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

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Microbial network
Abstract

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

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

Excessive salt concentrations in the rhizosphere remove water from the roots, consequently impeding plant growth. The effect of salinity on variation in microbial community has been studied in saline aquatic ecosystems (Benlloch et al., 2002; Crump et al., 2004; Mesbah et al., 2007; Bodaker et al., 2010), whereas few reports have examined the effects of salinity on the soil microbial community (Rath et al, 2016, 2019a, 2019b), particularly in the rhizosphere (Caton, 2004; Walsh et al., 2005).
Microbiota is believed to provide the host with adaptive capacities that influence its physiology and improve its fitness (Zilber-Rosenberg and Rosenberg, 2008). The rhizosphere microbiota may play an important role in plant nutrition and protection against biotic and abiotic stresses. However, certain stresses may severely alter plant development, thus requiring the integration of plant-beneficial microorganisms into agricultural practices to enhance plant growth and stress tolerance. Numerous studies have been devoted to experiments describing crop inoculation with PGPR (plant growth-promoting rhizobacteria) as a means of improving plant growth or providing protection against pathogens. Increasing soil salinity is an environmental concern for global agriculture (Coleman-Derr and Tringe, 2014), and yet very little is known about the effect of plant root-associated microbiota on the ability of plants to withstand saline stress (and their effect on restoring plant growth).

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

Material and methods

1.1. Biological material

Three bacterial strains were used, BLA7 identified in the present study by 16S rRNA gene sequencing, *Rhizobium leguminosarum* RhOF125 (Benidire *et al.*, 2017), and *Ensifer meliloti* (formerly *Sinorhizobium meliloti*) RhOL9 strain (El Khalloufi *et al.*, 2011, 2013). BLA7 and RhOF125 strains were isolated respectively from nodules and the rhizosphere of field-grown
V. faba cultivated in the region of Marrakech Haouz (Morocco), and RhOL9 strain was isolated from root nodules of local Medicago sativa plants collected from the Marrakech region. We identified the strain BLA7 by 16S rRNA gene sequencing (Supplementary data).

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

1.2. Experimental design

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

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

After 30 days of treatment, the roots of inoculated and non-inoculated V. faba plants were collected and ground in ultrapure water, following removal of the root-adhering soil by three
successive washes with sterile ultrapure water. The root homogenates and root-adhering soil suspensions were then centrifuged (9,000 rpm for 15 min), and the resulting pellets were retained until DNA extraction. Each replicate corresponds to two pooled seedlings. The roots analysed per replicate come from two seedlings, as does the root-adhering soil. The fresh weight of the shoots was measured after recovery of the roots for DNA extraction.

1.3. DNA extraction

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

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

The Operational Taxonomic Units (OTUs) were then defined by clustering at 97 % similarity. The OTUs were classified taxonomically using Blast (Altschul et al., 1990) and grouped into each taxonomic level into “counts” and “percentage” files. The phylogenetic position of bacteria in the different samples was characterised using taxa summary QIIME script until genus level (L6). OTU tables were rarefied at the lowest sequencing depth to control sequencing depth. OTU richness, diversity index and rarefaction curves were determined on a random picking of an equal sequence number in each sample (10,451 reads per sample) from the 36 samples through sequencing analysis by using the pipeline QIIME (Caporaso et al., 2010). After quality control trimming, a total of 238,757 sequences were generated from 36 samples and utilized for analysis. The average length of the sequences after processing was 400 bp for all samples. The clean sequences were clustered into OTUs at 97 % similarity levels (excluding the unclassified sequences at the phylum level), and sequences classified as chloroplast or cyanobacteria were also removed. The bulk soil, root-adhering soil and root tissues respectively displayed averages of 227, 242 OTUs and 372 OTUs, at 3 % divergence.
All statistical analyses were conducted using R version 3.1 (R Core Team, 2016). Unless otherwise specified, we determined statistical significance at $\alpha = 0.05$. PCoA and CAP analyses were conducted using the capscale function. PERMANOVA was conducted using the adonis function and Bray-Curtis dissimilarities were calculated using the vegdist function all from the Vegan package (Dixon, 2003).

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

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

Sequence data are available in NCBI SRA bank under accession number SRR5883473, experiment SRX3049495.
2. Results

2.1. Integrating phytobeneficial bacteria improves plant tolerance to saline stress

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

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

Bacterial inoculation significantly mitigated the effect of salt on plant growth, as shoot biomass was significantly higher when plants were inoculated (Fig. 1).

