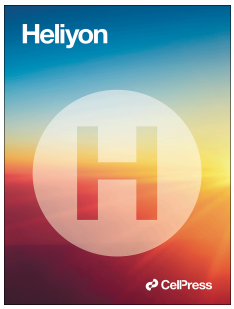

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Plant growth promoting activities of endophytic bacteria from *Melia azedarach* (Meliaceae) and their influence on plant growth under gnotobiotic conditions

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ABSTRACT

Bacteria that live asymptomatically within plant tissues are known as endophytes. Because of the close relation with the plant host, they have been a matter of interest for application as plant growth promoters. *Melia azedarach* is a widely distributed medicinal tree with proven insecticidal, antimicrobial, and antiviral activity. The aim of this study was to isolate and characterize endophytic bacteria from *M. azedarach* and analyze their plant growth promoting activities for the potential application as biological products. Bacteria were isolated from roots and leaves of trees growing in two locations of Northeastern Argentina. The isolates were characterized by repetitive extragenic palindromic sequence PCR and 16S rDNA sequence analysis. The plant growth-promoting activities were assayed *in vitro*, improvement of plant growth of selected isolates was tested on *M. azedarach* plantlets, and the effect of selected ACC deaminase producing isolates was tested on tomato seedlings under salt-stress conditions. The highest endophytic bacterial abundance and diversity were obtained from the roots. All isolates had at least one of the assayed plant growth-promoting activities and 80% of them had antagonistic activity. The most efficient bacteria were *Pseudomonas monteilii*, *Pseudomonas farsensis, Burkholderia* sp. and *Cupriavidus* sp. for phosphate solubilization (2064 µg P ml^{-1}), IAA production (94.7 µg ml^{-1}), siderophore production index (5.5) and ACC deaminase activity (1294 nmol α-ketobutyrate mg^{-1} h^{-1}). *M. azedarach* inoculation assays revealed the bacterial growth promotion potential, with *Pseudomonas monteilii*, *Pseudomonas farsensis* and *Cupriavidus* sp. standing out for their effect on leaf

area, leaf dry weight, specific leaf area, and total Chl, Mg and N content, with increases of up to 149%, 58%, 65%, 178%, 76% and 97.7%, respectively, compared to NI plants. Efficient ACC deaminase-producing isolates increased stress tolerance of tomato plants under saline condition. Overall, these findings indicate the potential of the endophytic isolates as biostimulant and biocontrol agents.

Keywords: endophyte; plant growth-promoting bacteria; *Melia azedarach* L.; medicinal plant; biological control.

1. Introduction

Plants live in association with a diverse microbial community that includes bacteria, fungi, and archaea. The plant host compartments provide diverse habitats in roots, rhizosphere, and above-ground organs, leading to the adaptation of niche specialized microbes [1–3]. The internal plant tissues integrate the endosphere, a more stable environment with ecological advantages, characterized by less competition for nutrients, protection from external biotic and abiotic factors, and direct plant interaction [4,5]. Plant traits, such as root morphology, metabolite production and immune response, play significant roles in plant-microbe interactions [6]. Flavonoids, for example, are chemoattractants that take part in the rhizobia-legumes symbiosis [7], but they also induce the colonization of rice roots by non-rhizobial endophytes, such as *Serratia* sp. [8]. Similarly, secondary defense metabolites, such as pyrrolizidine alkaloids, can affect the rhizosphere microbiota by favoring resistant or tolerant microorganisms [9].

In return, the presence of endophytic microorganisms in plant tissues can influence plant growth and development. Plant growth-promoting bacteria (PGPB) enhance plant nutrition efficiency through diverse mechanisms, such as nitrogen fixation [10,11], soil phosphorus and iron solubilization [12,13], and phytohormone production [14]. Under adverse environmental conditions, like drought, heat, and salinity, endophytes can mitigate the effects of the abiotic stresses by producing phytohormones [15,16], antioxidants [17], osmoprotectants and 1-aminocyclopropane-1-carboxylic acid (ACC) deaminase [18,19]. Endophytes can also be biological control agents and inhibit disease

symptoms caused by viral, fungal, and bacterial pathogens. Being able to colonize internal tissues, they can protect the plant directly by space competition against pathogens, and the production of antimicrobial compounds [20,21]. Besides, the close interaction and continuous crosstalk between endophytes and their plant host favor the induction of indirect control mechanisms, like plant systemic disease resistance [22,23] and the decrease of plant ethylene levels [24].

Plant growth-promoting bacteria were isolated from vegetative and reproductive structures of numerous plant species, and to a greater extent in agricultural crops [25]. Instead, there is limited information about PGPB of woody species, which represent an interesting potential given the unique ecological characteristics that occur especially in perennial and widely distributed species [26]. Most of the reports refer to nitrogen fixation and phytohormone production activities of bacterial endophytes inoculated into poplar and pine trees [27,28].

Melia azedarach is a tree native to Asia that has been widely distributed in tropical, sub-tropical and warm temperate regions of the world [29]. Leaf and fruit extracts have insecticidal, antimicrobial, and antiviral activity against human and plant pathogens [30-32]. Like other medicinal plants that produce unique bioactive secondary metabolites, *M. azedarach* is expected to harbor a distinctive microbiota [33,34], which may also participate in metabolic pathways or produce specific biologically active compounds [35,36]. In fact, previous works reported the isolation of endophytic fungi able to produce compounds with antifungal and antibacterial activity [14,37]. Based on this data, it is reasonable to think that the endophytic bacterial community would also have useful plant growth promoting traits. The aim of this study was, therefore, to isolate and characterize endophytic bacteria from *M. azedarach* and analyze their plant growth promoting activities for the potential application as bioinoculants for crop production.

2. Materials and methods

2.1. Plant sampling and endophytic bacteria isolation

Root and leaf samples were collected from four *Melia azedarach* trees growing in two locations, Santa Ana (San Cosme Department; 27°27′23.5"S, 58°41′13.6"W) (trees named 4 and 11) and Corrientes (Capital Department; 27°28′24″S, 58°46′56.1"W) (trees named 2 and 3), in Corrientes province, in the northeast of Argentina. The region is characterized by a humid subtropical

climate, with a mean annual rainfall of approximately 1,200 mm, distributed mainly during spring-summer period (November to March). The mean annual temperature is 22°C with scarce frosts [38]. The soil is an Entisol (Aquents suborder), sandy-textured, with a clear and narrow surface horizon and poor organic matter content [39]. Samplings were conducted in September (2018) and March (2019) (corresponding to spring and autumn, respectively). In each tree, three root and leaf samples were analyzed as previously described [12]. Each root sample consisted of five random 2 g-subsamples fully mixed, making 10 g-samples. In the case of leaf samples, 5 g-subsamples were mixed to obtain 25 g-samples.

