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

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ABSTRACT

Plant roots are inhabited by a large diversity of microbes, some of which are beneficial for the growth of plants and known as plant growth promoting rhizobacteria (PGPR). In this work, we designed a multispecies inoculum of PGPRs containing *Rhizobium phaseoli*, *Sinorhizobium americanum* and *Azospirillum brasilense* nitrogen-fixing strains and other plant-growth promoting bacteria such as *Bacillus amyloliquefaciens* and *Methylobacterium extorquens*. We evaluated the effect of this group of bacteria on the growth of one-month-old maize plants. The multispecies inoculum exerted a beneficial effect on maize plants that was greater than that obtained with single-bacteria. Using the same multispecies inoculant, acetylene reduction was recorded in 5-day-old roots indicating active nitrogen fixation by bacteria in maize. *Azospirillum* nitrogen fixation was lower than that obtained with the multispecies inoculum. We focused on the analysis of *R. phaseoli* gene expression in presence of other PGPRs. Many *R. phaseoli* up- regulated genes in roots in the presence of other bacteria are hypothetical, showing our poor knowledge of bacteria-bacteria interactions. Other genes indicated bacterial nutrient competition and *R. phaseoli* stress. Differentially expressed transcriptional regulators were identified that may be key in bacteria-bacteria interaction regulation. Additionally, gene expression was analyzed from *Azospirillum* but not from sinorhizobia and methylobacteria due to the low number of transcripts obtained from maize roots. The metatranscriptomic analysis from maize roots showed expression of *Azospirillum nif* genes in the presence of PGPR bacteria. Our hypothesis is that other bacteria stimulate *Azospirillum* capacity to fix nitrogen and this should be further explored.

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Introduction

In nature and agricultural fields there is a great diversity of bacteria associated with plants. Many plant-borne bacterial isolates when individually tested in plant trials are able to promote plant growth [20,26,27,33,38,41,42]. Plant growth promoting rhizobacteria (PGPR) can produce phytohormones or volatile substances, solubilize nutrients, fix nitrogen or inhibit pests and pathogens. Among them, nitrogen-fixing bacteria are in general a minority, possibly due to the metabolic load of nitrogen fixation [2,37,46].

Most trials with PGPRs are carried out with plants inoculated with a single species. However, considering that different bacterial species co-exist in soil and plants, recent inoculation trials have made use of more than single strains, and combinations of *Azospirillum* and rhizobia, or bacilli and rhizobia have been successful in plant-growth promotion [3,59,66,85]. The inoculation of *Azospirillum* and *Bradyrhizobium* improves nodulation and nitrogen fixation in legumes such as soybeans [40] and, in chickpeas a combined inoculation with *Mesorhizobium, Pseudomonas* and the fungus *Piriformospora indica* increased dry weight of plants by 31% [57]. Similarly, the co-inoculation of *Azospirillum* with rhizobacteria increased corn grain production [60] and the co-inoculation of *Azospirillum brasilense* Az 39 and *Bradyrhizobium japonicum* E109 improved growth and yield of soybean exposed to arsenic [11].

Among the PGPRs, *Azospirillum* deserves special attention. It was one of the first diazotrophs isolated from non-legumes [75,78]. A

Abbreviations: PGPR, plant growth promoting rhizobacteria; MI, multispecies inoculum; MCPs, methyl-accepting chemotaxis proteins.

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global analysis of 30 years of *Azospirillum* inoculation trials in agricultural crops, including corn, showed an average increase in yield of 10–30% [60]. In a controlled trial under water stress, *Azospirillum* in maize plants increased the number of shoots by 10% and the height of the plant by 7% [13]. Given its plant growth promoting properties, *Azospirillum* is now widely used as a commercial product. Three different enzymatic routes have been described to produce auxins in *Azospirillum* [72,75], and mutants that overproduce auxins are more efficient at promoting plant growth [31]. Thus, the production of auxins by *Azospirillum* is considered the main mechanism of plant growth promotion [20,72].

Bacillus is considered a PGPR as it has also been reported to improve plant growth [24,76,83], and produce auxins [43] as well as many antibiotics [7]. *Bacillus* is also known to increase corn plant resistance against drought [82], and osmotic stress [25]; as well as to increase plant biomass, relative water content and water potential of the leaf [82]. A great diversity of bacilli has been found in the roots of maize [64], and of *Phaseolus vulgaris* [55]. As *P. vulgaris* and maize are grown as associated crops [62], they presumably share similar root microbiomes, including *Bacillus* symbionts which have been found also to be seed-borne [50].

Methylobacteria are found in many plants in roots, leaves and stems [71], and are capable of producing cytokinins and metabolizing the methanol produced as a secondary product during plant wall synthesis [47]. Furthermore, they are known to promote growth in plants and mosses [47]. *Methylobacterium extorquens* is a bacterium with high potential to promote the growth of corn and other crops such as wheat, tomatoes, strawberries and tobacco [1]. *Methylobacterium* has also been shown to help maize plants cope with salinity conditions (150 mM NaCl) [45].

