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► To cite this version:

Loubna Benidire, Fatima El Khalloufi, Khalid Oufdou, mohamed Barakat, Joris Tulumello, et al.. Phytobeneficial Bacteria Improve Saline Stress Tolerance in *Vicia faba* and modulate microbial interaction network. *Science of the Total Environment*, 2020. hal-03065045

HAL Id: hal-03065045

<https://hal.science/hal-03065045>

Submitted on 14 Dec 2020

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1 **Phytobeneficial Bacteria Improve Saline Stress Tolerance in *Vicia***
2 ***faba* and modulate microbial interaction network**

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17 Running title: **Phytobeneficial Bacteria Improve Saline Stress Tolerance**

- 18 Key words: *Vicia faba*, **Phytobeneficial Bacteria**, **Saline Stress**, **Rhizosphere**, **Microbiota**,
- 19 **Microbial network**

20 **Abstract**

21 Increased global warming, caused by climate change and human activities, will seriously
22 hinder plant development, such as increasing salt concentrations in soils, which will limit
23 water availability for plants. To ensure optimal plant growth under such changing conditions,
24 microorganisms that improve plant growth and health must be integrated into agricultural
25 practices. In the present work, we examined the fate of *Vicia faba* microbiota structure and
26 interaction network upon inoculation with plant-nodulating rhizobia (*Rhizobium*
27 *leguminosarum* RhOF125) and non-nodulating strains (*Paenibacillus mucilaginosus* BLA7 and
28 *Ensifer meliloti* RhOL1) in the presence (or absence) of saline stress. Inoculated strains
29 significantly improved plant tolerance to saline stress, suggesting either a direct or indirect
30 effect on the plant response to such stress. To determine the structure of microbiota
31 associated with *V. faba*, samples of the root-adhering soil (RAS), and the root tissues of
32 seedlings inoculated (or not) with equal population size of RhOF125, BLA7 and RhOL1 strains
33 and grown in the presence (or absence) of salt, were used to profile the microbial
34 composition by 16S ribosomal RNA gene sequencing. The inoculation did not show a
35 significant impact on the composition of the root microbiota or the root-adhering soil
36 microbiota. The saline stress shifted the RAS microbiota composition, which correlated with
37 a decrease in *Enterobacteriaceae* and an increase in *Sphingobacterium*, *Chryseobacterium*,
38 *Stenotrophomonas*, *Agrobacterium* and *Sinorhizobium*. When the microbiota of roots and
39 RAS are considered together, the interaction networks for each treatment are quite different
40 and display different key populations involved in community assembly. These findings
41 indicate that upon seed inoculation, community interaction networks rather than their
42 composition may contribute to helping plants to better tolerate environmental stresses. The
43 way microbial populations interfere with each other can have an impact on their

44 functions and thus on their ability to express the genes required to help plants tolerate

45 stresses.

46

47 **1. Introduction**

48 The soil microbial community is fundamental to soil organic matter turnover, nutrient cycling
49 and plant productivity preservation (Haichar *et al.*, 2014). This central role illustrates the
50 importance of the microbial response to environmental stresses such as salinity,
51 temperature and water deficit. Stressors such as salinity can disrupt the microbiota structure
52 by decreasing the activity of surviving microorganisms, due to the energy required for the
53 induction of stress tolerance mechanisms, and the inactivation of sensitive bacteria (Schimel
54 *et al.*, 2007). In a hot and dry climate, when water deficiency and salinity occur at the same
55 time, they represent a significant threat to the soil microbiota. The same is true for crop
56 productivity thereby directly challenging human nutrition. Soils are considered saline when
57 the electrical conductivity (EC)_e reaches or exceeds 4 dS/m, which is equivalent to
58 approximately 40 mM NaCl and results in an osmotic pressure of 0.2 MPa (Munns and
59 Tester, 2008). As a result, around the world, 100 Mha (5 % of arable land) of agricultural land
60 are affected by salt at a level that causes osmotic stress, reducing agricultural production
61 (Lambers, 2003) and promoting land erosion and eutrophication of waterways (Chowdhury
62 *et al.*, 2011).

63 Excessive salt concentrations in the rhizosphere remove water from the roots, consequently
64 impeding plant growth. The effect of salinity on variation in microbial community has been
65 studied in saline aquatic ecosystems (Benlloch *et al.*, 2002; Crump *et al.*, 2004; Mesbah *et*
66 *al.*, 2007; Bodaker *et al.*, 2010), whereas few reports have examined the effects of salinity on
67 the soil microbial community (Rath *et al.*, 2016, 2019a, 2019b), particularly in the rhizosphere
68 (Caton, 2004; Walsh *et al.*, 2005).

69 Microbiota is believed to provide the host with adaptive capacities that influence its
70 physiology and improve its fitness (Zilber-Rosenberg and Rosenberg, 2008). The rhizosphere
71 microbiota may play an important role in plant nutrition and protection against biotic and
72 abiotic stresses. However, certain stresses may severely alter plant development, thus
73 requiring the integration of plant-beneficial microorganisms into agricultural practices to
74 enhance plant growth and stress tolerance. Numerous studies have been devoted to
75 experiments describing crop inoculation with PGPR (plant growth-promoting rhizobacteria)
76 as a means of improving plant growth or providing protection against pathogens. Increasing
77 soil salinity is an environmental concern for global agriculture (Coleman-Derr and Tringe,
78 2014), and yet very little is known about the effect of plant root-associated microbiota on
79 the ability of plants to withstand saline stress (and their effect on restoring plant growth).

80 Therefore, we have focused our study on the plant *V. faba*, which is cultivated in several
81 countries around the world. Specifically, we investigated the potential of phytobeneficial
82 bacteria inoculation to improve plant tolerance to saline stress, and we have evaluated the
83 impact of saline stress and bacterial inoculation (alone or in combination) on the rhizosphere
84 microbiota assemblage and interaction network.

85 Material and methods

86 1.1. Biological material

87 Three bacterial strains were used, BLA7 identified in the present study by 16S rRNA gene
88 sequencing, *Rhizobium leguminosarum* RhOF125 (Benidire *et al.*, 2017), and *Ensifer meliloti*
89 (formerly *Sinorhizobium meliloti*) RhOL9 strain (El Khalloufi *et al.*, 2011, 2013). BLA7 and
90 RhOF125 strains were isolated respectively from nodules and the rhizosphere of field-grown

91 *V. faba* cultivated in the region of Marrakech Haouz (Morocco), and RhOL9 strain was
92 isolated from root nodules of local *Medicago sativa* plants collected from the Marrakech
93 region. We identified the strain BLA7 by 16S rRNA gene sequencing (Supplementary data).

94 The seeds used in this study were a commercial variety of *V. faba* (Alfia 5). Seeds were
95 surface disinfected with hypochlorite (1.8 % NaClO) and aseptically germinated on sterile
96 sand incubated in the dark at room temperature for 4 days.

97

98 1.2. Experimental design

99 The evaluation of the effect of salinity stress on soil bacterial diversity was conducted in
100 polyethylene bags filled with agricultural soil collected from Aït Ourir (Marrakech region,
101 Morocco).