Disinfection was performed as described by Domecq *et al.* [40] with slight modifications. Roots and leaves were thoroughly washed with tap water, sequentially surface-sterilized with 70% ethanol and 2% (v/v) sodium-hypochlorite, and rinsed three times with sterile distilled water. Root and leaf sections were then placed on tryptone soybean agar (TSA) medium for one week at 28°C. To check the surface disinfection efficiency, aliquots of the last wash water were plated on TSA and examined for the presence of microbial-growing colonies.

Preliminary assays were performed in order to standardize the endophytic bacteria isolation method. Two protocols were tested, with and without cell enrichment steps, according to Ikeda *et al.* [41]. Due to the low quantity of colony forming units (CFU) obtained from the leaf samples, we decided to increase the amount of input material from 10 g to 25 g. The cell enrichment method resulted in a significantly higher number of cultivable bacteria, and was therefore selected for processing the samples, with slight modifications as follows. Leaf (25 g) or root (10 g) samples were homogenized in 100 ml of bacterial cell extraction (BCE) buffer in a blender for three 1-min periods. The homogenate was sequentially centrifuged at 110×g for 5 min at 10°C and twice at 435×g for 20 min at 10°C. The supernatant was filtered using a Whatman filter paper (101 fast) and then centrifuged at 4,900×g for 20 min at 10°C. The pellet was suspended in 12.5 ml of BCE buffer and centrifuged at 13,500 × g for 10 min at 10°C. The supernatant was again filtered with sterile 40 µM filter disk and centrifuged at high speed; this procedure was performed twice. Finally, the pellet was suspended in 1.5 ml of 50mM-Tris-HCl pH 7.5. Serial dilutions of this suspension were plated in TSA medium supplemented with 0.01% cycloheximide to prevent fungal growth.

Plates were incubated at 28° C for 96 h for the isolation and enumeration of cultivable endophytic bacteria. The bacterial colonies were counted on the plates containing 10–100 CFU. The number of CFU/g of plant was expressed as the logarithm at the base of 10. The viable cell count was conducted in triplicate. The bacterial isolation was performed on two replicates per sample; 20 colonies of the predominant morphologies were selected from each plate, subcultured in fresh medium and purified.

2.2. Identification of endophytic bacteria

The endophytic community was characterized at the strain level by repetitive extragenic palindromic sequence PCR (Rep-PCR) using ERIC1R-ERIC2 primers [42]. Bacterial DNA was purified using Chelex 100 resin (Bio-Rad) as described by Alippi and Aguilar [43]. Amplification and electrophoresis analysis were performed according to Versalovic *et al.* [42]. The digital images were analyzed with Gelcompare software version 4.0 (Applied Maths BVBA, Belgium). A total of 167 Rep-PCR profiles were generated with a similarity cutoff of 85%.

One isolate of each profile generated by Rep-PCR was selected for identification by 16S rDNA sequencing and phylogenetic analysis. Ribosomal 16S rRNA gene was amplified by PCR using rD1 and fD1 universal primers [44]. The resulting PCR products were purified with a commercial kit (AccuPrep® PCR/Gel Purification Kit, Bioneer) and sequenced by Macrogen Inc., Seoul, Korea. Nucleotide sequences were compared with the EzBioCloud 16S database, and pairwise sequence similarities were determined with the EzTaxon server [45].

The 16S rDNA sequences were clustered into operational taxonomic units (OTU) with a dissimilarity threshold of 3% using CD-HIT SUITE program [46], for assignment at the species level. Although sequence divergence is not evenly distributed in the 16S rRNA region, 3% dissimilarity is often chosen in practice as the cutoff value to define bacteria species [47,48]. The abundance of each OTU was determined by adding the number of isolates from each representative Rep-PCR profile for all the 16S rDNA sequences included in that OTU. Prior to comparative analyses, the abundance values were normalized using the totalgroup-based method described in mothur (http://www.mothur.org/wiki/Normalize.shared), by this method sequences were subsampled to the number of sequences in our smallest group (12 sequences) and then normalized across samples. Forty five OTUs were distinguished in the

bacterial community, 35 of them remained after normalization and were used for the abundance matrix (heat map in Fig. 2.B), and diversity and composition analyses.

Pseudomonas isolates were further identified by multilocus sequence analysis [49,50] because 16S rRNA gene sequences of related species are highly similar (98.2 to 99%), and not informative enough to reach species level identification [51,52]. Genomic DNA of six isolates representative of the three *Pseudomonas* OTUs (28, 29 and 32) were amplified using primers PsEG30F/PsEG790R [53] and gyrB-F/ gyrB-R [54] for *rpo*D and *gyr*B genes, respectively. Both genes were successfully amplified from isolates of OTUs 28 and 29, while for OTU 32 only the *rpo*D sequence was obtained. Neighbor-joining phylogenetic trees were constructed from the combined nucleotide sequences of *rpo*D (690 bp), *gyr*B (910 bp) and 16S rRNA (810 bp) for OTUS 28 and 29, and from of *rpo*D and 16S rRNA for the three OTUs together.

2.3. In vitro plant growth-promoting (PGP) activities

The endophytic community was assayed for *in vitro* plant nutrition, plant growth regulation and for antagonistic potential against phytopathogenic bacteria and fungi. Sixty five isolates were tested, including at least one representative of the 45 previously identified OTUs. All tests were carried out in triplicate.

2.3.1. Nitrogen fixation ability

The nitrogen fixation ability was tested according to Weber *et al.* [55], with modifications. The isolates were grown in liquid malate NFb medium [56] supplemented with yeast extract (0.005%) and incubated 72 h at 28ºC. Once growth was observed, an aliquot was used to inoculate fresh liquid NFb medium and incubated under the same growth conditions. This procedure was repeated at least three times. Finally, 0.1-ml aliquots were inoculated into vials containing semi-solid bromothymol blue NFb medium with (nitrogen supplemented condition) or without $(NH_4)_2SO_4$ (nitrogen fixation condition). Vials without $(NH_4)_2SO_4$ showing a veil-like surface layer were considered positive. The nitrogen fixing *Azospirillum brasilense* Cd was used as reference strain [57].

