1 Metatranscriptomics and nitrogen fixation from the rhizoplane of maize plantlets
2 inoculated with a group of PGPRs

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23

24 ABSTRACT

25 The free-living soil bacteria that are beneficial for the growth of plants are known as
 26 plant growth-promoting rhizobacteria (PGPR). In this work, a multi-species of PGPR
 27 bacteria inoculant was designed, which included nitrogen-fixing strains such as
 28 *Rhizobium phaseoli*, *Sinorhizobium americanum* and *Azospirillum brasilense*, as well as
 29 other plant growth promoting bacteria such as *Bacillus subtilis* and *Methylobacterium*
 30 *extorquens*. The multi-species community exerted a beneficial effect on plant seedlings
 31 when it was inoculated, greater than the effect observed when inoculating each bacteria
 32 individually. Acetylene reduction of maize roots was recorded with the multi-species
 33 inoculant, which suggests that nitrogen fixation occurred under these conditions. To
 34 analyze the contributions of the different nitrogen-fixing bacteria that were inoculated, a
 35 metatranscriptomic analysis was performed. The differential expression analysis
 36 revealed that the predominantly *nif* transcripts of *Azospirillum* are overexpressed,
 37 suggesting that it was responsible for nitrogen fixation in maize. Overall, we analyzed
 38 the interaction of a synthetic community, suggesting it as an option, for future
 39 formulations of biofertilizers.

40 IMPORTANCE

41 While nodulation processes and nitrogen fixation by rhizobia have been well studied,
 42 little is known about the interaction between rhizobia and non-leguminous plants such
 43 as maize, which is used as a model for this study. Nitrogen fixation in cereals is a long
 44 searched goal. Instead of single species inoculants, multi-species inoculation may be
 45 more efficient to promote plant growth and fix nitrogen. Metatranscriptomes allowed us
 46 to recognize the bacteria responsible for nitrogen fixation in plant rootlets. The study of
 47 the function of certain genes may help to understand how microorganisms interact with

the root plant, as well as allow a better use of microorganisms for the generation of novel biofertilizers using microbial consortia.

INTRODUCTION

In nature and in agricultural fields there is a large diversity of bacteria associated with plants. Once isolated in culture media and subsequently tested individually in plant assays, many plant-bacteria prove to be capable of promoting plant growth. Bacterial mechanisms of action are diverse, and some bacteria may exhibit more than one mechanism. Plant growth promoting rhizobacteria (PGPR) may produce phytohormones or volatiles, solubilize nutrients, fix nitrogen or inhibit pests/pathogens. Among them, nitrogen-fixing bacteria are in general a minority, possibly due to the metabolic charge of fixing nitrogen.

As bacteria are not alone in soil and in plants, recent inoculation assays have considered the use of more than single strains, and combinations of *Azospirillum* and rhizobia or *Bacillus* and rhizobia have been more successful than single-strain inoculants in promoting plant growth (reviewd in 1–3). For example, *Azospirillum* and *Bradyrhizobium* inoculation enhances nodulation and nitrogen fixation in legumes such as soybean (4) and, in chickpea a combined inoculation of *Mesorhizobium*, *Pseudomonas* and the *Piriformospora indica* fungi increased 31% plant dry weight. *Azospirillum* coinoculation with rhizobacteria may alleviate plant stress (5).

Multispecies inoculum bacteria

Azospirillum

Among the PGPRs, *Azospirillum* deserves special attention. It was one of the first diazotrophs isolated from non-legumes (6, 7) but its role for fixing nitrogen was critically dismissed. A revision of plant growth promotion mechanisms in *Azospirillum* has been reported (8). A global analysis of 30 years of *Azospirillum* trials in agricultural

73 crops including maize showed and average 10-30% increase in yield when inoculated
74 (9). In a controlled assay under hydric stress, *Azospirillum* in maize plants increased
75 10% the number of sprouts and 7% of plant height (10). *Azospirillum* is widely used as
76 a commercial product. Auxin production by *Azospirillum* is considered the main
77 mechanism to increase plant growth (11, 12), and three different enzymatic pathways to
78 produce auxins have been described in *Azospirillum* (7), mutants that overproduce
79 auxins have enhanced effects (13).