102 Pre-germinated *V. faba* seeds were co-inoculated with the three strains: RhOF125, RhOL9
103 and BLA7. Experiments were conducted in four replicates for biomass measurements and in
104 triplicates for 16S rRNA metabarcoding analyses. The inocula were prepared by culturing the
105 bacteria in a liquid mannitol yeast extract medium (Vincent, 1970) at a cell density of
106 approximately of 10^9 colony-forming units (cfu) mL⁻¹. The experiment was performed in
107 plastic bags containing 5 kg of soil with a density of two plants per bag, and the cultivation
108 was conducted under natural conditions. After appearance of the first leaves, bags were
109 exposed, in each irrigation, to NaCl stress at a concentration of 70 mM; control bags were
110 irrigated with sterile distilled water.

111 After 30 days of treatment, the roots of inoculated and non-inoculated *V. faba* plants were
112 collected and ground in ultrapure water, following removal of the root-adhering soil by three

113 successive washes with sterile ultrapure water. The root homogenates and root-adhering
114 soil suspensions were then centrifuged (9,000 rpm for 15 min), and the resulting pellets
115 were retained until DNA extraction. Each replicate corresponds to two pooled seedlings. The
116 roots analysed per replicate come from two seedlings, as does the root-adhering soil. The
117 fresh weight of the shoots was measured after recovery of the roots for DNA extraction.

118 1.3. DNA extraction

119 Total DNA was extracted from 36 samples including, bulk soil, root-adhering soil (RAS) and
120 root tissue samples. Sterile glass beads were added to tubes containing TE (Tris-EDTA) buffer
121 and roots, root-adhering soil samples or bulk soil (36 samples). Thereafter, each material
122 was ground into a homogeneous sample with a Mixer Mill (MM200, Retsch Technology).
123 These experiments are conducted in triplicates. Total DNA was extracted directly from
124 homogenates using TE buffer with the addition of proteinase K (20 mg/mL), lysozyme (10
125 mg/mL), SDS (10 %) and sarkosyl. After heating and shaking at 50 °C for 1 h in an incubator,
126 the samples were treated with phenol/chloroform/isoamyl alcohol (25:24:1, pH 8), briefly
127 mixed, and centrifuged 1 h at 4 °C. The supernatant was then transferred to sterile tubes
128 filled with chloroform/isoamyl alcohol (24:1), mixed, and centrifuged for 1 min at 4 °C.
129 Subsequently, the aqueous phase was collected. DNA was precipitated overnight using
130 absolute isopropanol at room temperature, pelleted by centrifugation (5,000 rpm for 20 min
131 at 4°C), washed in 70 % ethanol, dried for 1 h in a SpeedVac (Thermo Fisher Scientific), and
132 eluted in 250 µL TE buffer. All extracted crude DNA were first loaded onto PVPP-columns
133 (1000×g, 10 min, 10 °C) as described by Petric *et al.* (2011) and then purified using the
134 GENE CLEAN® II kit (MP BIOMEDICALS, Solon, OH, USA), according to the manufacturer's
135 instructions.

136 1.4. PCR procedure, bioinformatics, and statistical analysis

137 The 16S rRNA gene V4-V6 variable region PCR primers 530F/1100R were used for PCR
138 amplification as previously described (El Khalloufi *et al.*, 2016). Sequencing was performed at
139 MR DNA (www.mrdnalab.com; Shallowater, TX, USA) using Roche 454 FLX titanium
140 instruments following the manufacturer's guidelines Sequencing data were processed with
141 QIIME version 1.8 (Caporaso *et al.*, 2010). Sequences were trimmed of barcodes and
142 primers, and the short sequences (< 150 bp), and the ambiguous sequences were removed.

143 The Operational Taxonomic Units (OTUs) were then defined by clustering at 97 % similarity.
144 The OTUs were classified taxonomically using Blast (Altschul *et al.*, 1990) and grouped into
145 each taxonomic level into "counts" and "percentage" files. The phylogenetic position of
146 bacteria in the different samples was characterised using taxa summary QIIME script until
147 genus level (L6). OTU tables were rarefied at the lowest sequencing depth to control
148 sequencing depth. OTU richness, diversity index and rarefaction curves were determined on
149 a random picking of an equal sequence number in each sample (10,451 reads per sample)
150 from the 36 samples through sequencing analysis by using the pipeline QIIME (Caporaso *et*
151 *al.*, 2010). After quality control trimming, a total of 238,757 sequences were generated from
152 36 samples and utilized for analysis. The average length of the sequences after processing
153 was 400 bp for all samples. The clean sequences were clustered into OTUs at 97 % similarity
154 levels (excluding the unclassified sequences at the phylum level), and sequences classified as
155 chloroplast or cyanobacteria were also removed. The bulk soil, root-adhering soil and root
156 tissues respectively displayed averages of 227, 242 OTUs and 372 OTUs, at 3 % divergence.

157 All statistical analyses were conducted using R version 3.1 (R Core Team, 2016). Unless
158 otherwise specified, we determined statistical significance at $\alpha = 0.05$. PCoA and CAP
159 analyses were conducted using the capscale function. PERMANOVA was conducted using the
160 adonis function and Bray-Curtis dissimilarities were calculated using the vegdist function all
161 from the Vegan package (Dixon, 2003).

162 The core microbiota was determined by plotting OTU abundance in the core at 5 % intervals
163 (from 50 % to 100 % of samples) using QIIME.

164 We analyzed bacterial networks for control, inoculation, and salinity treatments separately.
165 All network analyses were performed in R, using the package igraph, as in Williams et al.
166 (2014). Interactions consisted of Spearman's rank correlations and co-occurrence networks
167 were constructed using only significant correlations ($p < 0.05$ as in Barberan *et al.*, 2012) of ρ
168 > 0.8 . Random networks were constructed as in Williams *et al.* (2014). Thereafter, we
169 detected network modules using the cluster_edge_betweenness and modularity functions
170 and calculated clustering coefficients using the transitivity function. We analyzed the
171 centrality of network nodes using the betweenness function, and analyzed the
172 connectedness of network nodes using the degree function. All bacterial networks were
173 significantly more clustered than random networks.

174 Sequence data are available in NCBI SRA bank under accession number *SRR5883473*,
175 experiment *SRX3049495*.

176

177 **2. Results**

178 *2.1. Integrating phytobeneficial bacteria improves plant tolerance to saline stress*

179 *V. faba* plants were grown in the presence or absence of NaCl 70 mM and inoculated with
180 three bacterial strains, the exopolysaccharide (EPS)-producing *Paenibacillus mucilaginosus*
181 BLA7 strain (99% identity), the *V. faba* -nodulating *Rhizobium leguminosarum* RhOF125
182 strain, and the non-nodulating *E. meliloti* RhOL9 strain. We observed nodulation of *V. faba*
183 inoculated with *R. leguminosarum* RhOF125, whereas *E. meliloti* RhOL9 and *P. mucilaginosus*
184 BLA7 were supposed to colonize the rhizoplane and the rhizosphere (root-adhering soil).

185 The impact of salt and bacterial inoculation on plant growth was evaluated (**Fig. 1**). After 30
186 days of salt treatment, the growth parameters, represented as fresh weight of the plant,
187 clearly indicate the adverse effect of salinity on *V. faba* growth, as the fresh matter of shoots
188 decreased significantly ($p < 0.05$) under 70 mM NaCl treatment (-40 %). In contrast, plants
189 that were inoculated with the mixt inoculation of RhOF125, RhOL9 and BLA7 strains were
190 less impacted by saline stress and displayed a decrease in fresh shoot weight of only 20 %.
191 Bacterial inoculation significantly mitigated the effect of salt on plant growth, as shoot
192 biomass was significantly higher when plants were inoculated (**Fig. 1**).

