain the enhanced growth and protection observed against the fungal wilt pathogens of tomato.

Evaluation of the bacterial collection enriched in the rhizosphere of tomato plants showed that strains from several genera displayed multiple plant-beneficial characteristics. A substantial number of *Bacillus*, *S. maltophilia*, *E. cloacae*, *Ochrobactrum* and *Chryseobacterium* strains inhibited *Vd* and *Fox* growth by producing DSM and/or VOCs (Fig. 1). The ability of *Bacillus* species to inhibit *Vd* and *Fox* growth both *in vitro* and *in vivo* has been extensively reported in the literature^{59–61}. Similarly, antagonistic activity of *E. cloacae*, certain *S. maltophilia* and *Chryseobacterium* strains against *Fox*, *Vd* or both pathogens has been reported in *in vitro*, greenhouse and field trials^{62–67} whereas some strains belonging to *Ochrobactrum* suppressed the incidence of Fusarium wilt in greenhouse experiments^{68,69}. Additionally, a large proportion of the tested isolates could produce IAA or displayed ACC deaminase activity *in vitro* (Fig. 3). Most *Bacillus* strains produced IAA, all *E. cloacae* exhibited ACC deaminase activity whereas strains of *Chryseobacterium*, *Ochrobactrum*, and *S. maltophilia* could produce both IAA and ACC deaminase. The potential of isolates belonging to the aforementioned genera or species to produce or modify hormonal signals is also well-documented^{68,70–72}.

Next, plant-bacterium binary-association experiments allowed us to assess the effect of each strain on plant growth, by using *in vitro*-grown *Arabidopsis*

310 plants^{23,42,43}. A substantial number of *Bacillus* strains increased *Arabidopsis* shoot
 311 fresh weight (Fig. 2a) and repressed primary root length and number of lateral roots
 312 (Fig. 2b, c). As shown in Fig. 3a, Bacilli are IAA producers and it was previously
 313 shown that microbes producing auxin or other molecules with auxin activity, can
 314 promote plant growth and affect root morphogenesis by inhibiting primary root
 315 elongation^{73,74}. Most *E. cloacae* strains increased shoot fresh weight of *Arabidopsis*
 316 (Fig. 2), most likely via their ACC deaminase activity (Fig. 3b) by which bacteria can
 317 lower plant ethylene levels and stimulate plant growth⁷⁵. Finally, certain *S.*
 318 *maltophilia* strains induced shoot growth, root length and number of lateral roots of
 319 *Arabidopsis* plants (Fig. 2) and these phenotypes could be attributed to IAA
 320 production and/or ACC deaminase activity (Fig. 3).

321 Release of various metabolites by the plants can attract beneficial microorganisms
 322 and enrichment of selected microbes in the rhizosphere has been shown to help plants
 323 deal with abiotic and biotic stresses^{76,77}. More specifically, root associations with
 324 PGPR can protect plants from soil-borne pathogens by antagonistic mechanisms
 325 and/or activation of ISR^{51,78}, and can help plants acquire more nutrients and grow
 326 bigger either by the production of phytohormones or the inactivation of environmental
 327 pollutants⁷⁹. In our previous work, we found enrichment of Bacilli strains in the
 328 rhizosphere of tomato plants that displayed both increased growth and enhanced
 329 protection against soil-borne pathogens²⁶. Therefore, we tested here whether
 330 individual Bacilli isolates retain properties that could benefit the plant. Apart from the
 331 antifungal activity and the growth-modulating compounds discussed earlier, we found
 332 that a large part of the *Bacillus* strains produced hydrolytic enzymes and possessed
 333 genes for CLPs biosynthesis (Fig. 4a). Previous studies have shown that hydrolytic
 334 enzymes, such as chitinases, proteases, cellulases, and glucanases, are involved in the