Rhizobia are the best-studied plant symbionts and have been used in agriculture for over 120 years. They are used in commercial inoculants for a variety of crops, especially soybeans [79]. Rhizobia form nodules and fix nitrogen. In nodules, rhizobia can fix more than 250 kg of N per ha per year, which is equivalent to a substantial chemical fertilization. In addition, rhizobia can colonize the rhizospheres of many plants and the internal tissues of non-legumes [45,79]. Rhizobia can exert beneficial effects on non-legume plants similar to those triggered by other PGPRs. *nif* genes necessary for the free-living nitrogen fixation were found in *Sinorhizobium* [28], suggesting that *Sinorhizobium* could be a candidate for nitrogen fixation in non-legumes.

Due to worldwide cereal consumption, the identification of mechanisms to increase yield and to reduce the cost of crop production is an active area of research. Cereals require large amounts of expensive nitrogenous chemical fertilizers resulting in a considerable cost to farmers [70]. Thus, the identification of methods or organisms that allow nitrogen fixation would be of benefit to the cereal farming industry [29], and would potentially reduce the impact of fertilizers on the environment.

Bacterial genes of *Bacillus* [84], *Azospirillum* [17], *Sinorhizobium* [51] and *Rhizobium* [86] have been shown to be differentially regulated when inoculated onto plants in trials of a single strain. Specifically, *Bacillus* grown in maize exudates showed increased expression of genes for nutrient utilization, synthesis of antimicrobial peptides, chemotaxis, motility [34] and biofilm formation [87]. *A. brasilense* showed increased expression of genes involved in biofilm formation, chemotaxis and nitrogen fixation (*nif* genes) in wheat roots [17]. In rice roots, *Azospirillum lipoferum* expressed genes related to detoxification of ROS and multidrug flow pumps, however, *nif* genes were not induced [32]. An antioxidant response has also been described in *Methylobacterium* when exposed to exudates from the soybean root [10]. In *Rhizobium phaseoli,* genes expressed in roots are involved in exudate transport and in the defense to plant phytoalexins [54].

In addition to changes in response to roots and exudates, bacterial gene expression can be further altered in the presence of other root bacteria [50]. Bacteria–bacteria interactions in roots have been rarely characterized by functional genomics. Since *R. phaseoli* is our main research interest, we focused on the analysis of its gene expression with the aim to detect genes that are expressed in the presence of PGPRs by using a metatranscriptomic study with native Mexican maize.

Materials and methods

Strains and bacterial cultures

R. phaseoli Ch24-10 was isolated from three-month-old corn plants in Cholula, Puebla. This strain was the most competitive corn endophyte in inoculation experiments [68]. *A. brasilense* sp.7 was isolated from *Digitaria decumbens* and proved to promote maize, rice and wheat growth in different assay [32,78]. *Bacillus amyloliquefaciens* CCGE2031 is an endophytic bacterium of *P. vulgaris* [55]. *M. extorquens* bacteria are common leaf epiphytes and capable of colonizing different plants, *M. extorquens* AM1 is one of the most studied methylobacterial strains and was kindly provided by ME Lidstrom, University of Washington, USA [9]. *Sinorhizobim americanum* CFNEI156 was isolated from *Acacia* nodules [80] and promotes *Acacia* plant growth [21].

Multispecies culture in PY medium and RNA extraction

Multibacterial cultures were started by inoculating 30 ml (final volume) of PY medium with *R. phaseoli* Ch24-10, *A. brasilense* Sp7, *M. extorquens* AM1, *B. amyloliquefaciens* CCGE2031 and *S. americanum* CFNEI 156. *R. phaseoli* Ch24-10 (single-strain) culture had an initial bacterial concentration of 5×10^8 in 30 mL of liquid PY. All treatments were in triplicate. After 24 h, RNAlater (Ambion) was added to attain a 10% final concentration, samples were centrifuged for 15 min at 464 g at 4 °C to obtain a bacterial pellet from which total RNA was extracted (Fig. S1). RNA extraction was performed using the Qiagen protocol [65]. RNA obtained was quantified by Nanodrop 2000 (Thermo Scientific). The integrity of the total RNA was verified by electrophoresis in 1% agarose gels as well as by Bioanalyzer 2100 (Agilent Technologies).