193 To determine whether the beneficial effect of plant inoculation is directly related to bacterial
194 inoculation or indirectly via the modulation of the root-associated microbiota after
195 inoculation, we investigated the composition of the microbial community in the rhizosphere
196 and in the roots of *V. faba*.

197 *2.2. V. faba rhizosphere and root-associated microbiota*

198 The diversity of the rhizosphere of *V. faba* microbiota was moderate as very few bacterial
199 phyla were presented, such as Proteobacteria, Bacteroidetes, and Actinobacteria. The root-
200 adhering soil was mainly inhabited by very high proportion of Enterobacteriaceae (66 %) and
201 Sphingobacteriaceae (20 %), whereas the roots hosted mainly Rhizobiaceae (55 %) and
202 Streptomycetaceae (14 %).

203 The dominant phyla represented by the 165 identified genera were Proteobacteria (78
204 genera), Actinobacteria (43 genera), and Bacteroidetes (17 genera). The 12 most dominant
205 genera in each compartment are reported in **Table 1**.

206 Phylogenetic analysis of the two compartments (root tissues vs root-adhering soil) showed
207 the presence of 26 bacterial families, with a predominance of families belonging to
208 Proteobacteria (> 70 %) compared to other phyla (**Fig. 2a** and **2b**). In the root tissue samples,
209 the three dominant phyla were Proteobacteria, Actinobacteria and Bacteroidetes, with
210 relative abundances of 75 %, 21 % and 3 %, respectively (**Fig. 2a**). Root-adhering soil samples
211 hosted mainly Proteobacteria (71 %) and Bacteroidetes (27 %) (**Fig. 2b**). At the class level,
212 members of Gammaproteobacteria represented the most abundant group of Proteobacteria
213 in root-adhering soil compartments (67 %), followed by several members of
214 Alphaproteobacteria (3 %). In contrast, root tissues were mainly colonized by
215 Alphaproteobacteria (63 %).

216 *2.3 Salinity and co-inoculation impact on V. faba rhizosphere and root-associated*
217 *microbiota*

218 To evaluate the contribution of the microbiota assemblage to the plant tolerance to saline
219 stress, we surveyed the bacterial diversity of bulk soil, root-adhering soil compartment, and
220 root tissues of *V. faba* plants grown in the presence and absence of salt (70 mM).

221 To visualize the effect of inoculation and saline stress on microbial community modulation in
222 our data, we used principal coordinates analysis (PCoA) in combination with permutational
223 multivariate analysis of variance (PERMANOVA) on Bray-Curtis dissimilarities between the
224 samples. Consistent with our previous results (Haichar *et al.*, 2008, El Khalloufi *et al.*, 2016,
225 Bressan *et al.*, 2009), this analysis revealed significant differences between the microbiota of
226 the bulk soil, and that of root-adhering soil and root tissues, denoting the rhizosphere effect
227 and the selection of specific populations from the surrounding soil by the roots (**Fig. 2c**, $R^2 =$
228 0.28 , $p < 0.001$). Another observation from the PCoA analysis was that RAS samples
229 clustered closer towards the bulk soil samples than did the root samples.

230 Considering the saline stress, the samples of RAS are clustered in two groups (inoculated vs
231 non-inoculated) and the one of RT in a single one (**Fig. 2d**, $R^2 = 0.20$, $p < 0.004$,
232 perMANOVA).

233 The effect of bacterial inoculation on the microbiota associated to the RT and RAS samples
234 of *V. faba* is the same in presence or absence of saline stress, as all the samples of RT are
235 grouped tightly together, and the same for the RAS data (**Fig. 2e**).

236 Tolerance to saline stress could be linked to shifts in bacterial community composition in the
237 root-adhering soil that correlate with an increase of plant salt tolerance. These changes
238 could be considered as good indicators of a relationship between mitigation of saline stress
239 and community composition. The LEfSe (Linear discriminant analysis Effect Size) analysis
240 shows differentially abundant taxa as biomarkers using Kruskal–Wallis test ($p < 0.05$) with

241 LDA score > 2.0. We examined which OTUs differentiate the microbiota of salt-stressed
242 plants from those without stress by surveying which bacterial groups have significantly
243 different relative abundances. The roots of inoculated seedlings were specifically colonized
244 by bacteria belonging to Betaproteobacteria (Oxalobacteraceae and Comamonadaceae) and
245 to Alphaproteobacteria (*Caulobacter*), while under saline stress, the roots specifically
246 recruited bacteria belonging to Verrucomicrobia (*Opitutus*), Gammaproteobacteria
247 (*Steroidobacter* and Sinobacteraceae) and Actinobacteria (*Microbacterium*) (**Fig. 3a and 3b**).
248 The RAS compartment was more impacted, as inoculation induced a selection of bacteria
249 belonging to Gammaproteobacteria (*Trabusiella*), while saline stress induced a selection of
250 bacteria belonging to Betaproteobacteria (Burkholderiales, Alcaligenaceae and
251 Achromobacter) and Alphaproteobacteria (Rhizobiaceae). When inoculated plants were
252 submitted to saline stress, the root-adhering soil hosted specifically Alphaproteobacteria
253 (*Agrobacterium*, Caulobacterales, *Mesorhizobium*, Pyllobacteriaceae and
254 *Erythrobacteraceae*) and to Gammaproteobacteria (*Arenimonas*, *Erwinia*,
255 *Pseudoxanthomonas*) (**Fig. 3c and 3d**).

256 These modulations of microbial composition were complemented by principal components
257 of the principal component analysis (PCA) illustrating the relative ordination of the various
258 treatments based on the relative abundance of OTUs. On root system, NaCl treatment
259 and/or bacterial inoculation led to an increase in the proportion of some taxa. Specifically,
260 the proportion of organisms belonging to Enterobacteriaceae, Streptomycetaceae,
261 Pseudomonadaceae, Xanthomonadaceae and Microbacteriaceae was increased when
262 compared to the control. In contrast, salinity had a negative effect on Rhizobiaceae
263 abundance; this decrease was less pronounced when *V. faba* was inoculated (**Fig. 4a**). The

264 roots of inoculated seedlings recruited mainly bacteria of the genus *Streptomyces* and
265 Enterobacteriaceae and surprisingly bacteria of the genus *rhizobium* are less abundant at the
266 level of the inoculated roots, suggesting that probably the inoculated rhizobia are
267 preferentially established at the rhizosphere level. In the RAS compartment, analyses at the
268 family level revealed a significant decrease in the proportion of Enterobacteriaceae in
269 uninoculated seedlings following saline treatment. The RAS of inoculated seedlings, in the
270 presence and absence of saline stress, are particularly colonized by members of the phylum
271 of *Bacteroidetes* (*Sphingobacterium* and *Chryseobacterium*) (**Fig. 4b**). Therefore, these OTUs
272 are probably involved in plant tolerance to salt. saline stress had less impact on community
273 structure in the RAS when the seedlings were inoculated.