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

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

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

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

2.3 Salinity and co-inoculation impact on *V. faba* rhizosphere and root-associated microbiota
To evaluate the contribution of the microbiota assemblage to the plant tolerance to saline stress, we surveyed the bacterial diversity of bulk soil, root-adhering soil compartment, and root tissues of *V. faba* plants grown in the presence and absence of salt (70 mM). To visualize the effect of inoculation and saline stress on microbial community modulation in our data, we used principal coordinates analysis (PCoA) in combination with permutational multivariate analysis of variance (PERMANOVA) on Bray-Curtis dissimilarities between the samples. Consistent with our previous results (Haichar *et al.*, 2008, El Khaloufi *et al.*, 2016, Bressan *et al.*, 2009), this analysis revealed significant differences between the microbiota of the bulk soil, and that of root-adhering soil and root tissues, denoting the rhizosphere effect and the selection of specific populations from the surrounding soil by the roots (Fig. 2c, $R^2 = 0.28$, $p < 0.001$). Another observation from the PCoA analysis was that RAS samples clustered closer towards the bulk soil samples than did the root samples. Considering the saline stress, the samples of RAS are clustered in two groups (inoculated vs non-inoculated) and the one of RT in a single one (Fig. 2d, $R^2 = 0.20$, $p < 0.004$, perMANOVA).

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

Tolerance to saline stress could be linked to shifts in bacterial community composition in the root-adhering soil that correlate with an increase of plant salt tolerance. These changes could be considered as good indicators of a relationship between mitigation of saline stress and community composition. The LEfSe (Linear discriminant analysis Effect Size) analysis shows differentially abundant taxa as biomarkers using Kruskal–Wallis test ($p < 0.05$) with
LDA score > 2.0. We examined which OTUs differentiate the microbiota of salt-stressed plants from those without stress by surveying which bacterial groups have significantly different relative abundances. The roots of inoculated seedlings were specifically colonized by bacteria belonging to Betaproteobacteria (Oxalobacteraceae and Comamonadaceae) and Alphaproteobacteria (*Caulobacter*), while under saline stress, the roots specifically recruited bacteria belonging to Verrucomicrobia (*Opitutus*), Gammaproteobacteria (*Steroidobacter* and Sinobacteraceae) and Actinobacteria (*Microbacterium*) (Fig. 3a and 3b).

The RAS compartment was more impacted, as inoculation induced a selection of bacteria belonging to Gammaproteobacteria (*Trabusiella*), while saline stress induced a selection of bacteria belonging to Betaproteobacteria (Burkholderiales, Alcaligenaceae and Achromobacter) and Alphaproteobacteria (Rhizobiaceae). When inoculated plants were submitted to saline stress, the root-adhering soil hosted specifically Alphaproteobacteria (*Agrobacterium*, Caulobaterales, *Mesorhizobium*, Pyllobacteriaceae and *Erythrobacteraceae*) and to Gammaproteobacteria (*Arenimonas*, *Erwinia*, *Pseudoxanthomonas*) (Fig. 3c and 3d).

These modulations of microbial composition were complemented by principal components of the principal component analysis (PCA) illustrating the relative ordination of the various treatments based on the relative abundance of OTUs. On root system, NaCl treatment and/or bacterial inoculation led to an increase in the proportion of some taxa. Specifically, the proportion of organisms belonging to Enterobacteriaceae, Streptomycetaceae, Pseudomonadaceae, Xanthomonadaceae and Microbacteriaceae was increased when compared to the control. In contrast, salinity had a negative effect on Rhizobiaceae abundance; this decrease was less pronounced when *V. faba* was inoculated (Fig. 4a). The
roots of inoculated seedlings recruited mainly bacteria of the genus *Streptomyces* and *Enterobacteriaceae* and surprisingly bacteria of the genus *rhizobium* are less abundant at the level of the inoculated roots, suggesting that probably the inoculated rhizobia are preferentially established at the rhizosphere level. In the RAS compartment, analyses at the family level revealed a significant decrease in the proportion of *Enterobacteriaceae* in uninoculated seedlings following saline treatment. The RAS of inoculated seedlings, in the presence and absence of saline stress, are particularly colonized by members of the phylum of *Bacteroidetes* (*Sphingobacterium* and *Chryseobacterium*) (Fig. 4b). Therefore, these OTUs are probably involved in plant tolerance to salt. saline stress had less impact on community structure in the RAS when the seedlings were inoculated.