Germination, inoculation and growth of one month plants

Creole black seeds from Hidalgo. Mexico, were superficially disinfected as described [68]. Disinfected seeds were germinated on agar-water plates in the dark for 48 h at 30 °C, and subsequently transplanted into flasks with sterile vermiculite, one seed per flask. Plants were inoculated with 1 mL of culture with 10^8 cells mL^{-1} of each bacterium, individual inoculants were used with each of the bacteria separately: *R. phaseoli* Ch24-10, *A. brasilense* Sp7, *M. extorquens* AM1, *B. amyloliquefaciens* CCGE2031 and *S. americanum* CFNEI 156. Maize plants were inoculated as well with all bacteria together (referred to as multispecies noculum or MI). Non-inoculated plants were negative controls. Plants were watered once a week with nitrogen-free Fahraeus solution (0.132 g/L $CaCl_2$, 0.12 g/L $MgSO_4 \cdot 7H_2O$, 0.1 g/L KH_2PO_4, 0.075 g/L $Na_2HPO_4 \cdot 2H_2O$, 5 mg/L Fe-citrate and 0.07 mg/L each of $MnCl_2 \cdot 4H_2O$, $CuSO_4 \cdot 5H_2O$, $ZnCl_2$, H_3BO_3 and $Na_2MoO_4 \cdot 2H_2O$) [36], adjusted to pH 7.5 before autoclaving, alternating with sterile deionized water. Plants were maintained at 28 °C with a light/dark period of 12 h. Thirty days after inoculation, shoot fresh and dry weight and chlorophyll content were measured (with the portable LI-6400XT photosynthetic systems) [48]. Data obtained from plant length, dry weight and chlorophyll content were analyzed with ANOVA variance and the Tukey comparison tests ($p = 0.05$) (Fig. 1).

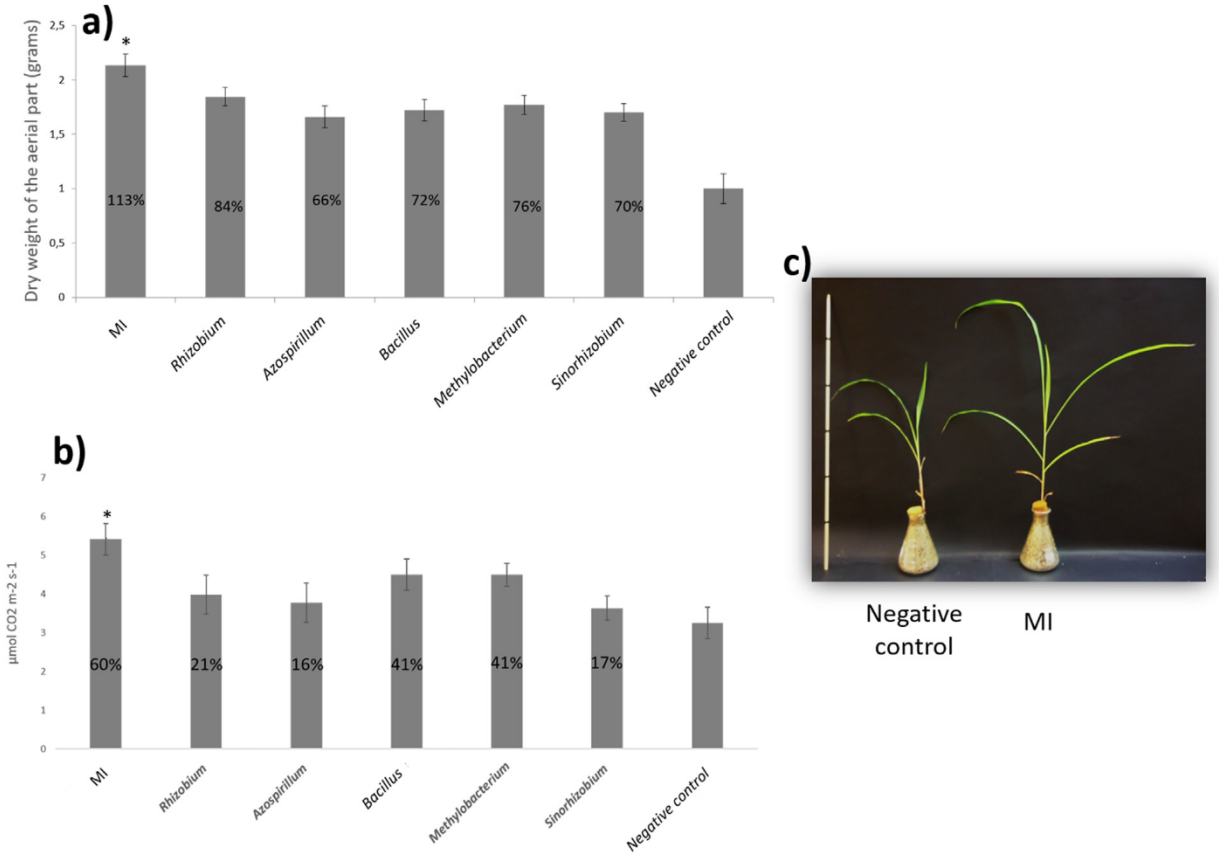

Fig. 1. (a) Average dry weight of ten shoots from thirty day-old plants inoculated with the multispecies inoculum or single strains, negative controls were non-inoculated plants; (b) Average photosynthetic rate of ten leaves from thirty day-old plants inoculated with the multispecies inoculum or single strains. Numbers within the bars are differences in percentage compared to the negative control. Asterisks indicate treatments that showed statistical significant differences obtained with the Tukey test (p value < 0.05). Lines represent ± standard deviation (SD). (c) Maize non-inoculated plant (negative control) and a plant colonized with MI 30 days after inoculation.