335 antagonistic activity of *Bacillus* sp. and other biological control agents against fungal
 336 pathogens^{14,15,80}. Production of antibiotics, including CLPs, is considered one of the
 337 most convincing properties of *Bacillus* spp. contributing to a broad-spectrum
 338 antimicrobial activity⁸¹. Besides their antimicrobial role, CLPs are also known to act
 339 as biosurfactants affecting the ecological fitness of the producing strains in terms of
 340 root colonization and biofilm formation⁵⁵. Several works have shown that a variety of
 341 CLPs can stimulate host immune responses and trigger ISR in certain host plants^{54,82}.
 342 In our study, the ability of the *Bacillus* isolates to form biofilm was verified *in vitro*
 343 (Fig. 4b). Previously, Chen *et al.*⁸³ suggested a positive correlation between the ability
 344 of *B. subtilis* to form robust biofilm and its biocontrol efficacy, and interestingly the
 345 formation of robust biofilms by *Bacillus* in defined medium was correlated with that
 346 on the plant roots. Another study also demonstrated the responsiveness of Bacilli
 347 towards plant cues, since root-associated biofilms of *B. subtilis* were triggered by
 348 exudates released from tomato roots⁸⁴. Taken together, the enrichment of Bacilli in
 349 the rhizosphere of tomato and the overall ability of the tested *Bacillus* strains to
 350 produce an array of lytic enzymes, possess CLPs biosynthetic genes and form
 351 biofilms may explain the beneficial effects observed on the plants and the suppression
 352 of soil-borne pathogens.

353 The contribution of each microbe to a plant phenotype is a major challenge as
 354 many processes with regard to host fitness are accomplished by microbial synergies.
 355 While the majority of biological control efforts has focused on single organisms in the
 356 past, it is now possible to extend the analysis of plant protection to the full breadth of
 357 microbiota and develop microbiome-based biocontrol strategies⁸⁵. Current research
 358 efforts focus on community rather than single-organism studies since the behavior of
 359 microorganisms in a community is different from that in pure cultures⁸⁶. Studies have

360 shown that single isolates with the ability to suppress fungal wilt pathogens under
361 laboratory experiments often fail to induce disease suppression in the field due to low
362 survival in the soil^{87,88}. Other studies have linked species-richness of communities to
363 higher resistance against pathogen invasions^{89,90}. Therefore, practices that increase the
364 size of the bacterial community may also increase the suppressiveness in soils and/or
365 composts⁸⁹.

366 Here, we constructed two SynComs using a bottom up approach by mixing
367 selected bacterial strains that were exclusively recovered from tomato roots grown in
368 a suppressive compost. The rationale behind these artificial assemblies was to achieve
369 a SynCom composition reflecting the proportion of different Phyla in the culturable
370 microbiome and in a next step to use those strains displaying *in vitro* antifungal
371 properties against *Fox* and *Vd* (Fig. 1). We reasoned that inclusion of the most
372 competitive bacterial isolates in terms of fungal growth inhibition and maintenance of
373 this attribute in a community context, might result in a reduction of fungal wilt
374 disease of tomato. For that, strain representation in SynCom1 was analogous to Phyla
375 representation in tomato rhizosphere (Supplementary Information Table S3), in order
376 to mimic the rhizosphere community features as closely as possible. SynCom2
377 contained only those *Bacillus* isolates that based on the *in vitro* pre-screening assays
378 showed antifungal and plant growth-promoting potential. Application of SynComs on
379 the roots of *Arabidopsis* and tomato plants in microcosm experiments produced
380 differential outputs. SynCom1 was able to promote growth of tomato (Fig. 6) but
381 compromised root length and fresh weight of *Arabidopsis* (Fig. 5). SynCom2 did not
382 affect growth of *Arabidopsis* (Fig. 5) but promoted tomato growth (Fig. 6).
383 Interestingly, SynCom1 that yielded the best growth-promoting effect on tomato, did
384 not protect plants against *Fox*, whereas SynCom2 was able to reduce the severity of

385 *Fusarium* wilt while displaying a moderate impact on plant growth (Figs. 6, 7). Our
 386 results suggest the critical involvement of *Bacillus* bacterial strains in the observed
 387 protection of tomato plants against *Fox*, while the combination of various different
 388 genera is required for the growth promotion of tomato. The phenotypic output of the
 389 *in vitro* experiments with SynComs and *Arabidopsis* plants were not consistent with
 390 the output of pot experiments with tomato plants suggesting that the effects produced
 391 my microbial consortia on host fitness are hard to predict only by *in vitro*
 392 experiments.

393 In conclusion, our results support the notion that growth promotion of plants
 394 and disease suppression are complex phenomena that are probably established by the
 395 activity of sophisticated microbial consortia^{19,91}. The creation of synthetic ecosystems
 396 using microbial mixtures that can influence plant properties such as plant growth and
 397 disease resistance is an emerging research field that can lead to promising solutions
 398 for sustainable agricultural practices⁴³. We propose that microbial synthetic
 399 communities can be used as compost inoculants to produce composts with desired
 400 characteristics e.g. predictive biocontrol of targeted pathogens. In that direction,
 401 further study is needed to understand how synthetic communities persevere in a
 402 compost and in the rhizosphere, what are the additive traits of a community compared
 403 to single strains and which host responses determine the outcome of the interaction.