80 ***Bacillus***

81 *Bacillus* strains may produce auxins (14) as well as many antibiotics, and has been
82 reported to enhance plant growth (15–17). A large diversity of *Bacillus* were found in
83 roots of *Phaseolus vulgaris*, derived from seeds (18). As *P. vulgaris* and maize are
84 grown in associated crops (19), we surmise that they share their *Bacillus* symbionts and
85 other root. *Methylobacterium* are found in many plants in roots, leaves and stems (20),
86 and are capable to produce cytokinins and metabolize methanol that is produced as a
87 side product during plant wall synthesis. It promotes growth in plants and mosses (21).

88 ***Rhizobia***

89 Rhizobia are the best studied plant symbionts which have been used in agriculture for
90 over 120 years. Rhizobia are used in commercial inoculants for different crops,
91 especially soybean. Rhizobia form nodules and fix nitrogen. In nodules, rhizobia may
92 fix over 250 kg of N per ha per year equivalent to a heavy chemical fertilization of
93 crops. Additionally, rhizobia may colonize the rhizospheres of many plants and the
94 inside tissues of non-legumes. Rhizobia may exert beneficial effects in non-legume
95 plants similar to PGPRs. In *Sinorhizobium*, *nif* genes that are needed for free living
96 nitrogen fixation were found (22) and thus we considered it as a good candidate for
97 nitrogen fixation in non- legumes.

98 Nitrogen fixation in cereals has been long pursued since cereals such as rice, maize and
 99 wheat became widely consumed worldwide (23), considering they use the largest
 100 amounts of chemical nitrogen fertilizers which represent a substantial cost for farmers,
 101 not to mention the high toll these fertilizers take on the environment. Maize originated
 102 and was domesticated in Mexico where many native races and varieties exist (24). Here
 103 we used a native Mexican race for the multispecies inoculation experiment.

104 A differential gene regulation of bacteria occurs in plants and has been reported in
 105 *Bacillus* (25), *Azospirillum* (26), *Sinorhizobium* (27), and *Rhizobium* (28) in single
 106 bacterium inoculation assays. *Bacillus* in maize exudates has increased expression of
 107 genes for nutrient utilization, synthesis of antimicrobial peptides and of chemotaxis and
 108 motility (29). *Azospirillum brasilense* showed upregulation of genes involved in biofilm
 109 formation, chemotaxis and nitrogen fixation in roots of wheat (26). In rice roots,
 110 *Azospirillum lipoferum* expressed genes related ROS detoxification and multidrug
 111 efflux pumps but *nif* genes were not induced (30). An antioxidant response has also
 112 been described in *Methylobacterium* when exposed to soybean root exudates (31). In
 113 rhizobia genes expressed in non-legume roots are involved in the transport of root
 114 derived nutrients and in legumes defense to plant phytoalexins (32). Furthermore,
 115 bacterial gene expression may be altered in presence of other root bacteria. Here we
 116 analyzed the metatranscriptome of a synthetic bacterial community in the roots of a
 117 native maize landrace. Considering that there were three nitrogen fixing bacteria in the
 118 inoculant it was of interest to determine which, if any would fix nitrogen in maize.

119

120 **MATERIALS AND METHODS**

121 **Bacterial strains and cultures**

122 *Rhizobium phaseoli* Ch24-10 (33), *Azospirillum brasilense* sp.7 (6), *Bacillus subtilis*
123 CCGE2031 (18), *Methylobacterium extorquens* am1 (34) and *Sinorhizobium*
124 *americanum* CFNEI156 (35), were grown in PY medium at 30°C for 12 h. Cells were
125 harvested by centrifugation at 6000 rpm for 15 min, resuspended in MgSO₄ 10mM and
126 adjusted to a final concentration of 10⁸ cfu ml⁻¹.

127 **Inhibition of growth between multi-species inoculum**

128 To verify that the chosen community could grow together and there was no inhibition of
129 growth among them we did antibiosis tests. Individual strains were grown in liquid PY
130 medium at 30°C for 12 h. Cells were harvested by centrifugation at 6000 rpm for
131 15 min, resuspended in MgSO₄ 10mM and adjusted to a final concentration of
132 10⁸ cfu ml⁻¹. Harvested cells from each strain were then dripped on solid PY medium at
133 30°C and monitored for 8 days. The treatment was done by biological triplicate and a
134 qualitative result was obtained.