ARA assay to determine nitrogen fixation in maize plantlets five days post-inoculation

Corn seeds were disinfected, germinated and maintained in hydroponic and axenic conditions. The disinfected seedlings were transferred to glass tubes (one per tube). Seedlings were inoculated with 1×10^8 bacteria resuspended in Fahraeus medium. Ten technical replicates were used per treatment, which consisted of inoculating each of the individual bacteria (*R. phaseoli* Ch24-10, *A. brasilense* sp7, *M. extorquens* AM1, *B. amyloliquefaciens* CCGE2031 and *S. americanum* CFNEI 156). Ten maize plants were also inoculated with the MI that contained 10^8 bacterial cells of each species. Positive control plants were inoculated with *Klebsiella variicola* [69] and negative control plants were not inoculated. Plants were kept for five days at 28 °C with a light/dark period of 12 h. Under those conditions plants fitted well in the containers without showing stress symptoms [54].

Nitrogenase activity was estimated by an acetylene reduction assay with ten roots per treatment, as described [74]. After five days, 5 g of roots (by treatment) were cut and sealed in 70 ml vials, one ml of air with acetylene and vials were incubated at room temperature. After 24 h, 1 mL of gas was removed from each vial and analyzed by gas chromatography to measure the ethylene concentration. The specific activity was expressed as nmol ethylene min^{-1} vol^{-1}. ANOVA and the Tukey comparison test ($p = 0.05$) were used to analyze variance.

RNA extraction from plant rhizoplane, construction of the RNA-seq library and sequencing

For RNA extraction, plants were grown under hydroponic and axenic conditions as described above. Disinfected seedlings were transferred to glass tubes (one seedling per tube) containing 10 mL of Fahraeus medium. Plants were inoculated with 1×10^8 bacteria for single inoculant trials while controls were without inoculant. Plants for the MI assay were inoculated with 10^8 cells from each species. Plants were kept at 28 °C with a light/dark period of 12 h.

Five days after inoculation, fifty rootlets were placed in 50 mL of $MgSO_4$ (10 mM) with 5 mL of RNAlater stabilization solution (Ambion) and bacteria were detached from roots using an ultrasonic cleaner by the indirect method for 20 min. The liquid was filtered with miracloth (pore size 22–25 µm) to remove plant debris and bacteria were recovered by centrifugation (464 *g*, 15 min, 4 °C). The cell pellet was resuspended in RNAlater solution. Total RNA was extracted as in [15] using a Qiagen RNeasy Mini Kit (50) and treated with DNAse (Qiagen) following the manufacturer's instructions. RNA high-throughput sequencing was performed in the Genomics and Bioinformatics Service of Texas A&M AgriLife. rRNA was eliminated using the Ribo-Zero rRNA Removal Kit (Bacteria) Protocol (Illumina). Sequencing libraries were prepared using the TruSeq Stranded protocol (Illumina) and sequenced with the Illumina HiSeq 2500 platform (125 nt paired-end reads) (Fig. S1).

In total, twelve libraries were sequenced: Six from PY media (three of *Rhizobium* alone, and three of the MI) and six from plants

(three of *Rhizobium* alone, and three of the MI, (Fig. S1). The raw sequencing data is available at NCBI's Sequence Read Archive (SRA), under bioproject number PRJNA499163.

Data analysis

The raw sequencing data were scanned for adapters and the quality was evaluated using FASTQC (v0.11.2) [8]. Sequencing adapters and low quality sequences were filtered using Trimmomatic (v0.38) [14] with the following parameters: 'ILLUMINACLIP: Adapter.fasta: 2:30:7 SLIDINGWINDOW:4:15 MINLEN:60'. Sequencing paired end reads were aligned to a composite fasta file containing the full genomes of all members of the community (versions NZ_CM003278.1 *R. phaseoli* Ch24-10 plasmid pRphCh2410a, whole genome shotgun sequence, NZ_CP012914.1 *A. brasilense* strain Sp7, complete sequence, NC_014551.1 *B. amyloliquefaciens* DSM7 complete genome, NC_012808.1 *M. extorquens* AM1, complete genome, NODE_1_length_389726_cov_11.7871_ID_2128270, downloaded from NCBI) as well as the latest version of the genome of *Zea mays* (version NC_024459.2 *Zea mays* cultivar B73 chromosome 1, B73 RefGen_v4, whole genome shotgun sequence, downloaded from NCBI) using Bowtie2 (v2.1.0) [49], with the following parameters: 'sensitive -rg 1000,1000 -rfg 1000,1000 1 -20'. Any single paired-end read was only considered as being transcribed from a specific genome if both ends of each fragment aligned to the same genome. Only uniquely mapping paired-end reads were used for quantification. Gene abundance was quantified using featureCounts from the Subread package (v1.6.2) [52] using the following parameters: '-T 8 -p -a -t CDS -g ID -o. /'. Genes that did not have at least one count per million (cpm) in at least one sample were removed from subsequent analyses (Fig. S2).