274 *2.4 Salinity and co-inoculation impact on V. faba rhizosphere and root-associated microbial* 275 *interaction*

276 The co-occurrence or co-exclusion patterns of bacterial groups in the RAS and root
277 compartments in response to inoculation, saline stress or both, were investigated and
278 compared by network analysis (**Fig. 5a**). OTUs with a large number of positive and negative
279 interactions indicating their importance in shaping bacterial groups are named hubs (Aglar *et*
280 *al.*, 2016; Layeghifard *et al.*, 2017). The shape of bacterial network of the different
281 treatments was distinctly different and more importantly hubs were quite different (**Fig. 5b**).
282 Although the network size is not significantly different between the different treatments, the
283 number of modules determined by the phylogenetic molecular ecological networks ranged
284 from 10 for control to 15 for NaCl treatment, indicating that the meta-community network
285 was more dispersed and less connected in the presence of salt. This is also corroborated by
286 the lower average connectivity observed for the saline treatment (**Table 1**). Proteobacteria

287 was dominated by Gammaproteobacteria which are highly reduced in abundance in the
288 presence of salinity whether the plant is inoculated or not (**Fig. 6a**). The most abundant
289 OTUs are not highly connected to the other members of microbial community, as nodes are
290 mainly represented by members of Alphaproteobacteria and Actinobacteria (**Fig. 6a and 6b**).
291 Therefore, hubs are primarily represented by alphaproteobacteria and actinobacteria,
292 indicating the importance of these bacterial groups in the microbial assemblage, except
293 under saline stress in uninoculated plants, where members of alphaproteobacteria are not
294 represented as hubs (**Fig. 6c**). We also notice that the proportion of hubs that are members
295 of Actinobacteria is significantly reduced in inoculated plants in the presence and absence of
296 saline stress (**Fig. 6c**). Venn diagrams show that a high proportion of OTUs and nodes are
297 shared by all treatments (**Fig. 6d and 6e**). However, only very few hubs are common to all
298 treatments (**Fig. 6f**). The relative number of positive (green) and negative (red) interactions
299 indicated for each hub in each treatment, shows that the different hubs are mainly involved
300 in co-occurrence interactions, and very few co-exclusion interactions are found (**Fig. 6g**).
301 Some shared hubs behaved differently such as *Sinorhizobium*, which in the absence of saline
302 stress displayed mostly negative interactions, while in the presence of saline stress and
303 bacterial inoculation, it was mainly involved in positive interactions (**Fig. 6g**). We can also
304 observe that the Gemm-5 OTU is essentially involved in co-exclusion interactions in the
305 absence and presence of saline stress, whereas in the concomitant presence of saline stress
306 and bacterial inoculation, this hub presents only co-occurrence interactions (**Fig. 6g**).

307 **3. Discussion**

308 Global climate change is presumed to have an impact on plant adaptation. To address this
309 issue, plant growth adaptation must be optimized by integrating root-associated microbiota

310 (especially bacterial species with beneficial effects on plant growth and health) into
311 agricultural practices (Busby *et al.*, 2017). Microbial inoculants are considered to be
312 environmentally friendly, and could provide a sustainable alternative technology for solving
313 agro-environmental issues. Specifically, beneficial microbes can improve crop productivity to
314 increase or support plant growth, nutrient availability and uptake, and plant fitness (Glick,
315 2014), thereby reducing the use of chemical fertilizers. The use of beneficial rhizobacteria
316 (PGPR) to improve plant growth and yield under saline stress has been extensively
317 investigated. It is acknowledged that bio-inoculation can increase tolerance, adaptation and
318 protection of plants against salinity stress (Hashem *et al.*, 1998; Fasciglione *et al.*, 2012;
319 Munns and Gilliam, 2015; Upadhyay and Singh, 2015; Qin *et al.*, 2016). Indeed, an increase
320 in plant biomass and the stimulation of root growth (allowing the enhanced uptake of
321 nutrient elements and consequently alleviate the effects of saline stress in different legume
322 species) has been attributed to different rhizobial strains that can establish a symbiotic
323 association under saline stress conditions (Nogales *et al.*, 2002; Bertrand *et al.*, 2015). In
324 addition, inoculation of maize plants with *P. fluorescens* containing ACC deaminase has been
325 reported to significantly enhance root elongation and plant biomass under saline conditions
326 (Kausar and Shahzad, 2006). Although most studies have focused primarily on changes in the
327 physiological and metabolic status of plants after inoculation with plant growth promoting
328 rhizobacteria (PGPR), a limited number of reports have investigated the effects of
329 inoculation on the diversity of soil microbial communities (Gupta *et al.*, 2014; Chihaoui *et al.*,
330 2015; Bharti *et al.*, 2016). We have examined here the effect of a combined application of
331 three selected bioinoculants (the *V. faba*-nodulating *R. leguminosarum* RhOF125, *E. meliloti*
332 RhOL9, and *P. mucilaginosus* BLA7) on bacterial diversity and bacterial interaction network in
333 the rhizosphere of *V. faba* cultivated under saline stress.

334 As expected, our glasshouse experiment revealed the positive effects of the PGPR
335 bioinoculants on the growth of *V. faba* plants. Specifically, we observed with 70 mM NaCl
336 treatment that shoot fresh matter was significantly increased ($p<0.05$) after bacterial
337 inoculation (mix of three strains) as compared to the control. These differences are thought
338 to be related to direct effects of PGPR in enhancing plant growth, as well as non-target
339 effects of bioinoculants on resident bacterial populations in the *V. faba* rhizosphere and
340 roots. In the present study, the relative abundance profiles suggested a shift in the bacterial
341 community composition of the inoculated plants in comparison with the initial bacterial
342 diversity, especially in the root-adhering soil compartment.

343 Rarefaction curves of the RAS compartment vary according to the treatments applied,
344 showing that salinity and bacterial inoculation increase the level of richness in this
345 compartment more than in the root tissues (**Fig. S1**). In RAS samples, an increase in bacterial
346 species richness was observed in response to salinity stress (and bacterial inoculation to a
347 lesser extent). It can be hypothesized that salinity stress has induced a decrease in the
348 population size of the dominant bacterial species, allowing access to a greater number of
349 sub-dominant species. A similar result was reported in the rhizosphere of halophytic plant
350 species, in which salt remarkably promoted the richness diversity of microbial communities
351 (Borruso *et al.*, 2014; Yang *et al.*, 2016). However, other authors have reported contrasting
352 results, indicating a significant decrease in species diversity and richness in high saline
353 rhizosphere soil (Nelson and Mele, 2007; Ibekwe *et al.*, 2017). As observed in this work,
354 inoculation appears to enhance the number of observed OTUs in RAS samples. Interestingly,
355 inoculation of *Phaseolus vulgaris* with *Agrobacterium sp.* 10C2 is reported to increase the
356 number of T-RFLP profiles in the rhizosphere by more than twofold (Chihaoui *et al.*, 2015). In

357 the present work, the rarefaction curves of roots microbiota displayed similar trends for all
358 samples, suggesting that the total species richness was weakly affected by salinity and
359 bacterial inoculation.