In addition, the presence of *nif*H gene, encoding the nitrogenase reductase subunit, was analyzed by PCR with PolF-PolR primers according to Poly *et al.* [58], and nested PCR as described by Yeager *et al.* [59].

2.3.2. P solubilization activity

Isolates were initially screened for their phosphate-solubilizing ability on NBRIP (National Botanical Research Institute's phosphate growth medium) solid medium with tricalcium phosphate as the exclusive P source according to Mehta and Nautiyal [60]. Four isolates were inoculated per plate and incubated at 28°C for three weeks. The solubilization index (SI) was calculated every two days by subtracting the colony diameter from the clear halo diameter; isolates displaying an SI equal or higher than 1.4 in three replicates were selected for quantitative P solubilization assay, performed as previously described by Collavino *et al.* [12]. Phosphorus solubilization was estimated using the molybdenum blue method [61]. Phosphate solubilizing *Pseudomonas* RHP3 was used as reference strain [60].

2.3.3. Indole acetic acid (IAA) production

The IAA production was determined as described by Patten and Glick [62]. Each isolate was inoculated in 10 ml of DF medium [63] supplemented with 200 µg ml^{-1} of L-tryptophan and incubated for 72 h at 28°C. The IAA content was assayed in the culture supernatant by reaction with Salkowski's reagent 1:2 (v/v) and measured by absorbance at 535 nm. The IAA producing strain, *Azospirillum brasilense* Cd, was used as reference [57].

2.3.4. Siderophore production

Siderophore production was estimated according to Schwyn and Neilands [64], with modified Chrome Azurol S (CAS) agar medium, as described by Alexander and Zuberer [65]. Four isolates were assayed per CAS agar plate by placing 20 µl of the bacterial suspension (approximately 10^8 CFU ml^{-1}) in each quadrant and incubating for 72 hours at 28ºC. The colony diameter and colored zone were measured daily. Siderophore producing index (SPI) was calculated as follows: total diameter (colony and colored zone)/ colony diameter. *Pseudomonas chlororaphis* RPAN1 was used as positive reference strain [66].

2.3.5. ACC deaminase activity

Isolates were screened for ACC deaminase activity on DF minimal medium with ACC (5 mM) as sole nitrogen source, according to Penrose and Glick [67]. The activity was quantified on the selected isolates by monitoring the amount of α-ketobutyrate produced by ACC deamination as described by Honma and Shimomura [68] with modifications [67]. *Pseudomonas putida* ATCC 17399 and the isogenic strain with plasmid pRKACC, carrying an ACC deaminase gene, were used as negative and positive reference strains, respectively [69].

2.3.6. Antagonist activity

In vitro antagonist activity was evaluated by dual culture assays against common phytopathogenic microorganisms of tomato, cassava and citrus, which are important crops of Corrientes province, Argentina. The following pathogens were used: *Fusarium oxysporum* [70], *Ralstonia solanacearum* [71], (Vegetable diseases laboratory, INTA EEA Bella Vista, Corrientes, Argentina), *Clavibacter michiganensis* subsp *michiganensis* [72] (kindly provided by Dr. Ana María Romero, Universidad de Buenos Aires, Argentina), *Xanthomonas axonopodis* pv. *manihotis, Xanthomonas citri* subsp. *citri, Xanthomonas axonopodis.* pv. *vesicatoria* and *Pseudomonas syringae* pv. *tomato* (Citrus plant pathology laboratory collection, INTA EEA Bella Vista, Corrientes, Argentina).

Bacterial antagonism was evaluated as previously described by Bach *et al.* [73] with modifications. Pathogenic and endophytic bacteria were grown in 10 ml of TS broth until OD_{600} = 1.00 (approximately 10^8 CFU ml^{-1}). 100 µl of each pathogenic culture were plated on TSA, followed by drop-inoculation (5 µl) of the endophytic bacteria. Nine isolates were inoculated per plate and the inhibition zone was examined after 24 h at 28°C.

Antagonism against *F. oxysporum* was evaluated as previously described by Comby *et al.* [74], with some modifications. Each bacterial suspension (OD_{600} = 1.00) grown on TS broth was used to saturate a sterile filter paper disk (5 mm) and placed at four equidistant points of a potato dextrose agar (PDA) plate. Then, a *F. oxysporum* seven-day culture mycelial pellet (0.5 cm^2) was inoculated at the center and incubated for 7 days at 28°C. Plates containing only *Fusarium* pellets served as control. The means of three independent repetitions were used to calculate the Inhibition index (Ii), being Ii = [(ø *Fusarium* alone – ø *Fusarium* with endophyte) / ø *Fusarium* alone]*100. Inhibition index values range from 0 (null inhibition) to 100 (complete inhibition).

2.3.7. Bacterial pathogenicity test

Hypersensitive response (HR) on tobacco and pathogenicity tests were done to discard potential plant pathogenic bacteria. Gram-negative bacteria were infiltrated in *Nicotiana tabacum* leaves to test the ability to induce HR after 48 h. This technique rapidly identifies Gram-negative heterologous pathogenic bacteria [75]. All HR positive isolates and Gram-positive bacteria were tested for pathogenicity on *M. azedarach* seedlings under greenhouse conditions. Each isolate was separately inoculated on leaves and roots so that different entrance

points were considered. For leaf inoculation, wounds were done with a scalpel at the leaf base and a drop of inoculum (20 µl, 10^6 CFU ml^{-1}) was applied. Previously wounded roots were inoculated by immersion in a 10^6 CFU ml^{-1} bacterial suspension. Negative controls were inoculated with PBS. Visual disease symptom appearance was registered until 30 days after inoculation.

2.4. In vivo plant growth promoting activities

2.4.1. Melia azedarach inoculation assays

The inoculation effects of PGP on plant growth were assayed on *M. azedarach* clone J2 plantlets obtained by *in vitro* propagation [76]. Rooted plantlets of similar height (7 cm to 10 cm) were transplanted into 250-ml plastic pots with sterilized perlite and watered with 1/4 Hoagland's nutrient solution [77]. After seven days, the plants were inoculated by root immersion for 1 h in a fresh bacterial suspension (2 x10^8 CFU ml^{-1}). The experimental design was in randomized complete blocks with 20 plants per treatment. The treatments included five single inoculations with isolates *Bacillus* sp. A101, *Pseudomonas* sp. A116, *Pseudomonas* sp. A60, *Burkholderia* sp. M55 and *Cupriavidus* sp. N1, and one non-inoculated (NI) control. The experiment was performed under controlled environmental conditions (28±1/24±2°C day/night temperature, 12-h photoperiod at 400 µmolm−2s−1).