The R package, edgeR (v.Bioconductor 3.7 v.R 3.5.1 v.edgeR_3.22.5 [67]), was used to estimate differential gene expression, considering that a gene was differentially expressed if it had an FDR < 0.05. Functional annotation was performed with Trinotate (v3.0.1), and GO term enrichment analysis was performed with the topGO R package (v2.32.0) [5] using a classic Fisher's test and establishing an enrichment threshold of p value < 0.05 (Fig. S2).

Results

Multispecies inoculum improves corn growth

We compared plants grown for 30 days with a single bacterial strain or with the MI (see methods). The weight of the aerial part of the plants and chlorophyll content were significantly larger in plants inoculated with the MI than those of plants inoculated with a single bacterial strain (Fig. 1). Using the same multispecies inoculum, at five days in hydroponics we detected acetylene reduction activity which was detected as well from roots inoculated with *Azospirillum* or *Klebsiella* (used as a positive control) (Fig. 2). The metatranscriptomic analysis was carried out with maize plants five days after inoculation (see below).

Differential gene expression in R. phaseoli *Ch24-10 in corn plants*

We carried out a metatranscriptomic analysis of bacteria in free-living conditions (in culture PY medium) and in maize roots. Each sample had three biological replicates (Fig. S1). We obtained on average 40 million reads per sample that after quality filtering were mapped to reference genomes. The Spearman correlation coefficient of the read counts for the inoculated libraries of *R. phaseoli* alone and *R. phaseoli* in the MI was 0.90 on average, indicating high reproducibility between biological replicates (Fig. S3).

When we compared samples of *R. phaseoli* Ch24-10 from PY medium and plants, 730 genes were found to be significantly up-regulated (log2 fold-change >1 and FDR ≤ 0.05) (Dataset S1) and 376 genes were significantly down-regulated (Dataset S2). Genes up-regulated in *R. phaseoli* in the presence of roots encoded transporters for putrescine, urea or cations (Dataset S1). Genes related to nitrogen fixation NifX and NifZ, nitrogenase cofactor biosynthesis protein NifB, and the nitrogenase stabilization protein NifW (Fig. 3) and cellular respiration were also found to be up-regulated in *R. phaseoli* including the genes encoding the FixP subunit of the type T cbb3 cytochrome c oxidase, the CcoS assembly protein of cytochrome oxidase type cbb3, cytochrome b, cytochrome c and cytochrome o ubiquinol oxidase subunit I.

Within the genes that were significantly down-regulated (when comparing *R. phaseoli* Ch24-10 grown in PY versus plants), were those encoding chemotaxis proteins CheR and CheW and the regulator of the chemotactic protein-glutamate methylesterase response or CheB. Down-regulated genes for flagella were those encoding the basal body protein (FliL), the basal rod proteins (FlgC and FlgG), the hook-basal protein complex (FliE), the motor switch protein FliN, flagellin, flagellin protein C, the regulator of flagellin synthesis and the repressor of flagellum biosynthesis (FlbT) (Dataset S2).

A particular relevant comparison was the one considering *R. phaseoli* Ch24-10 versus *R. phaseoli* Ch24-10 within the MI, both in roots that allowed us to identify genes expressed in the presence of other bacteria. Specifically, we found 110 genes to be up-regulated (Dataset S3) and 53 genes down-regulated (Dataset S4). Genes that increased their expression included those for transport which encode an ATP binding protein, sugar ABC transporter permease, sugar ABC transporter substrate binding protein, MFS transporter, ABC peptide transporter, ATP binding protein of the sugar ABC transporter and many hypothetical genes [53], (Dataset S3).

Notably, when *Rhizobium* is the only bacterial inoculant in maize roots, its machinery for nitrogen fixation and respiration was expressed (Fig. 3), while when *Rhizobium* is inoculated as part of the MI community, its nitrogen fixation machinery is repressed. Genes down-regulated in *R. phaseoli* in the MI in roots were those encoding the flavoprotein subunit of electron transfer proteins of the alpha/FixB family, as well as the nitrogen fixation protein NifZ, nitrogenase cofactor biosynthesis protein NifB, and nitrogenase, iron-molybdenum co-factor biosynthesis protein NifN (Dataset S4).