360 The bacterial diversity analysis of RAS and root tissues compartments at the phylum level
361 revealed the presence of 26 bacterial phyla, predominantly represented by Proteobacteria,
362 Actinobacteria and Bacteroidetes. This generally agrees with numerous previous reports
363 showing that these are the most common groups in different agricultural systems or other
364 soil types, based on pyrosequencing surveys (Haichar *et al.*, 2008; Lauber *et al.*, 2009; Blasiak
365 *et al.*, 2014; El Khalloufi *et al.*, 2016; Yaish *et al.*, 2016). Nevertheless, each of the dominant
366 phyla exhibited differences in average abundance level between the two studied
367 compartments (root tissue and RAS). Members belonging to the *Bacteroidetes* phylum are
368 reported to be widespread in numerous different ecosystems, where they can function in
369 the degradation of polymeric organic matter (Fierer *et al.*, 2007; Thomas *et al.*, 2011).
370 *Bacteroidetes* species have also been observed as the dominant phylum living in the
371 rhizosphere and proximal to the soil surface (Martínez-Alonso *et al.*, 2010). Actinobacterial
372 species are well known as members of the plant rhizosphere, but they also have a critical
373 role in the decomposition of complex biopolymers (*e.g.* lignocellulose, keratin, and chitin)
374 (Sykes and Skinner, 1973; Li *et al.*, 2016).

375 We used PCoA analysis to evaluate the main driver of bacterial diversity in response to
376 inoculation, salt or both. Among the selected variables, the compartment (RAS vs root
377 tissue), inoculation and saline stress, the latter was found to be the main driver of microbial
378 composition shift. If inoculation does not have any impact on microbiota shift that means
379 that it may have a direct impact on plant physiology by increasing its tolerance to salt and

380 also by creating a safer environment for the root-colonizing beneficial microbiota, probably
381 *via* root exudation. It has been suggested that such an effect might be due to root exudation
382 of specific molecules responsible for chemotactic attraction of specific bacterial populations
383 in the rhizosphere from the soil reservoir (Borruso *et al.*, 2014). Additionally, Trabelsi *et al.*
384 (2011) found that in *Phaseolus vulgaris*, rhizobacterial populations of Alphaproteobacteria
385 (Rhizobiaceae) and Gammaproteobacteria were enhanced by mixed inoculation with
386 *Sinorhizobium meliloti* 4H41 and *Rhizobium gallicum* 8a3. Likewise, Gupta *et al.* (2014)
387 proposed that this enhancement could be attributed to the fact that bioinoculants directly
388 and/or indirectly promote the growth of existing soil bacterial populations by
389 releasing/fixing/solubilising certain substrates that act as nutrients or inhibitors of
390 pathogenic microorganisms.

391 The saline stress is an important driver of the microbiota assemblage, acting through
392 selective enrichment of specific taxa, including *Microbacterium*, *Opitutus* and *Steroidobacter*
393 on the root tissues. In the root-adhering soil, members of Betaproteobacteria
394 (*Achromobater*), and *Bacteroidetes* (*Flavobacterium*) were specifically enriched. These
395 bacterial populations are whether the most tolerant to saline stress or could proliferate
396 because their antagonists are sensitive to saline stress. However, their enrichment did not
397 enable the plant to better tolerate saline stress, or to a lesser extent than may have done
398 taxa enriched under saline stress and inoculation, as members of Alphaproteobacteria
399 (*Agrobacterium*, *Mezorhizobium*...) and Gammaproteobacteria (*Erwinia*, *Arenimonas*).
400 Enterobacteriaceae seem to have shifted from the RAS to the roots in the presence of saline
401 stress. Similar results on the effects of NaCl in the rhizosphere of various agricultural crops
402 (Li *et al.*, 2016; Nie *et al.*, 2016; Hahm *et al.*, 2017; Ibekwe *et al.*, 2017) and on natural

403 halophytes (Liu *et al.*, 2016; Yang *et al.*, 2016) have been previously reported. However, the
404 length of 16S sequences obtained with current high-throughput sequencing techniques is relatively
405 short, and does not allow us to describe OTUs at the species level, and since within the same bacterial
406 genus, phytobeneficial bacteria and phytopathogens can co-exist, we can hardly claim that the plant,
407 under certain stress conditions, will select specifically beneficial microbiota.

408 The core microbiota regroups bacteria that are present in all samples whatever the
409 treatment. These bacteria are likely not sensitive to saline stress, however their presence in
410 not sufficient to help plants to tolerate the saline stress (**Fig. 6a, 6d**). This raises the question
411 of whether the core microbiota confers any benefit to the host and whether it improves the
412 plant tolerance to saline stress. Linking microbiome assembly and plant saline stress
413 tolerance is an overarching challenge.

414 It is possible to identify positive and negative co-occurrence correlations between bacterial
415 community members, which may reflect synergistic and antagonistic functional relationships
416 (Faust and Raes, 2012). Such interactions can be analyzed and visualized as networks and
417 may help understanding host-associated microbiome dynamics. We used network analysis to
418 explore complex interactions between microorganisms. Microbial networks are
419 spatiotemporal representations of ecosystems and are composed of nodes and edges.
420 Connectivity, represented by the number of links between a node and other nodes, is an
421 indicator of the strength with which an OTU is connected to other OTUs. OTUs having an
422 extraordinarily large number of positive and negative interactions and named hubs and thus
423 appear important drivers of bacterial communities shaping (Agler *et al.*, 2016; Layeghifard *et*
424 *al.*, 2017). Our network analysis evidenced that hubs are mainly members of
425 Alphaproteobacteria and Actinobacteria and also members of very rare bacterial groups

426 such as Gemmatimonadetes, Verrucomicrobia, Acidobacteria and Chloroflexi. Our analysis
427 indicates that a hub involved in positive interactions under certain conditions may behave
428 differently under stress conditions and establish mainly antagonistic interactions. Such
429 behavior might be linked to the expression of genes encoding antimicrobial compounds
430 concomitantly to those involved in stress tolerance, or at the contrary, the stress tolerance
431 mechanism requiring energy, may be expressed at the expense of genes encoding
432 antimicrobial compounds. The latter hypothesis is likely more common, as the case of
433 *Sinorhizobium* and an OTU member of Gemmatimonadetes, which are mainly involved in
434 positive interactions only in the presence of saline stress, while they exhibit antagonistic
435 interactions in the absence of saline stress.

436 The novel structure of bacterial communities might confer a benefit to the plant host by
437 improving its saline stress tolerance. Bacterial inoculation may have a direct or indirect
438 contribution via the modulation of the microbiota structure, thus enabling plants to
439 withstand salinity-derived drought. These results indicate that improving plant tolerance to
440 saline stress is not only based on certain microbial populations, but also on the interaction
441 and interplay of the entire microbiota. Understanding such associations will likely help
442 understanding plant salt tolerance and the implication of their associated microorganisms
443 (de Zelicourt *et al.*, 2013).

444 Several questions remain unresolved. How plants respond to a change in microbial networks
445 and assemblage might be assessed by metabolomics, transcriptomics and proteomics
446 approaches, but determining whether these modifications are imposed on plants or whether
447 rearrangements of the interaction network are induced by the plants is much more

448 challenging. In addition, we can hardly define the network in which microorganisms can
449 cooperate to thrive harsh conditions and help the plant to withstand such stresses.

450 Functional understanding of the microbiota still remains the biggest challenge and
451 integration of high-throughput profiling of the microbial transcriptome, metabolome, and
452 proteome is needed.