135 **Cultivation in PY medium of the multi-species inoculum for transcriptome**

136 Cultures were made by inoculating 30 ml of PY medium with *Rhizobium phaseoli*
137 Ch24-10, *Azospirillum brasilense* sp7, *Methylobacterium extorquens* AM1, *Bacillus*
138 *subtilis* CCGE2031 and *Sinorhizobium americanum* CFNEI 156, as well as *R. phaseoli*
139 Ch24-10 alone were inoculated only at a final bacterial concentration of 10⁸, treatments
140 were performed in triplicate. After 24 hours, 3 ml of RNAlater (Ambion) were added,
141 the samples were centrifuged for 15 minutes at 6000 rpm at 4 ° C, a pellet was obtained
142 from the cultures and RNA was extracted (Fig. 1).

143 From the pellet obtained from the PY medium, RNA extraction was performed through
144 the Qiagen protocol. The RNA obtained was quantified by Nanodrop 2000 (Thermo
145 Scientific). The integrity of the total RNA was verified by means of 1% agarose gels
146 and in Bioanalyzer 2100 (Agilent Technologies).

147 **Germination, inoculation and growth of seedlings**

148 Black Criollo maize seeds were surface disinfected with 70% ethanol (1 min), 30%
 149 sodium hypochlorite (20 min), 2% thiosulfate (2 min) and rinsed thoroughly with sterile
 150 water. Disinfected seeds were germinated in agar-water plates in the dark for 24 h at
 151 30°C, and subsequently transplanted into flasks with sterile vermiculite. Plants were
 152 irrigated with nitrogen free Fahraeus solution (0.132 g/L CaCl₂, 0.12 g/L MgSO₄·7H₂O,
 153 0.1 g/L KH₂PO₄, 0.075 g/L Na₂HPO₄·2H₂O, 5 mg/L Fe-citrate, and 0.07 mg/L each of
 154 MnCl₂·4H₂O, CuSO₄·5H₂O, ZnCl₂, H₃BO₃, and Na₂MoO₄·2H₂O, adjusted to pH 7.5
 155 before autoclaving (36), and inoculated with 10⁸ cfu ml⁻¹ of each bacteria; single
 156 inoculants and plants without inoculants were used as controls. Plants were maintained
 157 for 30 days at 28 °C with a 12 h dark/light period. At 30 days after inoculation, growth
 158 of the aerial part, fresh and dry weight and chlorophyll content were measured.

159 For RNA extraction, *in vitro* plant cultures were carried out under hydroponic and
 160 axenic conditions. Seedlings from disinfected seeds were transferred to glass tubes (one
 161 seedling in each tube) containing 10 mL of Fahraeus medium. Plants were inoculated
 162 with 10⁸ bacteria, and single inoculants and plants without inoculants were used as
 163 controls. Plants were maintained for 5 days at 28°C with a 12 h dark light/period. The
 164 data obtained from each of the treatments were analyzed with an analysis of variance
 165 (ANOVA) and the comparison test of means Tukey (p = 0.05) (Fig. 1).

166 **RNA extraction, RNA-seq library construction and sequencing**

167 Root-attached bacteria were recovered from hydroponic cultures 5 days after
 168 inoculation by sonicating roots for 15 min from 50 seedlings in 5 mL of RNAlater
 169 stabilization solution (Ambion), followed by centrifugation (6000 rpm; 15 min; 4°C).
 170 The cell pellet was resuspended in RNAlater solution. Total RNA was extracted using a
 171 Qiagen kit and treated with DNase (Qiagen) following the manufacturer instructions.