Azospirillum *differential gene expression*

When we compared *Azospirillum* genes expressed from the MI in plants to MI in PY, we found that *Azospirillum* in roots of corn over-expressed 1147 genes (Dataset 5) and 1376 genes were down-regulated (Dataset 6). Among the over-expressed genes, we found those involved with ABC type transporters and carbohydrate ABC transporter permease. Some of the notable over-expressed genes are for toxin biosynthesis, such as the HicB toxin-antitoxin system. The toxins of all known TA systems are proteins, while the antitoxins are proteins or small RNAs (sRNAs). The TA systems act on crucial cellular processes that include translation, replication, cytoskeleton formation, membrane integrity and cell wall biosynthesis [81]. Other up-regulated genes are those involved in nitrogen fixation, like Fe-S cluster assembly family protein NifU, nitrogen fixation proteins NifZ, NifW, and NifU family protein (Fig. 4). These proteins are responsible for carrying out biological nitrogen fixation in free-living conditions. Also over-expressed were genes for the production of auxins such as indolepyruvate/phenylpyruvate decarboxylase, indole-3-glycerol phosphate synthase (TrpC), which would have a role in plant-growth promotion [73]. Within the genes that were down-regulated in *Azospirillum* from plants, we found those involved with chemo-

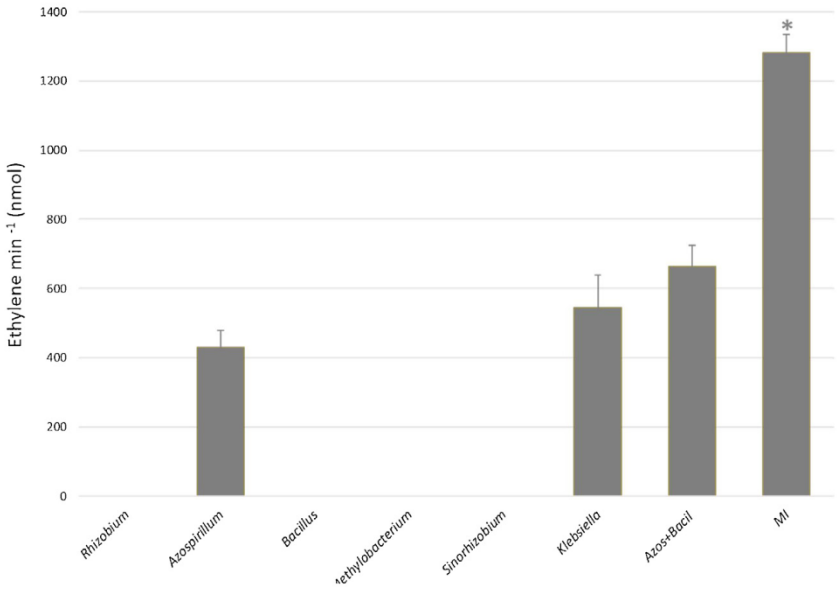

Fig. 2. Ethylene produced in maize roots by the acetylene reduction assay (ARA) using different single species, the MI and *Klebsiella* positive control, *Azos + Bacil* was the combination of *Azospirillum* plus *Bacillus*. The asterisk shows the statistically significant treatment obtained with the Tukey test (p value < 0.05). Lines indicate ± standard deviation (SD).

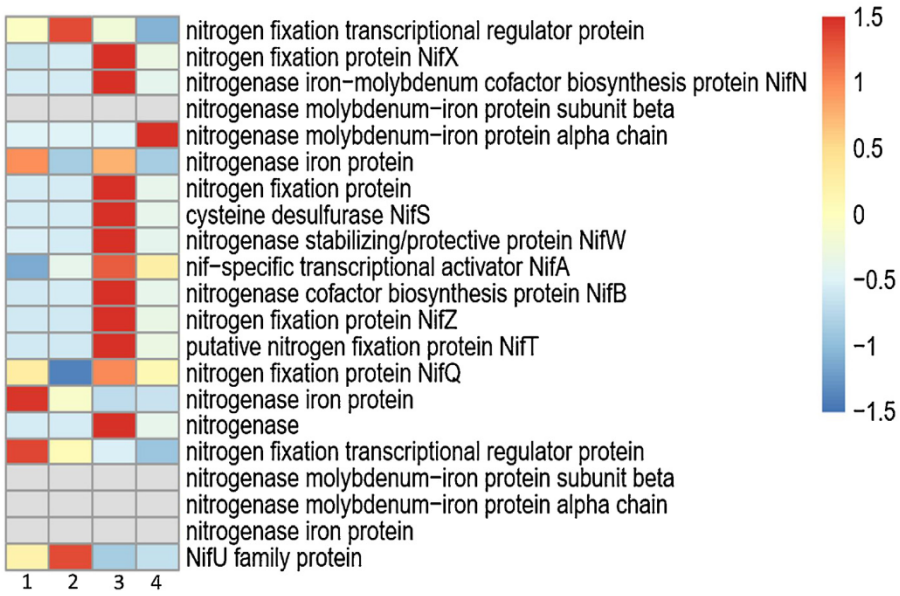

Fig. 3. Fold change of *nif* gene expression in different comparisons. Column (1) *R. phaseoli* in the multispecies inoculum (MI) in PY to *R. phaseoli* in PY; (2). *R. phaseoli* in MI in plant to *R. phaseoli* in plant; (3) *R. phaseoli* in plant to *R. phaseoli* in PY, (4) *R. phaseoli* in MI in plant to *R. phaseoli* in MI in PY.

taxis and flagellar assembly, and some transcriptional factors such as GntR, TetR/AcrR, XRE, Fis, LysR, and ArgP.