404

405 **Methods**

406 **Fungal species and bacterial strains**

407 Race 1 isolate Fol004 of *F. oxysporum* f. sp. *lycopersici* (*Fox*)²⁷ and race 1 isolate
 408 70V of *V. dahliae* (*Vd*)²⁸ were used. Studied bacterial strains (Supplementary

Information Table S1) were isolated from the rhizosphere of tomato plants grown in a suppressive compost²⁶. Both fungal and bacterial isolates were cryopreserved as an aqueous 20% glycerol suspension at -80°C. Fungal strains were activated on potato dextrose agar (PDA, Merck) at 25°C, while bacterial strains were grown on tryptic soy agar (TSA, BD) at 25°C.

***In vitro* antifungal activity of bacterial isolates**

The antagonistic activity of the diffusible secondary metabolites (DSM) of the bacterial isolates against *Vd* and *Fox* was determined using the *in vitro* dual-culture assay. PDA medium (20 ml) was poured into sterile Petri dish (90 mm diameter) and a 5 mm mycelial plug, taken from 6-d-old fungal culture, was placed on the center of the plate. Single bacterial colonies were streaked at ~1 cm from the plate's rim and then plates were incubated for 7 d at 25°C. The inhibitory activity of bacterial isolates was expressed as the percent growth inhibition, compared to the control (plates inoculated with fungal plugs alone), according to the formula:

$$\text{Growth inhibition (\%)} = (D_C - D_T) / D_C \times 100$$

where, D_C , diameter of control and D_T , diameter of fungal colony in dual culture

Each bacterial isolate was tested three times.

The effect of bacterial isolates VOCs on *Vd* and *Fox* growth was assessed using the bottoms of two Petri dishes. A 5-mm mycelium plug was added in one dish as above. Each bacterial isolate was inoculated into 25 ml tryptic soy broth (TSB, BD) in a 50 ml falcon tube and incubated for 48h at 25°C under agitation (180 rpm). A 100-μl bacterial culture ($OD_{600}=0.5$) was spread on the second dish, containing 1x

432 Murashige and Skoog medium (MS, Sigma-Aldrich) with 1.5% sucrose, 0.4% TSB
433 and 1.5% agar²⁹. The dish with the bacterial culture was inverted over the dish with
434 the fungal mycelium plug, allowing physical separation between them and both dishes
435 were sealed with Parafilm and incubated at 25°C. Petri dishes containing *Vd* or *Fox*
436 exposed to a Petri dish containing only bacterial medium served as controls. Fungal
437 growth inhibition was calculated as described above and each bacterial isolate was
438 tested 4 times.

439

440 ***In vitro* plant growth assays**

441 *Arabidopsis thaliana* Col-0 accession (hereafter *Arabidopsis*) was used in these
442 assays. Seeds were surface sterilized³⁰ and sown on 1x MS agar medium with 0.5%
443 sucrose in square plates (120 x 120 x 17 mm). After 2 d of stratification at 4°C, plates
444 were transferred in growth chamber (22°C; 16h light, 8h dark; light intensity 100
445 $\mu\text{mol m}^{-2} \text{s}^{-1}$) and positioned vertically. Uniform 5-d-old seedlings were transferred to
446 new square plates containing 1x MS agar with 0.5% sucrose. Each bacterial isolate
447 was cultured on TSB and after 24–48 h of growth, cells were centrifuged at 2,600 *g*
448 for 7 min at 4°C, washed twice in 10 mM MgSO_4 and finally resuspended in 10 mM
449 MgSO_4 . Ten μl of bacterial suspensions (final $\text{OD}_{600}=0.002$) were spotted just below
450 the root tip of each plant 2 d after transferring the seedlings to new plates. After 8 d of
451 co-cultivation, shoot fresh weight, primary root length and number of lateral roots
452 were measured as previously described³¹.

453

454 **Indole acetic acid (IAA) quantification assay**

455 Production of IAA by the bacterial strains was measured in culture supernatants using
456 Salkowski's reagent³². Absorbance was measured at 535 nm with a Tecan Infinite
457 M200 Pro plate reader (Tecan, Männedorf, Switzerland). The concentration of IAA
458 was determined for each sample after comparison with a standard curve ranging
459 between 5 and 50 $\mu\text{g ml}^{-1}$.