172 Sequencing was done at the Genomics and Bioinformatics Service Texas A&M
 173 AgriLife. rRNA was depleted using the Ribo-Zero Bacteria Protocol (Illumina).
 174 Sequencing libraries were prepared using the protocol TruSeq Stranded (Illumina) and
 175 sequenced with Illumina HiSeq 2500 (2x125).
 176 Lastly, twelve libraries were obtained three from rhizobium alone and three from
 177 rhizobium with the community in PY and three from rhizobium alone and three from
 178 rhizobium with the community in plant. The sequencing data are available in the Texas
 179 A&M AgriLife website.
 180 ([https://download.txgen.tamu.edu/Mateos/150804_D00572_0126_AC79VYANXX_150](https://download.txgen.tamu.edu/Mateos/150804_D00572_0126_AC79VYANXX_15035Mts/)
 181 [35Mts/](https://download.txgen.tamu.edu/Mateos/150804_D00572_0126_AC79VYANXX_15035Mts/)).

182 **Data analyses**

183 Raw sequencing data were scanned for adapters and quality assessed using FASTQC
 184 (v0.11.2) (37). Sequencing adapters and low quality sequences were removed using
 185 Trimmomatic (v0.38) (38) with the parameters ILLUMINACLIP:Adapter.fasta:2:30:7
 186 SLIDINGWINDOW:4:15 MINLEN:60. Paired-end sequencing reads were then
 187 mapped to concatenated genome sequence that contains all the members of the
 188 community using Bowtie2 (v2.1.0) (39), with the following parameters --sensitive --rdg
 189 1000,1000 --rfg 1000,1000 1 -k 20. Gene abundance was quantified using
 190 featureCounts from the Subread software (v1.6.2) (40) using the following parameters -
 191 T 8 -p -a -t CDS -g ID -o ./ . Genes that did not have at least 1 cpm in at least one sample
 192 were removed from further analysis (Supplementary 2).

193 The R package edgeR (v3.22.3)(41), was used to test for differential gene expression,
 194 considering a gene as differentially expressed at FDR < 0.05. Functional annotation was
 195 performed using Trinotate (v3.0.1), and GO term enrichment was performed using the
 196 topGO R package (v2.32.0) (42), using a Fisher classic test and setting an enrichment

197 threshold of p-value < 0.05 (Supplementary 2).

198 RESULTS

199 The multi-species inoculum improved growth of maize

200 Before designing the multispecies inoculum, we evaluated whether the microbial
201 consortium was able to grow together and was cultivated in solid PY medium,
202 observing that there was no inhibition between any of the chosen bacteria (data not
203 shown).

204 A model of plant-bacterial interaction was set up under axenic conditions. Maize
205 seedlings were incubated at 28°C for 5 days with a light cycle of 12 hours and then
206 inoculated with bacterial suspension containing 10^8 of the inoculum multi-species, other
207 treatment with only strain as inoculum and controls were non-inoculated maize
208 seedlings.

209 Shoot weight was higher in plants inoculated with the multispecies group in comparison
210 to non-inoculated plants or plants inoculated with single bacterial species (Fig. 2), with
211 increases up to over 100%. Chlorophyll content increased 60%, as well as in plants with
212 the multispecies inoculum in comparison to non-inoculated plants or plants inoculated
213 with single bacterial species (Fig. 2).

214 RNA-seq profiling of *Rhizobium phaseoli* Ch24-10 interacting with maize seedlings

215 We used the FastQC tool to visualize the quality of the sequences, and in general the
216 samples were of good quality. The RNA-Seq generated approximately 40 million reads
217 on average for each sample, of which 98% were confirmed to be valid after filtering
218 reads using Trimmomatic (supplementary 1).

219 For gene differential expression analyses, reads were first mapped to the *R. phaseoli*
220 genome or the concatenated sequence of the genomes of the members of the community
221 using Bowtie2, with the featureCounts software, we counted the reads mapped in the

different contigs and obtained the matrix of accounts that was used for the differential expression. The Spearman correlation coefficient of the transcriptome data for the inoculated libraries of *R. phaseoli* alone and *R. phaseoli* with community was 0.95 on average, indicating that the biological and technical replicas had a high reproducibility.