Bacillus *differential gene expression*

When analyzing *Bacillus* transcripts from MI in maize roots, we found 362 over-expressed genes (Dataset 7) and 254 sub-expressed genes in comparison to *Bacillus* in MI in PY medium (Dataset 8). Many of the over-expressed genes are involved with cell motility, flagellum synthesis, biofilm formation [16,23,34]. Genes for iron, magnesium and sugar transporters were also over-expressed. Down-regulated genes mainly encoded components of ABC type transporters and enzymes for carbohydrate metabolism, as well as many hypothetical proteins.

Discussion

Although it is known that microorganisms favor the growth of crops, less is known about the interactions between microbes in plants and how this influences plant growth promoting properties [3,4,19,20,57,66,72]. Notably, we found that plants with not available fixed-nitrogen and inoculated with MI were significantly larger than plants inoculated with a single species or non-inoculated plants. The transcriptome results showed that *Azospirillum* is the main nitrogen-fixing bacterium in the MI in roots as *Azospirillum nif*

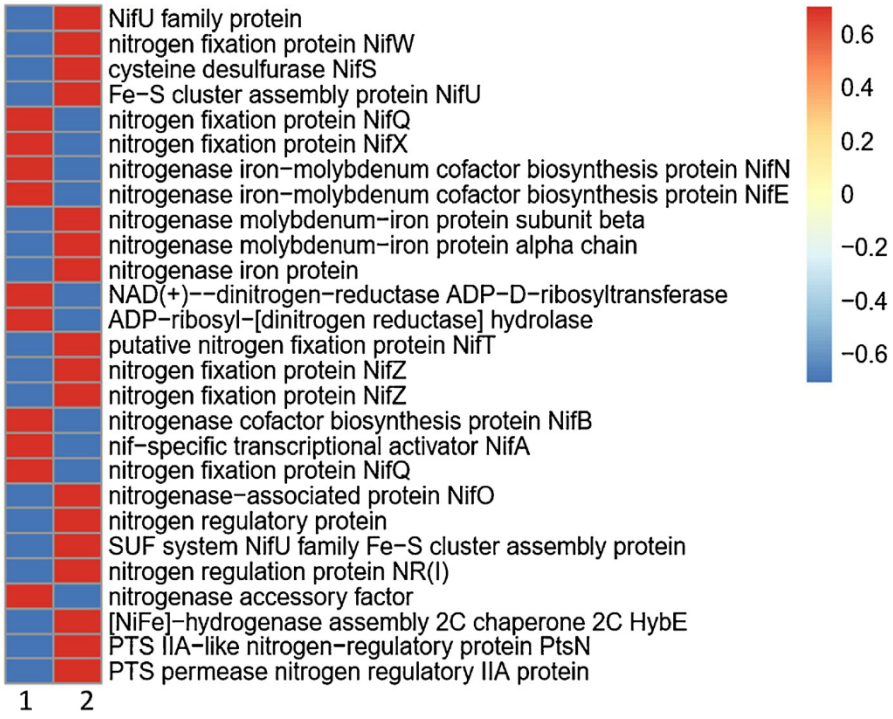

Fig. 4. Fold change of *Azospirillum nif* genes expressed in the presence of other bacteria either in PY medium or in plant. (1) *Azospirillum* in MI in PY, compared to *Azospirillum* in MI in plant (2) in MI in plant, compared to *Azospirillum* in MI in PY. Down-regulated genes are in blue, in red up-regulated. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

genes were found highly expressed. In congruence acetylene reduction was detected in maize plants inoculated only with *Azospirillum*.

The few reads assigned to *Methylobacterium* and *Sinorhizobium* precluded a differential expression study to be performed for these bacteria (Fig. S4). In roots with the MI, most transcript-reads corresponded to *Azospirillum* and in one plant *Rhizobium* transcripts (not analyzed here) were as abundant as those from *Azospirillum* (Fig. S4), suggesting that these bacteria were successful maize-root colonizers in one-month-old plants. We also tested an inoculum of *Azospirillum* and *Bacillus* in hydroponics as *Bacillus* transcripts were abundant in MI in PY, but this double inoculant did not attain the nitrogen fixation levels obtained with the MI (Fig. 2). Thus, it seems plausible that the community as a whole provides suitable conditions for *Azospirillum* to fix nitrogen. Additional plant growth promotion tests using bacterial mixtures eliminating one by one the bacterial members of the MI would help to determine the minimum number of strains to achieve maximum nitrogen fixation in maize. Biofilm formation induced in *Bacillus* by root exudates [22] and *Azospirillum* forming aggregates [41], may provide favorable nitrogen-fixing conditions by protecting bacteria from oxygen. Bacteria in the MI may help to maintain low oxygen conditions or may provide nutrients that stimulate *Azospirillum* nitrogen fixation. There are examples of metabolic complementation that can occur between different symbiotic species in laboratory assays [58]. The functional synergism that can lead to the promotion of growth by the community tested on the roots may reflect long term bacterial interactions in the rhizosphere [36].