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

The co-occurrence or co-exclusion patterns of bacterial groups in the RAS and root compartments in response to inoculation, saline stress or both, were investigated and compared by network analysis (Fig. 5a). OTUs with a large number of positive and negative interactions indicating their importance in shaping bacterial groups are named hubs (Agler et al., 2016; Layeghifard et al., 2017). The shape of bacterial network of the different treatments was distinctly different and more importantly hubs were quite different (Fig. 5b). Although the network size is not significantly different between the different treatments, the number of modules determined by the phylogenetic molecular ecological networks ranged from 10 for control to 15 for NaCl treatment, indicating that the meta-community network was more dispersed and less connected in the presence of salt. This is also corroborated by the lower average connectivity observed for the saline treatment (Table 1). Proteobacteria
was dominated by Gammaproteobacteria which are highly reduced in abundance in the presence of salinity whether the plant is inoculated or not (Fig. 6a). The most abundant OTUs are not highly connected to the other members of microbial community, as nods are mainly represented by members of Alphaproteobacteria and Actinobacteria (Fig. 6a and 6b). Therefore, hubs are primarily represented by alphaproteobacteria and actinobacteria, indicating the importance of these bacterial groups in the microbial assemblage, except under saline stress in uninoculated plants, where members of alphaproteobacteria are not represented as hubs (Fig. 6c). We also notice that the proportion of hubs that are members of Actinobacteria is significantly reduced in inoculated plants in the presence and absence of saline stress (Fig. 6c). Venn diagrams shows that a high proportion of OTUs and nods are shared by all treatments (Fig. 6d and 6e). However, only very few hubs are common to all treatments (Fig. 6f). The relative number of positive (green) and negative (red) interactions indicated for each hub in each treatment, shows that the different hubs are mainly involved in co-occurrence interactions, and very few co-exclusion interactions are found (Fig. 6g). Some shared hubs behaved differently such as Sinorhizobium, which in the absence of saline stress displayed mostly negative interactions, while in the presence of saline stress and bacterial inoculation, it was mainly involved in positive interactions (Fig. 6g). We can also observe that the Gemm-5 OTU is essentially involved in co-exclusion interactions in the absence and presence of saline stress, whereas in the concomitant presence of saline stress and bacterial inoculation, this hub presents only co-occurrence interactions (Fig. 6g).

### 3. Discussion

Global climate change is presumed to have an impact on plant adaptation. To address this issue, plant growth adaptation must be optimized by integrating root-associated microbiota
(especially bacterial species with beneficial effects on plant growth and health) into agricultural practices (Busby et al., 2017). Microbial inoculants are considered to be environmentally friendly, and could provide a sustainable alternative technology for solving agro-environmental issues. Specifically, beneficial microbes can improve crop productivity to increase or support plant growth, nutrient availability and uptake, and plant fitness (Glick, 2014), thereby reducing the use of chemical fertilizers. The use of beneficial rhizobacteria (PGPR) to improve plant growth and yield under saline stress has been extensively investigated. It is acknowledged that bio-inoculation can increase tolerance, adaptation and protection of plants against salinity stress (Hashem et al., 1998; Fasciglione et al., 2012; Munns and Gilliham, 2015; Upadhyay and Singh, 2015; Qin et al., 2016). Indeed, an increase in plant biomass and the stimulation of root growth (allowing the enhanced uptake of nutrient elements and consequently alleviate the effects of saline stress in different legume species) has been attributed to different rhizobial strains that can establish a symbiotic association under saline stress conditions (Nogales et al., 2002; Bertrand et al., 2015). In addition, inoculation of maize plants with P. fluorescens containing ACC deaminase has been reported to significantly enhance root elongation and plant biomass under saline conditions (Kausar and Shahzad, 2006). Although most studies have focused primarily on changes in the physiological and metabolic status of plants after inoculation with plant growth promoting rhizobacteria (PGPR), a limited number of reports have investigated the effects of inoculation on the diversity of soil microbial communities (Gupta et al., 2014; Chihaoui et al., 2015; Bharti et al., 2016). We have examined here the effect of a combined application of three selected bioinoculants (the V. faba-nodulating R. leguminosarum RhOF125, E. meliloti RhOL9, and P. mucilaginosus BLA7) on bacterial diversity and bacterial interaction network in the rhizosphere of V. faba cultivated under saline stress.
As expected, our glasshouse experiment revealed the positive effects of the PGPR bioinoculants on the growth of *V. faba* plants. Specifically, we observed with 70 mM NaCl treatment that shoot fresh matter was significantly increased (*p*<0.05) after bacterial inoculation (mix of three strains) as compared to the control. These differences are thought to be related to direct effects of PGPR in enhancing plant growth, as well as non-target effects of bioinoculants on resident bacterial populations in the *V. faba* rhizosphere and roots. In the present study, the relative abundance profiles suggested a shift in the bacterial community composition of the inoculated plants in comparison with the initial bacterial diversity, especially in the root-adhering soil compartment.