Genes involved with nitrogen fixation are activated when *Rhizobium* interacts with the corn plant

Our analyses revealed 730 up-regulated (fold-change higher than 1 and $FDR \leq 0.05$) and 376 down-regulated genes when comparing samples of *R. phaseoli* Ch24-10 grown in PY medium against *R. phaseoli* Ch24-10 grown in plants. Within the up-regulated genes, there are genes involved with transport, and encode ABC transporter permease, urea ABC transporter ATP-binding protein UrtD and cation transporter.

Genes involved with cellular respiration were also found as up-regulated in *R. phaseoli*. They encode T cbb3-type cytochrome c oxidase subunit FixP, cbb3-type cytochrome oxidase assembly protein CcoS, cytochrome b, cytochrome c, cytochrome o ubiquinol oxidase subunit I, cytochrome o ubiquinol oxidase subunit IV, cytochrome-c oxidase, cbb3-type subunit II, and cytochrome P450.

Many of the genes that were up-regulated in this condition are associated with nitrogen fixation. They encode, for example, nitrogen fixation protein NifX, nitrogen fixation protein NifZ, nitrogenase, nitrogenase cofactor biosynthesis protein NifB, nitrogenase iron-molybdenum cofactor biosynthesis protein NifN, nitrogenase stabilizing/protective protein NifW were found to be upregulated when *R. phaseoli* was grown in seedlings (Supplementary 3).

Within the genes that were found down-regulated were genes involved with chemotaxis and encode chemotaxis proteins CheR and CheW as well as chemotaxis response regulator protein-glutamate methylesterase; and genes involved in flagellar synthesis

247 processes, such as those encoding flagellar basal body protein FliL, flagellar basal body
248 rod protein FlgC, flagellar basal body rod protein FlgG, flagellar hook-basal body
249 complex protein FliE, flagellar motor switch protein FliN, flagellin, flagellin C protein,
250 flagellin synthesis regulator protein and flagellum biosynthesis repressor protein FliB
251 (Supplementary 3).

252 When comparing *R. phaseoli* Ch24-10 in plant against *R. phaseoli* Ch24-10 that was
253 inoculated with the bacterial community in plant seedlings, we found 110 up-regulated
254 and 53 down-regulated genes. Encompassed among up regulated genes were genes
255 involved with transport, encoding ABC transporter ATP-binding protein, ABC
256 transporter permease, ABC transporter substrate-binding protein, MFS transporter,
257 peptide ABC transporter, sugar ABC transporter ATP-binding protein, and many
258 hypothetical genes (Supplementary 3).

259 Among the downregulated genes were genes involved with respiration such as those
260 encoding cytochrome P450 and electron transfer flavoprotein subunit alpha/FixB family
261 protein, as well as fixing nitrogen proteins such as nitrogen fixation protein, nitrogen
262 fixation protein NifZ, nitrogenase cofactor biosynthesis protein NifB and nitrogenase
263 iron-molybdenum cofactor biosynthesis protein NifN (Supplementary 4).

264 Notably when *Rhizobium* is the only bacterial inoculant its machinery for nitrogen
265 fixation is expressed. However, when *Rhizobium* is inoculated along the community,
266 this machinery is repressed, and the role is overtaken by other members of the
267 community. The results imply that *Azospirillum* overtakes the nitrogen fixation
268 function, observed mainly in the overexpression of *Azospirillum* genes involved in said
269 function in both PY medium or in plants (Fig. 3).

270 This differentially expressed genes enriched with the topGO tool and represented with
271 REVIGO are shown in the Figure 4 and 5.

272

273 **DISCUSSION**

274 The functional synergism that may lead to growth promotion by the tested community
275 in roots may reflect interactions resulting from a long evolutionary history of bacteria
276 which might have been selected as advantageous for plants in roots. Metatranscriptomic
277 analyses seem justified to try to understand the bacterial interactions on roots, and for
278 simplicity we designed an inoculum with few species to be used.

279 We found that the plants with the multi-species inoculum were statistically larger in
280 comparison with the non-inoculated control and with the plants inoculated with a single
281 organism or with non-inoculated controls. These results suggest that there was
282 synergism between the strains inoculated in corn, which favors the biomass gained by
283 the plant with the multispecies inoculum. In the single plant assays with rhizobia, our
284 results confirm previous experiments with rhizobia in non-legumes that show growth
285 promotion in plants. (43, 44).