Nitrogen fixation is a key service in the ecology of plants, but it is an energy-expensive process. Therefore, diazotrophs are usually a minority among bacterial communities in plants [61], and nitrogen fixation is a tightly regulated process that is deactivated when fixed nitrogen is available. Here fixed nitrogen (which would inhibit nitrogen fixation) was not added to the Fahraeus solution and mainly root exudates (which contain sugars, organic acids, amino acids and vitamins) would support bacterial growth. Nitrogen fixation would support plant and bacterial growth. It is known that corn exudates contain carbohydrates, amino acids and organic acids that serve as carbon sources for bacteria in the rhizosphere [18]. Van Deynze et al. reported that biological nitrogen fixation was observed by adding corn mucilage to two nitrogen-fixing bacteria, *Herbaspirillum* and *Azospirillum* [30]. Notably wheat plants that have the capacity to excrete large amounts of organic acids showed increased nitrogen fixation [26], indicating that some substances from root exudates are favorable for nitrogen fixation. The selection of crop plants that overproduce them would allow increased nitrogen fixation in agriculture.

To promote nitrogen fixation, we included three different diazotrophs in the bacterial corn inoculant. In the single-species inoculation trial, our results confirmed previous experiments showing growth promotion in maize plants with *R. phaseoli* [22,70]. In previous studies to identify diazotrophs in non-legume roots with a culture-independent approach, *nif* genes of *Rhizobium* were found expressed in rice and sugar cane [35], suggesting that nitrogen fixation by rhizobia occurred in non-legume plants [44]. We showed here that *R. phaseoli* turned on its *nif* gene machinery on maize roots, in addition *Rhizobium* expressed bacterial respiration genes which are directly related to the supply of ATP necessary to provide enough energy for nitrogen fixation to take place. Nitrogen-fixing bacteria use cbb3-type oxidases encoded by the *fixNOQP* operon and this was also expressed [88,56]. Although *Rhizobium* nitrogen fixation genes were expressed, no acetylene reduction was detected in these bacteria associated with the plants even when they were inoculated as single species. Clearly, there are other elements that are provided by the nodule that are necessary for *Rhizobium* to fix nitrogen or the ARA assay is not sensitive enough to detect low levels of nitrogen fixation.

Exudates, mucilage and border cells are nutrient providers that support bacterial growth on roots. When in presence of other bacteria, *R. phaseoli* seemingly has to compete for root nutrients and showed enhanced expression of transporters which could serve to

uptake sugars and other molecules [53], (Dataset 1 and 3). Iron competition seems to occur under those conditions as genes for iron uptake were upregulated in *R. phaseoli* as well.

The osmolality of a typical soil water has been estimated as less than 50 mOsm/kg [61]. Rapid bacterial adaptation to increased osmolarity would facilitate rhizosphere colonization [61]. Genes to tolerate hyperosmolar conditions were found expressed in *Azospirillum* sp. B510, *Burkholderia phytofirmans* PsJN, *Methylobacterium populi* BJ001, and *Pseudomonas putida* W619 when associated with different plants [63].We had previously detected the expression of *R. phaseoli* genes for osmotolerance in corn roots [54] and in the presence of other bacteria, this stress seems to be accentuated. An ectoine biosynthetic gene was found up-regulated and ectoine has a role in protection from osmotic stress in rhizobia [77] and may inhibit melanin production [87]. Additional evidence that *R. phaseoli* is encountering stress (in the presence of other bacteria) is its upregulation of universal stress protein, oxygenases, and oxidases. *R. phaseoli* produces melanin in nodules and *in vitro* with added tryptophan and copper. The *R. phaseoli* genes overexpressed related to spermidine and melanin synthesis would be indicative of their bacterial production in roots in the presence of other bacteria. Transcripts for melanin synthesis were reported in *P. vulgaris* nodules in a recently published transcriptomic study that compares *Rhizobium leguminosarum* expression in determinate and non-determinate nodules [39]. Melanin may serve as antioxidant.

Growth on surfaces versus liquid medium has major effects on rhizobial gene expression [6]. Exopolysaccharides and adhesins may come into contact with the host and may be necessary for bacterial early binding on the surfaces of the roots [12]. The transcriptome analysis which was performed in liquid medium would not allow nutrient gradients to form and consequently chemotaxis and motility would not have an important role therein. Under those conditions down-regulated genes were those that involve motility encoding flagellar synthesis.

Notably the very large number of hypothetical genes up-regulated in the MI (Dataset 1 and 3) evidence our very poor knowledge of genes that function in bacterial interactions which is certainly an area that needs to be studied and this work is a basis for it. Transcriptional regulators (Dataset 1 and 3) may participate in orchestrating the expression of *R. phaseoli* genes in the presence of other bacteria and the differentially expressed transcriptional regulators may be key to continue studying bacteria-bacteria interactions.

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