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

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

We used PCoA analysis to evaluate the main driver of bacterial diversity in response to
inoculation, salt or both. Among the selected variables, the compartment (RAS vs root
tissue), inoculation and saline stress, the latter was found to be the main driver of microbial
composition shift. If inoculation does not have any impact on microbiota shift that means
that it may have a direct impact on plant physiology by increasing its tolerance to salt and
also by creating a safer environment for the root-colonizing beneficial microbiota, probably via root exudation. It has been suggested that such an effect might be due to root exudation of specific molecules responsible for chemotactic attraction of specific bacterial populations in the rhizosphere from the soil reservoir (Borruso et al., 2014). Additionally, Trabelsi et al. (2011) found that in Phaseolus vulgaris, rhizobacterial populations of Alphaproteobacteria (Rhizobiaceae) and Gammaproteobacteria were enhanced by mixed inoculation with Sinorhizobium meliloti 4H41 and Rhizobium gallicum 8a3. Likewise, Gupta et al. (2014) proposed that this enhancement could be attributed to the fact that bioinoculants directly and/or indirectly promote the growth of existing soil bacterial populations by releasing/fixing/solubilising certain substrates that act as nutrients or inhibitors of pathogenic microorganisms.

The saline stress is an important driver of the microbiota assemblage, acting through selective enrichment of specific taxa, including Microbacterium, Opitutus and Steroidobacter on the root tissues. In the root-adhering soil, members of Betaproteobacteria (Achromobacter), and Bacteroidetes (Flavobacterium) were specifically enriched. These bacterial populations are whether the most tolerant to saline stress or could proliferate because their antagonists are sensitive to saline stress. However, their enrichment did not enable the plant to better tolerate saline stress, or to a lesser extent than may have done taxa enriched under saline stress and inoculation, as members of Alphaproteobacteria (Agrobacterium, Mezorhizobium...) and Gammaproteobacteria (Erwinia, Arenimonas). Enterobacteriaceae seem to have shifted from the RAS to the roots in the presence of saline stress. Similar results on the effects of NaCl in the rhizosphere of various agricultural crops (Li et al., 2016; Nie et al., 2016; Hahm et al., 2017; Ibekwe et al., 2017) and on natural
halophytes (Liu et al., 2016; Yang et al., 2016) have been previously reported. However, the length of 16S sequences obtained with current high-throughput sequencing techniques is relatively short, and does not allow us to describe OTUs at the species level, and since within the same bacterial genus, phytobeneficial bacteria and phytopathogens can co-exist, we can hardly claim that the plant, under certain stress conditions, will select specifically beneficial microbiota.

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

It is possible to identify positive and negative co-occurrence correlations between bacterial community members, which may reflect synergistic and antagonistic functional relationships (Faust and Raes, 2012). Such interactions can be analyzed and visualized as networks and may help understanding host-associated microbiome dynamics. We used network analysis to explore complex interactions between microorganisms. Microbial networks are spatiotemporal representations of ecosystems and are composed of nodes and edges. Connectivity, represented by the number of links between a node and other nodes, is an indicator of the strength with which an OTU is connected to other OTUs. OTUs having an extraordinarily large number of positive and negative interactions and named hubs and thus appear important drivers of bacterial communities shaping (Agler et al., 2016; Layeghifard et al., 2017). Our network analysis evidenced that hubs are mainly members of Alphaproteobacteria and Actinobacteria and also members of very rare bacterial groups.
such as Gemmatimonadetes, Verrucomicrobia, Acidobacteria and Chloroflexi. Our analysis indicates that a hub involved in positive interactions under certain conditions may behave differently under stress conditions and establish mainly antagonistic interactions. Such behavior might be linked to the expression of genes encoding antimicrobial compounds concomitantly to those involved in stress tolerance, or at the contrary, the stress tolerance mechanism requiring energy, may be expressed at the expense of genes encoding antimicrobial compounds. The latter hypothesis is likely more common, as the case of *Sinorhizobium* and an OTU member of Gemmatimonadetes, which are mainly involved in positive interactions only in the presence of saline stress, while they exhibit antagonistic interactions in the absence of saline stress.