286 Though it is known that various microorganisms favor the growth of crops, less is
287 known on the cooperative interactions among them to produce additional effects on
288 plant growth (2, 5, 15, 45, 46).

289 From the metatranscriptomic analysis, we found genes encoding transporters showing
290 differential expression under distinct experimental conditions. Transporters belonging to
291 the ATP-binding cassette (or ABC) superfamily couple the energy released from ATP
292 hydrolysis to the translocation of a wide variety of substances into or out of cells
293 (47). We found up regulated genes in *R. phaseoli* in plant encoding ABC transporter
294 for putrescine, which is a polyamine involved in response to osmotic stress. Osmolarity
295 is expected to be high in the rhizoplane. Rapid bacterial adaptation to the increase in
296 osmolarity would facilitate rhizosphere colonization (48)) and allows the adaptation of

297 bacteria to hyperosmolar environments. Accordingly, this transporter has been reported
298 under hyperosmolar conditions in several bacteria such as *Azospirillum* sp. B510,
299 *Burkholderia phytofirmans* PsJN, *Methylobacterium populi* BJ001, *Pseudomonas*
300 *putida* W619 when they are associated with different plants (49).

301 Receptor signaling in bacterial chemotaxis occurs within stable complexes between
302 membrane receptors and the CheA kinase, CheB, CheR and CheY kinases. This family
303 of methyl-accepting chemotaxis proteins (MCPs) seem to be important elements in the
304 chemotaxis process (50) When comparing *R. phaseoli* grown in PY medium to *R.*
305 *phaseoli* Ch24-10 grown in plants after 5 days some down-regulated genes were those
306 involved with chemotaxis, such as those encoding chemotaxis protein CheR,
307 chemotaxis protein CheW. Once on plant roots, rhizobia may no longer need
308 chemotaxis to other sources of nutrients. Additional down-regulated genes were those
309 encoding flagellar synthesis. It is known that molecules of the bacterial envelope such
310 as flagellar proteins, exopolysaccharides, adhesins and lipopolysaccharides are the first
311 structures that contact the host and may be required for bacterial attachment on root
312 surfaces at early times (51).

313 **Nitrogen fixation and bacterial adaptation to microoxic environment**

314 Nitrogen fixation is a key service in plant ecology but an energetically expensive
315 process. Thus, diazotrophs are normally a minority among bacterial communities in
316 plants (52, 53), and nitrogen fixation is a tightly regulated process which is turned off
317 when fixed nitrogen is available. Therefore, we used a nitrogen free medium to grow
318 maize plants to allow bacterial nitrogen fixing genes to be fully expressed. To promote
319 nitrogen fixation, we included three different diazotrophs in the bacterial maize
320 inoculant.

321 In previous studies, when *nif* gene expression was analyzed to identify diazotrophs in
 322 non-legume roots with a culture independent approach, rhizobial *nif* genes sequences
 323 were found to be expressed in rice and sugarcane (54), suggesting that nitrogen fixation
 324 by rhizobia may occur out of nodules in non-legume plants (55) As these experiments
 325 were performed in field conditions, there were most probably other bacteria that
 326 colonized roots in addition to rhizobia (54), but the role of such bacteria on rhizobial *nif*
 327 gene expression was not explored. The synthetic ecology approach that we followed
 328 here was an attempt to answer this question. Surprisingly, our results showed that
 329 *Bacillus* and *Azospirillum* were the front-runners in the interstrain competition for the
 330 maize rhizosphere colonization under the conditions tested. Remarkably in rhizobial
 331 research, interstrain competition assays for nodule formation are the most valuable tests
 332 to define symbiotic defects. However, low levels of nitrogen fixation may occur with *R.*
 333 *phaseoli* Ch24-10 in maize though not detected by acetylene reduction, since Ch24-10
 334 promotes plant growth in the absence of added nitrogen.
 335 *R. phaseoli* *nif* genes were found expressed in plant when it was inoculated by itself.
 336 Furthermore, bacterial respiration genes under low oxygen were found expressed as
 337 well. Bacterial respiration is directly related to the supply of ATP; this ATP is
 338 necessary to give enough energy for the nitrogen fixation to take place. Nitrogen-fixing
 339 bacteria use *cbb3*-type oxidases encoded by the *fixNOQP* operon and this was expressed
 340 as well (56, 57). (58). In spite of rhizobium nitrogen fixation being transcriptionally
 341 active no acetylene reduction was detected from these plants associated bacteria. Clearly
 342 there are other elements that are needed for *Rhizobium* to fix nitrogen out of the nodule.
 343 It was unfortunate that when *Rhizobium* was inoculated with the other bacteria, its
 344 nitrogen-fixation machinery was not expressed. Only one of the nitrogen-fixing bacteria
 345 from the multispecies inoculum proved functional in plants, namely *Azospirillum*.