453

454 **4. Conclusions**

455 Our results demonstrate that the soil microbial community is complex and dynamic, with a
456 varying composition between the root-adhering soil and root tissue compartments.
457 Furthermore, our findings show that inoculation causes changes in the number and
458 composition of the bacterial taxonomic groups present in these compartments. These
459 changes could possibly modulate plant saline -tolerance and beneficial soil functions.
460 Altogether these data indicate that whether the plant recruits a specific microbiota, which
461 composition is driven by the root exudates, or the saline stress is the main driver of bacterial
462 community assemblage. This questions to what extent the plant itself, actively drives such
463 changes of the microbiota. The mechanisms by which plant hosts select their microbiota
464 from the reservoir are not fully understood.

465

466 **Acknowledgements**

467 This study was financially supported by Hubert Curien Partnerships (PHC), project PRAD 11-
468 01-Egide N° 24186QM and project TOUBKAL 16-21 Campus France n° 34717WA.

469

470 **Conflict of Interest:** The authors declare that they have no conflict of interest.

471

472

473 **Author Contributions:**

474 LB, FK, KO, TH, and WA designed the study. LB and FK, performed the experiments. JT, PO
475 and MB performed bioinformatics analysis. LB, WA, TH and KO wrote the paper.

476

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657 **Figure legends**

658 **Figure 1. Effect of salt treatment on the fresh weight of *Vicia faba* shoots.** T: non-
659 inoculated plants. Inoculation was performed with the three bacterial strains, BLA7, RhOL9
660 and RhOF125. Values are means \pm SD of four replicates. Bars with different letters are
661 significantly different at $P \leq 0.05$ according to the SNK test.

662 **Figure 2: Beta diversity of the rhizosphere of *Vicia faba* in response to inoculation and salt**
663 **stress.** Relative abundance of major OTUs at family level (a) in the root compartment (RT) ;
664 (b) in the root-adhering soil compartment (RAS). Community structure of bacteria, in the 36
665 samples was determined using principal-component analysis. The first two dimensions of a
666 principal-component analysis are plotted based on Bray-Curtis distances. Samples are color
667 coded according to the compartment, and are depicted with different colours. Clustering by
668 compartment (c), by salt treatment (d) and by inoculation treatment (e).

669

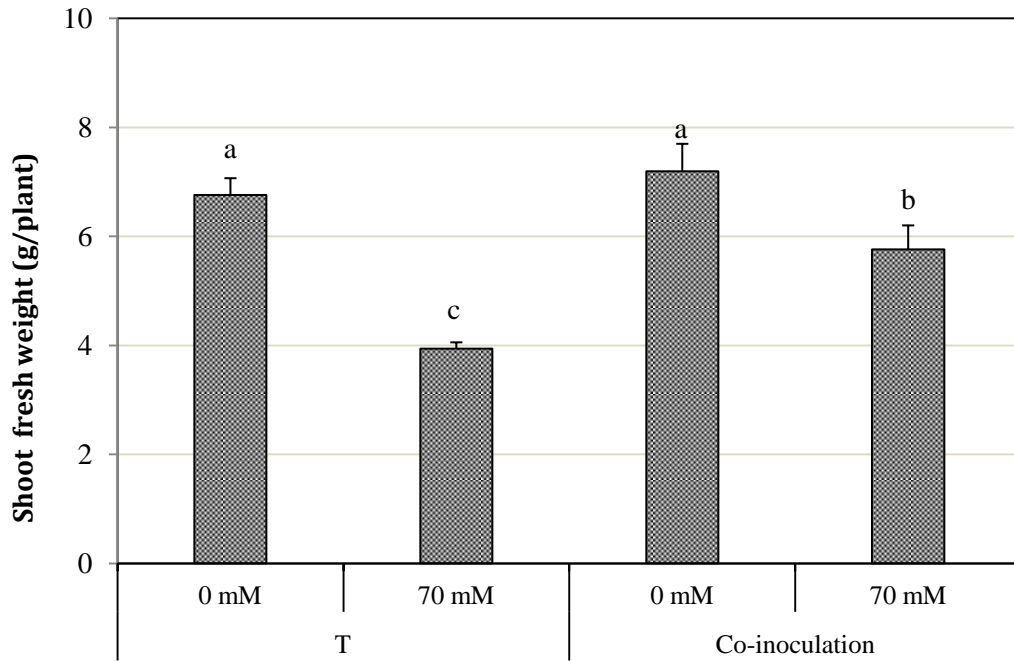
670 **Figure 3. Specific shift of beta diversity mediated by inoculation and salt stress.**
671 Phylogenetic cladogram of OTUs specific (a) to roots of inoculated seedlings and to roots of
672 seedlings submitted to saline stress, (b) to RAS of seedlings inoculated, under salt stress or
673 submitted to both treatments. (c, d) Linear discriminant analysis effect size (LEfSe) analysis
674 shows differentially abundant taxa as biomarkers using Kruskal–Wallis test ($P < 0.05$) with
675 Linear Discriminant Analysis (LDA) score > 2.0 . OTUs. The circles from inside to outside
676 indicate bacterial taxonomic levels from phylum to genus. Yellow dots represent bacteria not
677 significantly varying in abundance among treatments. Biomarker bacteria are colored
678 according to their corresponding class colors on the right.

679 **Figure 4: Relationship between diversity and inoculation and /or salt stress.** First PC1 and
680 second PC2 principal components of the principal component analysis (PCA) illustrating the
681 relative ordination of the various treatments based on the relative abundance of OTUs (a) in
682 the roots-adhering soil samples, (b) in the root samples. Taxa correlated with each
683 treatment are indicated.

684 **Figure 5. Figure 5: Bacterial Network of the *Vicia faba* rhizosphere and roots microbiota.**
685 (a) Correlation-based network of *V. faba* rhizosphere and roots-associated microbial OTUs
686 detected in response to inoculation and salt stress treatments. Each node corresponds to an
687 OTU, and edges between nodes correspond to either positive (green) or negative (red)
688 correlations inferred from OTU connectivity profiles using Pearson correlations. OTUs
689 belonging to different microbial kingdoms have distinct color codes, and node size reflects
690 their connection degree. (b).“Hub”microbes were identified as those which were
691 significantly more central based on all three measurements of centrality.

692 **Table 1:** Topological properties of the empirical phylogenetic molecular ecological networks
693 of microbial communities in the rhizosphere of *V. faba* under salt stress, inoculation or both
694 treatments.

695 **Figure 6. Beta diversity and interaction network features.** Impact of salt, inoculation and
696 both treatment on the relative abundance at phylum level and class level for Proteobacteria
697 of (a) OTUs, (b) nodes and (c) hubs in each treatment in the rhizosphere of *V. faba*. Venn
698 diagram representing the shared and specific (d) OTUs, (e) nodes and (f) hubs in the different
699 treatments. (g) Relative number of positive (green) and negative (red) interactions (edges
700 from network) for each hub in each treatment.