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

Several questions remain unresolved. How plants respond to a change in microbial networks and assemblage might be assessed by metabolomics, transcriptomics and proteomics approaches, but determining whether these modifications are imposed on plants or whether rearrangements of the interaction network are induced by the plants is much more
challenging. In addition, we can hardly define the network in which microorganisms can cooperate to thrive harsh conditions and help the plant to withstand such stresses.

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

4. Conclusions

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

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

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

References


Chihaoui, S. A., Trabelsi, D., Jdey, A., Mhadhbi, H., Mhamdi, R. (2015). Inoculation of *Phaseolus vulgaris* with the nodule-endophyte *Agrobacterium* sp. 10C2 affects richness
and structure of rhizosphere bacterial communities and enhances nodulation and

43(6), 1229–1236. doi: 10.1016/j.soilbio.2011.02.012

Coleman-Derr, D., and Tringe, S. G. (2014). Building the crops of tomorrow: advantages of
symbiont-based approaches to improving abiotic stress tolerance. *Front. Microbiol.* 5,
283. doi: 10.3389/fmicb.2014.00283

along an estuarine salinity gradient: combined influences of bacterial growth and


plant stress tolerance by *Enterobacter* sp. SA187 is mediated by 2-keto-4

https://doi.org/10.1371/journal.pgen.1007273

927–930.

Microbiote shift in the *Medicago sativa* rhizosphere in response to cyanotoxins extract

Physiological and antioxidant responses of *Medicago sativa*-rhizobia symbiosis to


Figure legends

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

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

Figure 3. Specific shift of beta diversity mediated by inoculation and salt stress. Phylogenetic cladogram of OTUs specific (a) to roots of inoculated seedlings and to roots of seedlings submitted to saline stress, (b) to RAS of seedlings inoculated, under salt stress or submitted to both treatments. (c, d) Linear discriminant analysis effect size (LEfSe) analysis shows differentially abundant taxa as biomarkers using Kruskal–Wallis test (P < 0.05) with Linear Discriminant Analysis (LDA) score > 2.0. OTUs. The circles from inside to outside indicate bacterial taxonomic levels from phylum to genus. Yellow dots represent bacteria not significantly varying in abundance among treatments. Biomarker bacteria are colored according to their corresponding class colors on the right.
Figure 4: Relationship between diversity and inoculation and/or salt stress. First PC1 and second PC2 principal components of the principal component analysis (PCA) illustrating the relative ordination of the various treatments based on the relative abundance of OTUs (a) in the roots-adhering soil samples, (b) in the root samples. Taxa correlated with each treatment are indicated.

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

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

Figure 6: Beta diversity and interaction network features. Impact of salt, inoculation and both treatment on the relative abundance at phylum level and class level for Proteobacteria of (a) OTUs, (b) nods and (c) hubs in each treatment in the rhizosphere of V. faba. Venn diagram representing the shared and specific (d) OTUs, (e) nods and (f) hubs in the different treatments. (g) Relative number of positive (green) and negative (red) interactions (edges from network) for each hub in each treatment.
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<table>
<thead>
<tr>
<th>Treatment</th>
<th>network size</th>
<th>Link</th>
<th>average connectivity</th>
<th>average geodesic distance</th>
<th>average clustering coefficient</th>
<th>modularity</th>
<th>number of modules</th>
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<tbody>
<tr>
<td>Control</td>
<td>181</td>
<td>951</td>
<td>11.41</td>
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<td>1114</td>
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<tr>
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<td>1167</td>
<td>11.95</td>
<td>3.66</td>
<td>0.79</td>
<td>0.65</td>
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<tr>
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<td>1145</td>
<td>11.37</td>
<td>3.2</td>
<td>0.8</td>
<td>0.63</td>
<td>10</td>
</tr>
</tbody>
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