460

461 **1-aminocyclopropane-1-carboxylate (ACC) deaminase activity**

462 Each bacterial isolate was grown from mid-up to late-log phase in TSB in a shaking
463 incubator (200 rpm, 25°C) and ACC deaminase activity was assessed by determining
464 α -ketobutyrate produced from ACC cleavage by ACC deaminase and comparing the
465 absorbance at 535 nm to a standard curve of α -ketobutyrate ranging between 0.01 and
466 0.1 μmol^{10} .

467

468 **Quantification of biofilm formation**

469 Biofilm quantification was performed in 96-well plates as described before³³ by
470 staining biofilms with 0.1% (w/v) crystal violet (CV). CV concentration was
471 determined by measuring the OD at 595 nm in a microtiter plate reader (Tecan). The
472 samples were assayed in quadruplicate.

473

474 **Phosphate solubilization**

475 Qualitative screening of phosphate-solubilizing bacterial isolates was performed on
476 Pikovskaya agar medium medium containing 0.5% (w/v) $\text{Ca}_3(\text{PO}_4)_2$. Bacterial isolates

were streaked into Pikovskaya's agar and phosphate solubilization was determined by the appearance of a clear halo around the colonies after 3 d of incubation at 28°C³⁴.

Determination of hydrolytic enzymes

Production of protease, chitinase, cellulose, and pectinase was detected on agar plates containing skim milk, colloidal chitin³⁵, carboxy-methylcellulose and pectin, respectively^{34,36,37}. Bacterial strains were inoculated on the respective agar plates and incubated at 28°C for 3–5 d. Production of clearing zone around the colonies indicated enzyme production. Zones of pectinase and cellulose were observed after staining with fresh Gram's iodine solution^{38,39}.

Detection of cyclic lipopeptides (CLPs) genes

Total genomic DNA was extracted from bacterial isolates as described previously²⁶. Primers used for the amplification of CLP biosynthesis genes^{40,41} are listed in Supplementary Information Table S2. PCR amplifications were carried out using Kapa Taq (Kapa biosystems) in a C100™ Thermal Cycler (Bio-Rad, Hercules, CA, USA) according to manufacturer's recommendations (Supplementary Information Table S2).

Synthetic community experiments

Design of the synthetic communities

Two simplified (partially overlapping) synthetic communities (SynComs) of bacteria were designed (Supplementary Information Table S3) using representative rhizospheric bacteria isolated previously²⁶. SynCom1 consisted of 25 isolates, selected to reflect the composition of the bacterial community, isolated from the rhizosphere of tomato plants grown in a suppressive compost (Supplementary Information Table S3). SynCom2 composed of 25 *Bacillus* isolates (from the same bacterial collection) was designed in order to test if the increased representation of the Bacilli compared to other genera in the rhizosphere of tomato plants (33%, Supplementary Information Table S1) can explain by itself the beneficial effects on plant fitness.

508

509 **Preparation of SynComs and treatments**

Each bacterial isolate was grown for 48 h in TSB (180 rpm, 25°C). Cultures were rinsed with a 10 mM MgCl₂ sterile solution and then centrifuged (2,600 g, 8 min). This process was repeated twice and cells were resuspended in 10 mM MgCl₂ solution. The OD₆₀₀ of each suspension was adjusted to 0.5 (~2.75 x 10⁸ cfu ml⁻¹). SynComs were obtained by mixing the isolates in equal ratios. For the *Arabidopsis* experiments, each SynCom suspension was diluted to a final concentration of 10⁵ cfu ml⁻¹ ^{42,43}. *Arabidopsis* seedlings were inoculated by applying 10 µl of a bacterial suspension to the primary root of each seedling, immediately below the hypocotyl⁴⁴. For SynComs tomato pot experiments, 2 ml of each SynCom suspension (OD₆₀₀=0.5) was added to 50 ml sterile 10mM MgCl₂ and mixed with 200 gr sterilized potting substrate (~2.75 x 10⁶ cells per gr substrate) directly before the sowing of surface sterilized tomato seeds.

522

523 **Plant growth conditions**

524 *Arabidopsis* seeds were grown as described above. Uniform 6-d-old seedlings were
525 transferred on 1x MS agar plates without sucrose, inoculated with a SynCom or
526 MgCl_2 (control) and were further grown for 20 d in a growth chamber (22°C; 16h
527 light, 8h dark; light intensity $100 \mu\text{mol m}^{-2} \text{s}^{-1}$).