The following parameters were evaluated 120 days after inoculation: plant height, leaf area, and leaf, stem and root dry weight. Total dry weight, aerial/root biomass ratio and specific leaf area were calculated. The chlorophyll content (Chl-*a*, Chl-*b*, and total Chl) was determined by spectrophotometry [78], performing the extraction in 0.1 g leaf, with 4 random samples for each treatment. Leaf content of major nutrients (N, P, K, Ca, Mg) was analyzed. Among them, K, Na, Ca, and Mg were determined after wet digestion, K and Na content was determined by flame photometry, and Ca and Mg by complexometric titration with EDTA [79]. Kjeldahl [80] and Murphy and Riley [61] methods were applied to determine N and P content, respectively.

2.4.2. Gnotobiotic root elongation assay in tomato seedlings

The gnotobiotic root elongation assay was based on the ACC deaminase producing bacteria ability to reduce ethylene levels of plants under stress conditions [67, 81]. The ACC deaminase producing bacteria were tested for their effect on the growth of tomato (*Solanum lycopersicum*) seedlings under salt-stress conditions. The assay was performed using the root elongation test

described by Penrose and Glick [67], with modifications [82]. Seeds were surface-sterilized and germinated in the dark on water agar plates at 28°C. Two-day-old seedlings were incubated for 1 h with fresh bacterial suspension in sterile 30-mM $MgSO_4$, at a density of about 2 $x10^8$ CFU ml^{-1}. The following isolates were selected for the plant assay: *Burkholderia* sp. M55, *Burkholderia* sp. M57, *Paraburkholderia* sp. N147, *Cupriavidus* sp. N1, *Variovorax* sp. N4, *Variovorax* sp. N133. Seedlings inoculated with sterile 30-mM $MgSO_4$ were used as negative control. After inoculation, 20 seedlings were placed in glass bottles with sterile filter paper, and watered with half strength N-free Hoagland's solution [79] supplemented with 100 mM NaCl. The bottles were placed in a completely randomized design with four replications for each treatment. The experiment was performed under controlled environmental conditions (28±1/24±2°C day/night temperature, 12-h photoperiod at 400 µmolm−$2s^{-1}$). After 9 days, the primary root and stem length were measured.

2.5. Statistical data analysis

Alpha diversity of the endophytic community was measured using Shannon (H') and Simpson (1-D) indices, analyzed with Past3 program [83]. To determine abundance, diversity and significance of plant growth parameters across samples/treatments, one-way ANOVA or non-parametric Kruskal–Wallis H tests were applied, according to their normality. The correlations between the plant parameters were calculated using Pearson's correlation coefficient. The variation analysis was performed with the statistical package InfoStat version 2011 [84].

3. Results

3.1. Endophytic community of Melia azedarach

The number of endophytes was significantly higher in root samples and interaction with the sampling time was observed ($F = 28$, $p = 0.0001$). The number of bacteria was higher in spring than in autumn leaf samples ($F = 158.66$, $p < 0.0001$); no significant difference was observed between spring and autumn roots (Fig. 1A).

A total of 507 isolates were obtained, 252 from leaves (137 and 115 from spring and autumn, respectively) and 255 from roots (110 and 145 from spring and autumn, respectively). Alpha diversity was significantly higher in roots, showing a strong effect of plant organ (H´ $F = 9.56$, $p = 0.006$; 1-D $F = 7.81$, $p = 0.012$) (Fig. 1B). Sampling time did not affect diversity ($p \geq 0.8$). 16S rRNA analysis

showed that most of the endophytes belonged to the phyla Proteobacteria (Pseudomonadales, Enterobacterales, Burkholderiales and Rhizobiales) and Firmicutes (Bacillales). Actinobacteria and Bacteroidetes were present in low proportion (Fig. 2A). The leaf community was mostly composed of Pseudomonadales (44%), Enterobacterales (36%) and Bacillales (18%). In contrast, a greater diversity of groups was observed in root samples, with abundance of Bacillales (31%), Burkholderiales (30%), Rhizobiales (17%) and Pseudomonadales (13%). As regards seasonal variation in the endophytic root community, spring samples had the highest proportion of Bacillales while Burkholderiales and Rhizobiales predominated in autumn (Fig. 2A).

The phylotype distribution among samples also reflected the differential bacterial composition within plant organs (Fig. 2B). Only 5 of the 35 OTUs observed were present in root and leaf samples. All Burkholderiales, Rhizobiales, and most of Bacillales phylotypes were detected only in roots while Enterobacterales and Pseudomonadales were found almost exclusively in leaves (Fig. 2B). The most abundant OTUs belonged to *Pseudomonas* (OTU 28) and *Kosakonia* (OTU 35), representing 18 and 16% of total isolates, respectively. OTU 28, closely related to *P. monteilii*, had wide distribution in root and leaf samples (present in 65% of the samples) while OTU 35, related to *Kosakonia cowanii*, was found exclusively and in almost all leaf samples (Fig. 2B).

The *Pseudomonas* OTUs were further identified to the species level through the combined analysis of 16S rRNA, *rpo*D and *gyr*B genes. In the generated tree, OTUs 29 and 32 clustered with *P. oryzihabitans* and *P. stutzeri,* respectively. Similar results were obtained from the phylogenetic trees generated by single or multiple gene sequences (16S rRNA, *gyr*B and *rpo*D genes for OTU 29, and 16S rRNA and *rpo*D for OTU 32), supporting the strains affiliation (Fig. 3A, B; Fig. S1). The isolates of OTU 28 were separated in two clusters, strains A1 and A116 grouped with *P. farsensis* while A60 and 2A10 grouped with *P. monteilii* and *P. parafulva*, with A60 being highly related with *P. monteilii* in all the phylogenetic trees, except the one inferred from the 16S rDNA sequences alone (Fig. 3A, B; Fig. S1). *P. farsensis* related isolates had low abundance and were found in only two samples (leaves of trees 2 and 3, warm season) while the isolates related with *P. monteilii* had the abundance and distribution described for cluster 28 (Fig. 2B).