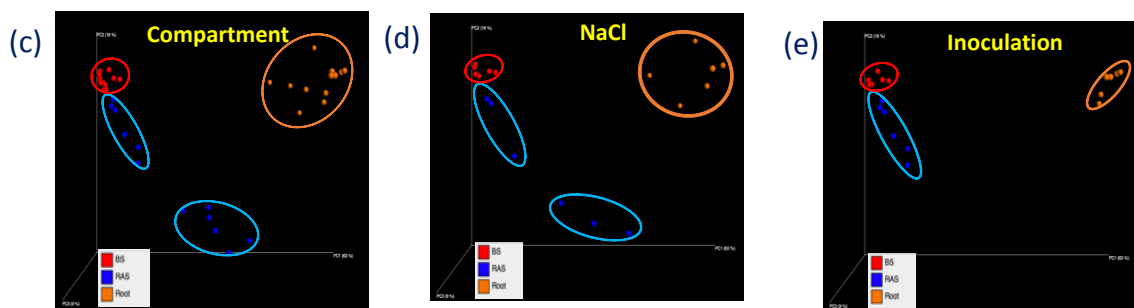
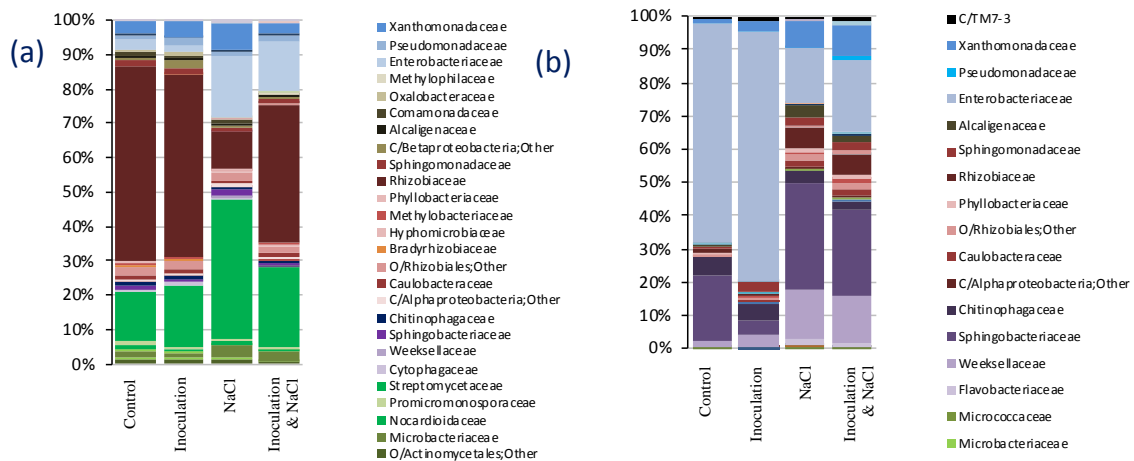
701

702 **Figure 1:** Effect of inoculation and salt treatment on the fresh weight of *Vicia faba* shoots. T:
 703 non-inoculated plants. Inoculation was performed with the three bacterial strains, BLA7,
 704 RhOL9 and RhOF125. Values are means \pm SD of four replicates. Bars with different letters are
 705 significantly different at $P \leq 0.05$ according to the SNK test.

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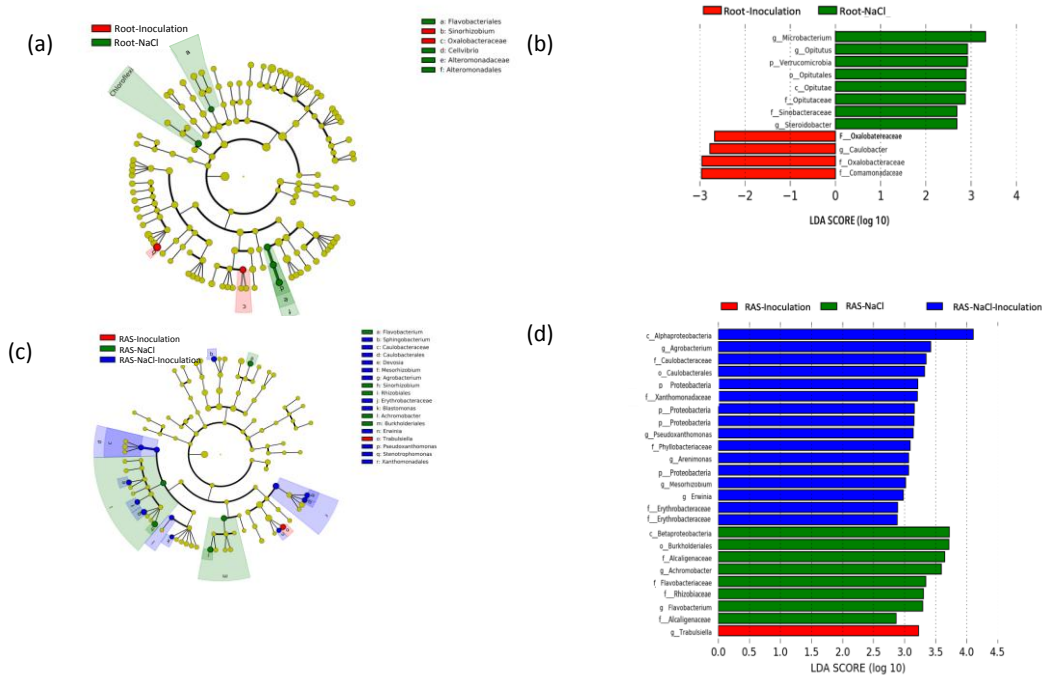


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710 **Figure 2:** Beta diversity of the rhizosphere of *Vicia faba* in response to inoculation and salt
 711 stress. Relative abundance of major OTUs at family level (a) in the root compartment (RT) ;
 712 (b) in the root-adhering soil compartment (RAS). Community structure of bacteria, in the 36
 713 samples was determined using principal-component analysis. The first two dimensions of a
 714 principal-component analysis are plotted based on Bray-Curtis distances. Samples are color
 715 coded according to the compartment, and are depicted with different colours. Clustering by
 716 compartment (c), by salt treatment (d) and by inoculation treatment (e).

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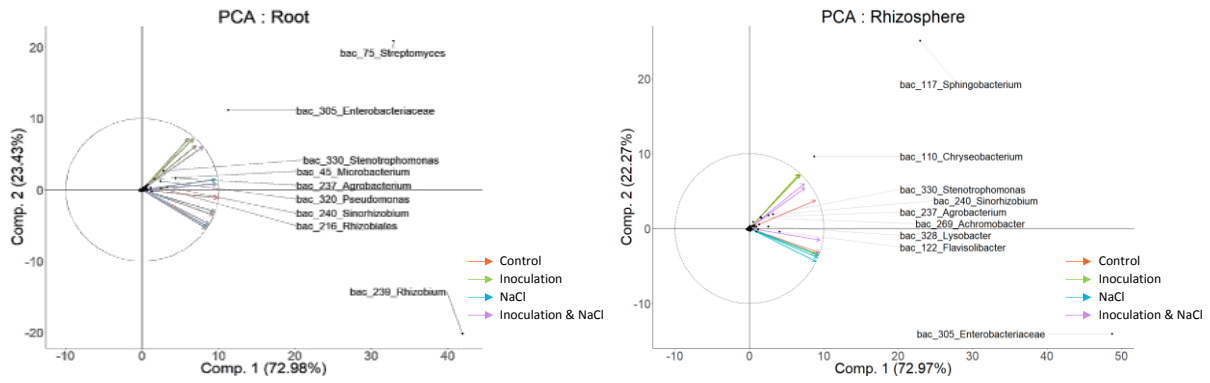


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720 **Figure 3. Specific shift of beta diversity mediated by inoculation and salt stress.**

721 Phylogenetic cladogram of OTUs specific (a) to roots of inoculated seedlings and to roots of
 722 seedlings submitted to saline stress, (b) to RAS of seedlings inoculated, under salt stress or
 723 submitted to both treatments. (c, d) Linear discriminant analysis (LDA) effect size (LEfSe)
 724 analysis shows differentially abundant taxa as biomarkers using Kruskal–Wallis test ($P < 0.05$)
 725 with LDA score > 2.0 . OTUs. The circles from inside to outside indicate bacterial taxonomic
 726 levels from phylum to genus. Yellow dots represent bacteria not significantly varying in
 727 abundance among treatments. Biomarker bacteria are colored according to their
 728 corresponding class colors on the right.