528 Tomato (*Solanum lycopersicum*) cv. Ailsa Craig seeds were surface-
529 sterilized⁴⁵ and sown into 10.5 cm diameter pots, each containing 200 gr sterilized
530 peat-based potting substrate (Plantobalt substrate 2, Plantaflor)²⁶, alone or with a
531 SynCom. Plants were grown in a controlled growth chamber (25°C; 16h light, 8h
532 dark; 65–70% RH; light intensity $450 \mu\text{mol m}^{-2} \text{s}^{-1}$ at pot height) and received
533 watering individually every second day with equal volume of water.

534

535 **Experiments for the determination of plant morphological parameters**

536 In the *Arabidopsis*-SynComs experiments, measurements of shoot and root fresh
537 weight and primary root length were performed after 20 d of co-cultivation with the
538 SynComs. For root hair length measurements, 5 individual roots per treatment were
539 obtained and in each root the length of 27–34 root hairs was quantified by ImageJ
540 (<https://imagej.nih.gov/ij/>). Totally, 307 root hairs were measured per treatment. Root
541 hair density was determined as the average root hair number in a 1 mm^2 root segment
542 located 1 cm above the root tip. Root hair length and density were estimated using
543 photographs taken by a binocular microscope (Olympus SZX16) with an attached
544 digital camera (ColorView II, Olympus Soft Imaging System GmbH).

545 In the tomato-SynComs experiments, leaf area, plant height and number of
546 leaves were determined 14 d after seedling emergence. For leaf area measurements, 5
547 plants were grown in sterile potting substrate alone or with a SynCom as described
548 earlier, individual leaves were dissected and their area was measured with ImageJ
549 software.

550

551 **Pathology studies**

552 Pathogenicity experiments were performed on tomato plants at the four-leaf stage.
553 Plants were grown in sterile potting substrate alone or with a SynCom as described
554 above. A 5-d-old *Fox* liquid culture grown in sucrose sodium nitrate (SSN)⁴⁶ in an
555 orbital shaker (140 rpm) at 25°C, was passed through cheesecloth to remove mycelia.
556 Conidial concentration was adjusted to 10⁷ conidia ml⁻¹ and each seedling was
557 inoculated with 10-ml suspension via root drenching. Control plants were mock-
558 inoculated with 10 ml sterile distilled water. Disease severity was calculated by the
559 number of leaves showing typical symptoms (wilting, yellowing, necrosis, stem
560 collapse) as a percentage of the total number of leaves of each plant. Symptoms were
561 periodically recorded for 22 d post inoculation (dpi) with the pathogen. Disease
562 ratings were plotted over time to generate disease progress curves. The area under the
563 disease progress curve (AUDPC) was calculated by the trapezoidal integration
564 method⁴⁷.

565

566 **Statistical analysis**

567 Data were analyzed by one-way ANOVA (Tukey's test with $P \leq 0.05$ or Dunnett's
568 test with $*P < 0.05$, $**P \leq 0.01$, $***P \leq 0.001$, $****P \leq 0.0001$), or two-way

569 ANOVA (Sidak's test with $P \leq 0.05$) using GraphPad Prism 7 for Windows
570 (GraphPad Software, La Jolla, CA, United States).

571

572 **Data Availability**

573 All data generated or analysed during this study are included in this published article
574 (and its Supplementary Information files).

575

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830

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835 **Author Contributions**

836 IAS and ISP conceived and designed the research. MDT, NFS, SP, AT and ISP
837 performed experiments. MDT, IAS and ISP analyzed and interpreted the data. IAS
838 and ISP wrote the manuscript. MDT and IAS contributed equally. All authors have
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840

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845

846 **Figure Legends**

847 **Figure 1: Antifungal potential of the rhizospheric microbial isolates.** Heat maps
848 illustrating the antifungal activity of (a) diffusible secondary metabolites and (b)
849 volatile organic compounds (VOCs) of the studied bacterial isolates against the fungal
850 pathogens *Verticillium dahliae* (*Vd*) and *Fusarium oxysporum* f. sp. *lycopersici* (*Fox*).
851 Color of each box corresponds to the mean inhibition percentage recorded for each
852 bacterial isolate as calculated from three and four replicate measurements, for
853 diffusible secondary metabolites and VOCs respectively. Color scale ranges from
854 green to red, with green, black and red representing negative values (promotion),
855 baseline value (no effect) and negative values (inhibition), respectively.
856 Measurements of antifungal activity were made 7 d after challenging *Vd* and *Fox* with
857 the diffusible secondary metabolites or VOCs produced by the bacterial isolates.