3.2. *Melia azedarach* endophytic community has diverse and highly efficient PGP activities

The potential plant growth promoting activities were assayed in 65 endophytic isolates, representing the 24 identified genera. The most common traits were P solubilization and IAA production, found in 97% and 89% of the isolates, respectively. N-fixing activity, assayed by growth on N-free media and detection of the *nif*H gene, had lower frequency (38.4%), but were equally distributed within all endophytic classes (Fig. 4). The efficiency of these activities differed among groups. Most isolates of Pseudomonadales and Enterobacterales showed high P solubilization activity, with maximum between 407 and 2064 µg P ml^{-1}, reaching three times higher activity than the reference strain (*Pseudomonas* RHP3, 685 µg P ml^{-1}). Also IAA production was high in *M. azedarach* endophytes; some isolates of *Pseudomonas*, *Pantoea* and *Bacillus* produced more IAA than the reference strain (*Azospirillum brasilense* Cd, 44 µg ml^{-1}), with maximum levels of 94.7, 87.5, and 79 µg ml^{-1}, respectively.

Other PGP activities, such as siderophore production (60%), bacterial antagonism (38.5%) and ACC deaminase (15.4%), had uneven distribution among bacterial orders (Fig. 4). The production of siderophores was detected mainly in Burkholderiales and Pseudomonadales, with the highest values in *Burkholderia* isolates, which doubled (5.5) those of the reference strain (*P. chlororaphis subs. aurantiaca* RPAN1, 2.2). Burkholderiales also had the highest number of isolates with ACC deaminase activity, with maximum of 1294, 1210 and 967 nmol α-ketobutyrate mg^{-1} h^{-1} production in *Cupriavidus, Paraburkholderia* and *Burkholderia*, respectively, similar to the reference strain (*P. putida* ATCC 17399/pRKACC, 1346 nmol α-ketobutyrate mg^{-1} h^{-1}).

The antagonistic activity was assayed by dual culture against six phytopathogenic bacteria and *F. oxysporum*. Bacterial antagonism was observed in 25 isolates, mainly *Pseudomonas*, *Burkholderia* and *Kosakonia* (Fig. 4; Table S1). The highest biological control activity was found against *X. axonopodis* pv. *manihotis*, with a total of 12 isolates from 8 genera, nine of which also showed antagonism against *X. axonopodis* pv. *vesicatoria*. In contrast, *X. citri* subsp *citri* and *C. michiganensis* subsp *michiganensis* were mainly controlled by *Pseudomonas* strains. *Ralstonia solanacearum* was inhibited only by Enterobacterales while *P. syringae* pv. *tomato* was controlled by *Paenibacillus* and *Burkholderia* isolates (Fig. 4; Table S1).

On the other hand, a large and diverse population had fungal antagonistic activity against *F. oxysporum*. Significant inhibitory activity (> 15%) was observed in 40 of the 65 isolates, with representatives of all genera and, in a greater proportion, *Bacillus*, *Kosakonia*, *Paenibacillus* and *Pseudomonas*. The highest inhibition (> 60%) was observed in isolates from *Pseudomonas* (100%), *Rhizobium* (77%), *Sphingobium* (77%) and *Paenibacillus* (64%) (Fig. 4; Table S1). Some bacteria that did not grow on PDA were also tested on TSA medium. Interestingly, four strains of the genus *Paenibacillus* and *Bacillus* presented an inhibition capacity greater than 75% under these conditions (data not shown).

3.3 Endophytic bacteria promote growth of Melia azedarach plants under gnotobiotic conditions

Five isolates, selected for their *in vitro* PGP performance, were assayed on their ability to promote *M. azedarach* plants growth. Among them, *Bacillus* sp. A101 and *P. farsensis* A116 showed the highest IAA production*, P. monteilii* A60 *and Burkholderia* sp. M55 were highly efficient in solubilizing P and siderophore production while *Cupriavidus* sp. N1 showed the highest ACC deaminase activity. All of them had antagonistic activity, were negative for pathogenicity tests, and most, except for *Cupriavidus* sp. N1, were N-fixers.

All the assayed isolates significantly increased plant growth, especially foliar parameters, i.e., leaf area, leaf dry weight and leaf specific area, with increases of up to 149%, 58% and 65%, respectively, compared to NI plants. The highest values were obtained in plants inoculated with *P. monteilii* A60, *Bacillus* sp. A101 and *Cupriavidus* sp. N1 (Fig. 5.A, B; Table S2). Likewise, all inoculated plants had higher chlorophyll content (b and total), and significantly lower Chl a/b ratio. The highest Chl total content was obtained with *P. monteilii* A60 and *Cupriavidus* sp. N1, with increases of up to 178% compared to NI plants (Figure 5.C). These plants, as well as those inoculated with *P. farsensis* A116, had significantly higher N and Mg content compared to the NI controls (Figure 5.D).

The principal component analysis (PCA) showed a clear separation between NI plants and all inoculated treatments with 59.6% of variability explained by plant height, leaf area, leaf dry weight, and Chl-*b* (Figure 5.E). Chl-*b* content was found positively correlated with leaf area (r= 0.7, p= 0.0002) and dry plant biomass, i. e. leaf (r= 0.7, $p < 0.0001$), aerial (r= 0.62, p= 0.0017), root (r= 0.42, p= 0.042) and total dry weight (r= 0.59, p= 0.0034). Regarding nutrient content, higher specific leaf area (SLA) and chlorophyll contents were correlated with increases

in P (SLA: r= 0.48, p= 0.016; Chl-*a*: r= 0.52, p= 0.015), Mg (r= 0.48, p= 0.017; Chl-*b*: r= 0.51, p= 0.02), K (SLA*:* r= 0.46, p= 0.024; Chl-*b* and total ChL: r= 0.62, p= 0.002) and N (SLA*:* r= 0.50, p= 0.043; total Chl: r= 0.49, p= 0.0442), while N was strongly correlated with Mg (r= 0.77, p= 0.007) and K levels (r= 0.71, p= 0.031) (Fig. 6).

3.4 ACC deaminase-producing endophytes increased tomato salinity tolerance

Six isolates that showed the highest α-ketobutyrate production (> 500 nmol α-ketobutyrate mg^{-1} h^{-1}) were selected for testing their effect on tomato seedlings growth under saline stress conditions. Non-inoculated stressed (NI stressed) seedlings showed a significant decrease of root (~70% lower) and shoot length (~40%) compared to those observed in normal growth conditions (NI unstressed) (Fig. 7.A, B). All the isolates, except for *Burkholderia* M55, had significant effect on plant growth under saline conditions; inoculated seedlings showed significantly higher root and shoot length than NI (p<0.0001); the highest increase was observed in plants inoculated with *Burkholderia* M57 and *Paraburkholderia* N147, where the growth parameters were comparable to those of the unstressed plants (Fig. 7.A, B).