346 Acetylene reduction was detected in *Azospirillum* cultures and when *Azospirillum* was
 347 as a single inoculum in plant and more activity was recorded when in combination with
 348 *Bacillus*. We surmise that in plant the large *Bacillus* populations attained inhibited *R.*
 349 *phaseoli* Ch24-10, *S. americanum* and *Methylobacterium*, but not *Azospirillum*.
 350 However, *in vitro* tests in culture medium this was not the case, since there was no
 351 antagonism amongst bacteria. Maybe in the presence of plant roots or in the presence of
 352 other bacteria *Bacillus* produces a distinct profile of antibiotics antagonistic to rhizobia
 353 and methylobacteria, but further experimentation is needed to test this hypothesis.

354 It seems plausible that *Bacillus* provides suitable conditions for *Azospirillum* to fix
 355 nitrogen, perhaps by inhibiting other bacteria that may compete for nutrients or by
 356 making a combined biofilm or by providing low oxygen conditions. This should be
 357 further explored. We may even speculate that *Bacillus* may stimulate the excretion of
 358 fixed nitrogen by *Azospirillum*. Notably excretion ammonium mutants of nitrogen
 359 fixing bacteria are capable of promoting plant growth in nitrogen deficient conditions.
 360 Enhanced biofilm formation was observed in multispecies biofilms in marine systems
 361 (59), and nitrogen fixation may be stimulated in interspecies biofilms. There are
 362 examples of metabolic complementation that may occur between distinct symbiont
 363 species in laboratory flow chambers (60). Our results are a basis to further evaluate
 364 *Bacillus/Azospirillum* inoculum in maize fields to promote nitrogen fixation.

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557 Figure 1. Overview of the workflow to determine changes within the transcriptomes of
558 *Rhizobium phaseoli* Ch24-10 grown PY medium and plant with and without a bacterial
559 community. After 24 hours, for cultures in PY medium and 5 days in cultures made
560 with plants after inoculation, RNA extraction was carried out, extracted RNA was
561 sequenced and the analysis of the data was performed as described in the Methods
562 section.

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565 Figure 2. Effect of multi-species inoculum on maize growth (a) Aerial mass of the plant
566 thirty days after inoculation (b) Improvement in photosynthetic rate in maize plants
567 inoculated 30 days after colonization (c) Comparison of an uninoculated plant and a
568 plant colonized with the multi-species inoculum thirty days after inoculation (d)
569 Increase in root mass in plants colonized with the multi-species inoculum. The numbers
570 inside the bars represent the percentage of gain compared to the control statistically
571 significant Tukey (value of $p < 0.05$).

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573 Figure 3. Heatmap of global gene expression profiles of *nif* genes in *Azospirillum*

574 *brasilense* sp7. Expression values were represented as a Z-score due to the difference of

575 expression among genes.

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579 Figure 4. Visualization of GO terms. Treemaps of enriched Biological Process GO
580 terms obtained using REVIGO when comparing *R. phaseoli* grown in PY medium
581 against *R. phaseoli* Ch24-10 grown in plants, using a) up-regulated genes b) down-
582 regulated genes.

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585 Figure 5. Visualization of GO terms. Treemaps of enriched Biological Process GO
586 terms obtained using REVIGO when comparing *R. phaseoli* grown in plant against *R.*
587 *phaseoli* Ch24-10 with community grown in plants, using a) up-regulated genes b)
588 down-regulated genes.

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