858

859 **Figure 2: Effects of bacterial isolates on plant growth and root system**
860 **architecture of *Arabidopsis* seedlings.** Boxplots showing variation around the
861 median for (a) shoot biomass production, (b) primary root length and (c) number of
862 lateral roots, measured after 8 d of co-cultivation with each bacterial isolate ($n = 21$
863 plants). In each graph, grey box corresponds to control, red boxes to isolates with
864 significant negative effect compared to control and green boxes to isolates displaying
865 significant positive effect compared to control. White boxes represent strains with
866 effect similar to control. Asterisks indicate statistically significant differences from
867 control plants: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$, one-
868 way ANOVA, Dunnett's test.

869

870 **Figure 3: Production of IAA and cleavage of ethylene precursor ACC by**
 871 **bacterial isolates.** Indole acetic acid (IAA) (a) and ACC deaminase activity (b) by
 872 bacterial isolates. Bars show the mean from three replicates of each isolate and are
 873 colored based on genus classification of the isolates. Error bars represent SE.
 874 Different letters indicate significant differences between the isolates ($P < 0.05$, one-
 875 way ANOVA, Tukey's test).

876

877 **Figure 4: Enzymatic potential and biofilm formation of selected bacterial**
 878 **isolates.** (a) Results of the different enzymatic activity assays and of the PCR
 879 targeting genes responsible for the production of cyclic lipopeptides in isolates of the
 880 genus *Bacillus*. Grey color indicates the presence and white color indicates the
 881 absence of the phenotype or gene in a bacterial isolate. TCP, Tricalcium phosphate
 882 [$\text{Ca}_3(\text{PO}_4)_2$]; *srfAA*, surfactin synthetase subunit 1; *srfAB*, surfactin synthetase subunit
 883 2; *fend*, fengycin synthetase; *bmyB*, bacillomycin synthetase; *ituC*, iturin A synthetase
 884 C; *bacA*, bacilysin biosynthesis protein; *spaS*, lantibiotic subtilin. (b) Boxplot of
 885 biofilm formation by each *Bacillus* isolate. Cells were cultured for 24 h in 96-well
 886 plates and the biofilm was quantified by crystal violet staining. Black dots represent
 887 the values of the 4 replicates per treatment. Asterisks indicate statistically significant
 888 differences from blank control: $*P < 0.05$, $**P < 0.01$, $***P < 0.001$,
 889 $****P < 0.0001$, one-way ANOVA, Dunnet's test.

890

891 **Figure 5: Effects of synthetic communities (SynComs) on *Arabidopsis* plant**
 892 **growth and root system architecture.** Effect of bacterial synthetic communities on

893 (a) shoot weight, (b) primary root length and (c) root weight of *Arabidopsis* plants (n
894 = 21). Asterisks indicate statistically significant differences between treatments:
895 *** $P \leq 0.001$, **** $P \leq 0.0001$, ns, not significant, one-way ANOVA, Tukey's
896 test. (d) Representative pictures of seedlings growing in control plates and plates with
897 SynCom1 and SynCom2. Pictures were taken 20 d post co-cultivation.

898

899 **Figure 6: Beneficial effects of synthetic communities (SynComs) on tomato**
900 **growth.** SynComs-mediated effect on (a) leaf area (in cm²), (b) plant height (in cm)
901 and (c) number of leaves. Data are means of 5 biological replicates and error bars
902 indicate SE of mean. Asterisks indicate statistically significant differences: * $P < 0.05$,
903 ** $P \leq 0.01$, *** $P \leq 0.001$, **** $P \leq 0.0001$, one-way ANOVA, Tukey's test.
904 (d) Representative pictures of tomato plants taken at 14 d after seedling emergence.

905

906 **Figure 7: SynCom2 has a protective effect on tomato plants against *F. oxysporum***
907 **f. sp. *lycopersici*.** (a) Disease progress over time and (b) amount of disease expressed
908 as relative AUDPC. Error bars indicate the SE of mean ($n = 8$ replicates). Asterisks
909 indicate statistically significant differences: * $P < 0.05$; ns, not significant, one-way
910 ANOVA, Tukey's test. (c) Fusarium wilt symptoms on tomato plants grown in sterile
911 peat (left), sterile peat inoculated with SynCom1 (middle) and with SynCom2 (right),
912 at 22 d post inoculation.

**Inhibition of fungal growth
by bacterial metabolites**

**Effects of bacterial VOCs
on fungal growth**

Bacterial Isolates

a

b

a **b** **c** **d**

a **b** **c** **d**