4. Discussion

In search of plant growth promoting bacteria, we have characterized the culturable endophytic bacterial community of *Melia azedarach* trees from two localities of Northeastern Argentina. In the assayed trees, the endophytic bacterial community was composed predominantly of Proteobacteria and Firmicutes, represented mainly by Pseudomonadales and Enterobacterales, and Bacillales, respectively. These groups have been found in diverse plant tissues and environmental conditions, indicating physiological versatility that allows them to adapt to various plant internal microenvironments [85,86]. The bacterial community was also similar to other medicinal plants, as shown in a recent review that indicated Bacillales, Enterobacterales and Pseudomonadales as the most common orders found in 40 medicinal plant families [87]. The community structure was mainly affected by the organ source. Abundance and diversity were higher in roots, suggesting that the leaf niche is more restrictive for the entry and colonization of endophytes. This result agrees with previous reports in relation to plant colonization. Mishra *et al.* [88] found that endophytes were distributed in

internal niches depending on their ability to colonize, overcoming plant defenses, but also on the allocation of plant resources. This generally results in a decreasing endophytic diversity from the root towards the upper parts of the plant [89,90]. Analysis of several forest species, such as *Populus*, *Alnus* and *Betula*, showed that bacterial diversity increased from the leaves to the root, as opposed to the behavior observed in endophytic fungi, explained by the different colonization strategies [91,92]. The endophytic composition was also affected by the plant compartment. More than 85% of the observed OTUs were not shared between tissues; Rhizobiales, Burkholderiales and most Bacillales were detected only in roots. In contrast, the leaf endophytic configuration was more stable, with Gammaproteobacteria (Pseudomonadales/ Enterobacterales) representing 80% of the total isolates. Several works have shown the effect of compartmentalization on the endophytic assembly in plants [92-94]. In *Populus*, Burkholderiales were found enriched in the rhizospheric soil, and *Pseudomonas* in leaves and stems, while Rhizobiales dominated the root bacterial community [95]. Further analyses, including different sampling sites and a higher number of trees, would be necessary in order to establish the core microbiota of *M. azedarach* and the key factors that shape it.

The endophytic bacteria isolated from *M. azedarach* showed diverse and efficient functional activities. All of them had at least one PGP activity. For instance, isolates affiliated to *P. monteilii* (cluster 28) had most of the PGP functions analyzed (except for ACC deaminase), with highly efficient IAA production, P solubilization and bacterial and fungal antagonistic activity (Fig. 4). Moreover, a high proportion of the endophytes showed antagonistic activity, providing a collection of potential biological controllers of *F. oxysporum* and six plant pathogenic bacteria. Like *M. azedarach*, other medicinal plants have been postulated as a source of diverse antimicrobial compound-producing endophytes [96-98]. In previous works, actinobacteria isolated from *Thymus roseus* efficiently inhibited *F. oxysporum* and *Verticillium dahliae* growth [34]. The endophytic community of the medicinal plants *Dodonaea viscosa*, *Fagonia indica*, *Caralluma tuberculata*, and *Calendula arvensis* have been also reported to inhibit *Phytophthora parasitica* growth, mediated by secondary metabolites production [99]. Plant pathogens were not the only target for biocontrol, endophytic bacteria from *Origanum vulgare* have been tested against human pathogens and demonstrated antimicrobial activity against antibiotic resistant bacteria [100].

These results support the hypothesis that endophytic bacteria extend the potential of medicinal plants as bio product sources beyond the production of bioactive compounds.

Efficient ACC deaminase activity was detected only in Burkholderiales. This group was found exclusively in roots, which could suggest a higher ACC deaminase activity in this organ. ACC is an ethylene precursor, involved in plant development, defense, and symbiosis [101]. In response to stress conditions, ACC is exuded by the roots and can be taken up by ACC deaminase-producing bacteria to be used as carbon and nitrogen sources, reducing plant ACC levels, and thus the "stress ethylene". Bacteria with ACC deaminase activity have been found in soils and associated with plants, on the surface and inside roots, leaves, and seeds [18,102]. However, there are few reports about their relative abundance in the plant endophytic community. Rhizosphere and root compartments have shown higher frequency and expression of the ACC deaminase encoding gene (*acd*S) than bulk soil [103]. Likewise, the abundance of these bacteria increased in the roots of plants under stress conditions [104-106]. It has been postulated that plants selectively recruit ACCd-producing soil bacteria to integrate into their microbiome [107]. Indeed, ACC is a strong chemoattractant for PGPB [103,106,108,109]. Roots are the main pathway for the entry of microorganisms and exhibit a high microbial diversity and abundance [92,93,110]. Higher ACCd activity in the roots would favor plant microbial colonization, rapid response to soil stress conditions, and prevent ACC/ethylene signaling.

Plant inoculation assays showed the efficiency of endophytic bacteria as growth promoters of *M. azedarach. P. monteilii* A60, *P. farsensis* A116 and *Cupriavidus* sp. N1 strains standed out for their effect on plant growth and physiological parameters, such as plant height, leaf area, leaf dry weight, specific leaf area, and Chl-*b*, Mg and N content. Besides, highly efficient ACC deaminase-producing isolates were able to increase stress tolerance of tomato plants under saline condition. *M. azedarach* inoculated plants showed higher leaf area and specific leaf area (SLA), which were found directly correlated with Chl-*b* and leaf macronutrient content. These foliar traits contribute importantly to plant photosynthesis, growth rate and productivity [111-113]. The increase of aerial/root biomass ratio, chlorophyll, N and Mg content indicated a nutritional effect of the endophytic bacteria on *M. azedarach* plants, regardless of the

bacterial isolate. It has been widely observed that PGP activity increased plant growth and consequently crop yields, especially under adverse conditions [114]. However, these effects were not frequently evident under optimal growth conditions [115]. It is interesting to note that, at least to our knowledge, the PGP effect was evidenced in *M. azedarach* plants under optimal environmental and nutritional conditions. Further studies will be needed to validate the promoting effects of inoculation under field conditions.