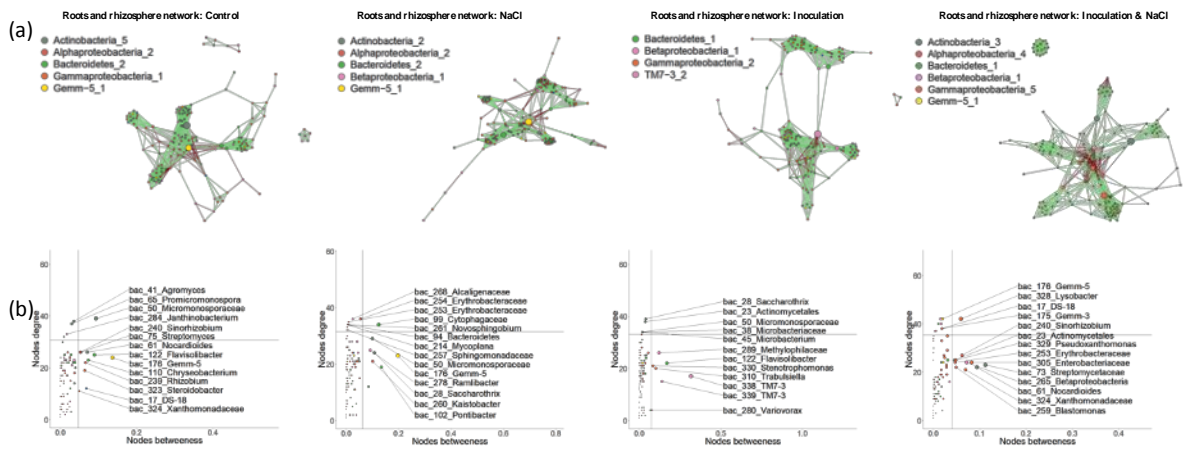
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731 **Figure 4: Relationship between diversity and inoculation and /or salt stress.** First PC1 and
 732 second PC2 principal components of the principal component analysis (PCA) illustrating the
 733 relative ordination of the various treatments based on the relative abundance of OTUs (a) in
 734 the roots-adhering soil samples, (b) in the root samples. Taxa correlated with each
 735 treatment are indicated.

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738 **Figure 5:** Bacterial Network of the *Vicia faba* rhizosphere and roots microbiota. (a)

739 Correlation-based network of *V. faba* rhizosphere and roots-associated microbial OTUs

740 detected in response to inoculation and salt stress treatments. Each node corresponds to an

741 OTU, and edges between nodes correspond to either positive (green) or negative (red)

742 correlations inferred from OTU connectivity profiles using Pearson correlations. OTUs

743 belonging to different microbial kingdoms have distinct color codes, and node size reflects

744 their connection degree. (b).“Hub”microbes were identified as those which were

745 significantly more central based on all three measurements of centrality.

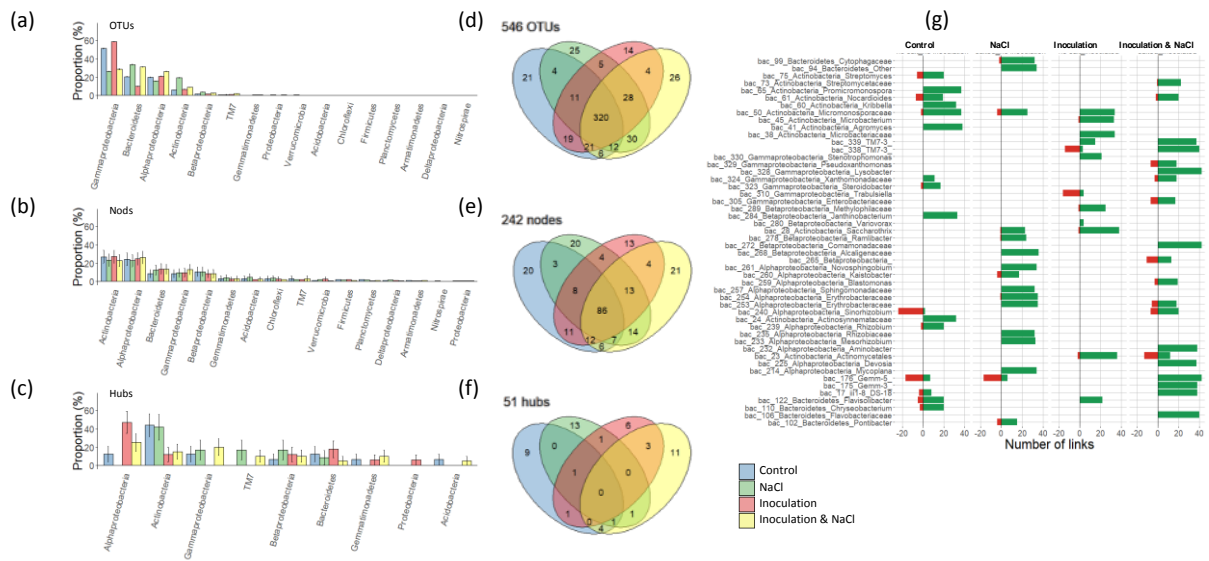
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747 **Table 1:** Topological properties of the empirical phylogenetic molecular ecological networks
 748 of microbial communities in the rhizosphere of *V. faba* under salt stress, inoculation or both
 749 treatments.

Treatment	network size	Link	average connectivity	average geodesic distance	average clustering coefficient	modularity	number of modules
Control	181	951	11.41	3.05	0.83	0.64	10
NaCl	179	1114	10.75	3.39	0.79	0.68	15
Inoculation	179	1167	11.95	3.66	0.79	0.65	12
Inoculation & NaCl	178	1145	11.37	3.2	0.8	0.63	10

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753 **Figure 6** : Beta diversity and interaction network features. Impact of salt, inoculation and
 754 both treatment on the relative abundance at phylum level and class level for Proteobacteria
 755 of (a) OTUs, (b) nods and (c) hubs in each treatment in the rhizosphere of *V. faba*. Venn
 756 diagram representing the shared and specific (d) OTUs, (e) nods and (f) hubs in the different
 757 treatments. (g) Relative number of positive (green) and negative (red) interactions (edges
 758 from network) for each hub in each treatment.

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