Among the bacterial activities that could explain the plant performance improvement, all the inoculated strains, except N1, were able to fix N_2. However, our results indicated that a bacterium may directly affect plant growth by one or more of PGP mechanisms. Apart from N_2 fixation, *P. farsensis* A116 and *Bacillus* sp. A101 had high IAA production, *P. monteilii* A60 and *Burkholderia* sp. M55 were efficient in siderophore production and P solubilization. On the other hand, *Cupriavidus* sp. N1 was not able to fix N_2, but had high ACC deaminase activity, bacterial antagonism and siderophore production. Besides promoting the growth of *M. azedarach*, *Cupriavidus* sp. N1 promoted shoot and root growth of tomato seedlings under salinity conditions, showing the ability to synthesize ACC deaminase *in vivo*. The use of ACC deaminase-producing bacteria in several plant species has resulted in increased tolerance to different biotic and abiotic stresses [81,116,117]. The PGP traits of *C.* N1, particularly its remarkable efficiency in the synthesis of ACC deaminase, as well as its ability to interact with different plant species, would point it as a potential inoculant to be used under normal and growth-limiting conditions. Interestingly, this strain was found most closely related with *C. numazuensis* and *C. necator*, species that have been found nodulating *Mimosa* spp. [118].

5. Conclusion

This study revealed that *Melia azedarach* trees host endophytic bacteria with significant plant growth-promoting potential, as evidenced by their diverse and efficient functional activities. Notably, *Pseudomonas monteilii*, *Pseudomonas farsensis* and *Cupriavidus* sp. demonstrated a high capacity to enhance *M. azedarach* growth. Additionally, five efficient ACC deaminase producing strains enhanced the salt tolerance of tomato plants. Overall, these findings underscore the utility of medicinal plant-associated endophytes as biostimulant and biocontrol agents.

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Data availability statement

The sequences obtained in this study were deposited in the GenBank nucleotide sequence database under accession numbers OQ429106 to OQ429274 (for 16S rRNA sequences) and OR497792-OR497802 (for *rpo*D and *gyr*B sequences).

CRediT authorship contribution statement

Carolina Ramírez: Methodology, investigation, writing – original draft. Marina Cardozo: Methodology, investigation, writing – original draft. Maura López Gastón: Methodology, investigation. Ernestina Galdeano: Conceptualization, formal analysis, writing – original draft, writing – review & editing, funding acquisition. Mónica M. Collavino: Conceptualization, formal analysis, writing – original draft, writing – review & editing, funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Ethics statement

Review and approval by an ethics committee was not needed for this study because the work did not involve materials, tissues, data, or human or animal subjects.

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TABLE AND FIGURE CAPTIONS

Fig. 1. Abundance and α-diversity. A. Number of viable bacterial cells found in disinfected roots (red boxes) and leaves (light blue boxes) of *Melia azedarach* trees sampled at spring (striped boxes) and autumn (solid boxes). **B.** Calculated alpha-diversity, using Shannon (H´) and Simpson (1-D) indices, of the endophytic bacterial community isolated from leaves (red boxes) and roots (light blue boxes) samples. Different letters indicate significant difference between means at $p < 0.05$ according to Tukey HSD test (ANOVA).

Fig. 2. Taxonomic composition. A: Proportion of endophytic bacterial orders found in each sample type of *Melia azedarach*. **B.** Neighbor-joining analysis of partial 16S rRNA gene sequences showing the relationship between the 35 endophytic phylotypes. OTU number and its closest sequence are indicated in each branch. Shading area in the tree indicates the organ source, root (green), leaf (blue), or both (not shaded). Only bootstrap values ≥50 (based on 1000 pseudoreplicates) are indicated.

The heatmap in the right margin illustrates the relative abundance of the sequences in each sample according to the plant organ (leaves and roots) and sampling-time (spring and autumn). Original abundance values are ln(x+1)-transformed. No scaling was applied to rows.

Fig. 3. Phylogenetic analysis of *Pseudomonas* isolates. Six isolates representative of the three *Pseudomonas* OTUs, 28 (names in red), 29 (names in green) and 32(names in light blue), identified by partial 16S rRNA, *rpo*D and *gyr*B nucleotide sequence analysis. **A.** Neighbor-joining phylogenetic trees based on a concatenated alignment of *rpo*D (690 bp), *gyr*B (910 bp) and 16S rRNA (810 bp) sequences for *Pseudomonas* OTUS 28 and 29 and closest related species. **B.** Neighbor-joining phylogenetic trees based on a concatenated alignment of *rpo*D and 16S rRNA for the three OTUs together and closest related species. Bootstrap values >50% are shown on branches (1000 replications).

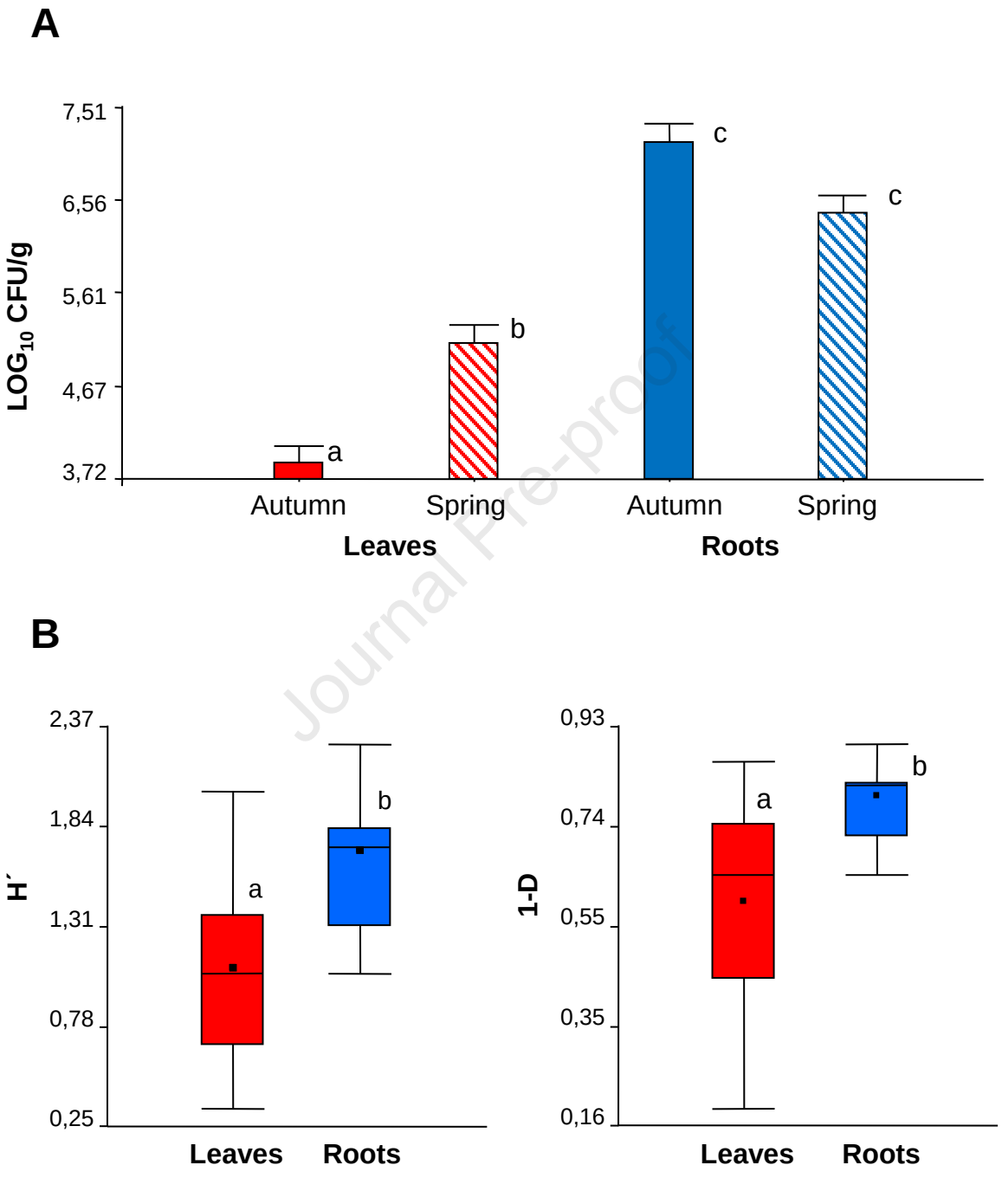
Fig. 4. *In vitro* plant growth-promoting (PGP) activities. Heatmap showing the PGP activities analyzed in 65 endophytic isolates. IAA production (IAA), P

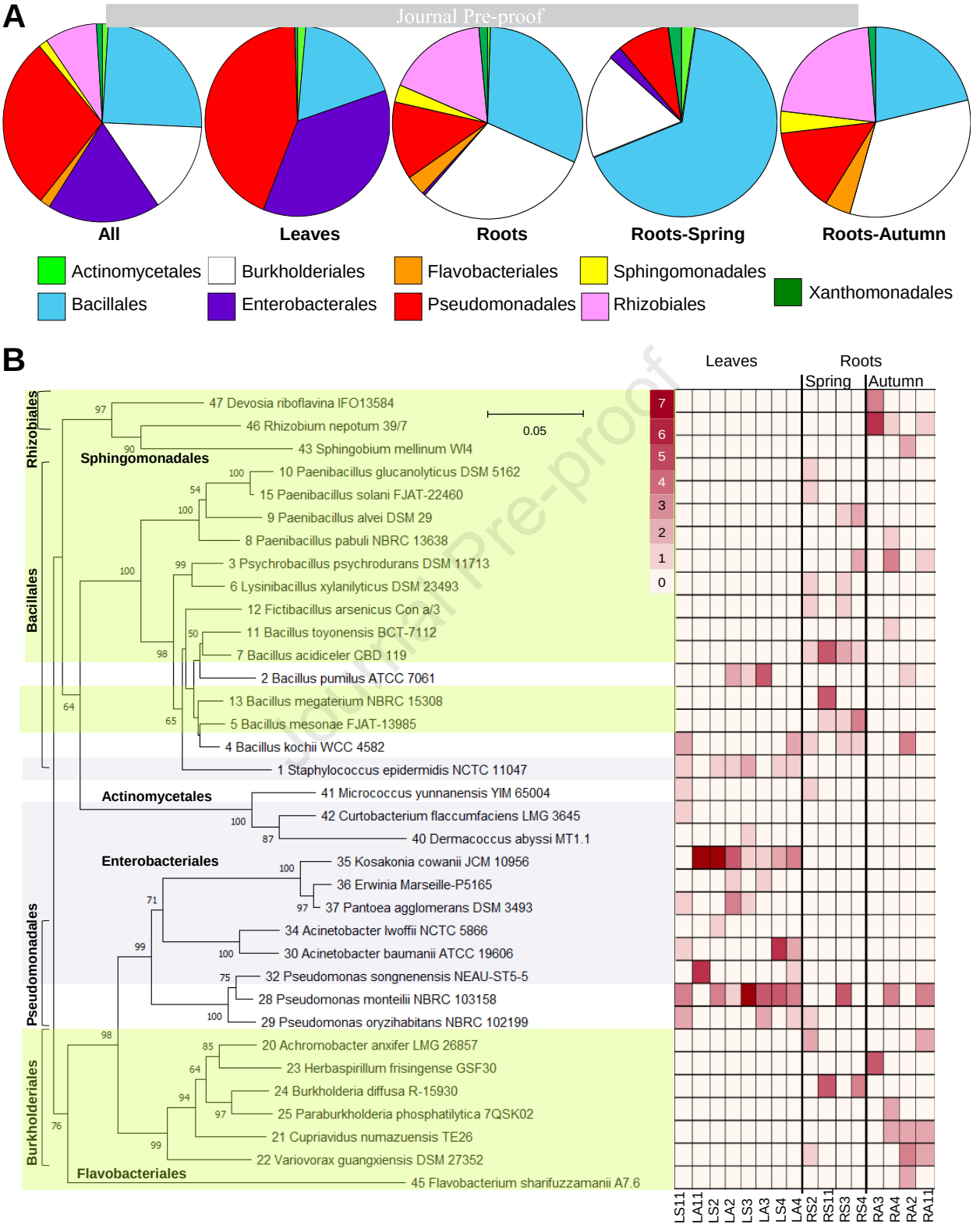
solubilization (P), ACC deaminase activity (ACC) and siderophore production (S) are shown as percentage activity relative to the maximum activity found (100%); Inhibition index (Ii) values are indicated for *Fusarium* antagonism (FA) while nitrogen fixation (Fix) and bacterial antagonism (BA) are indicated as positive (+) or null (-) activity.

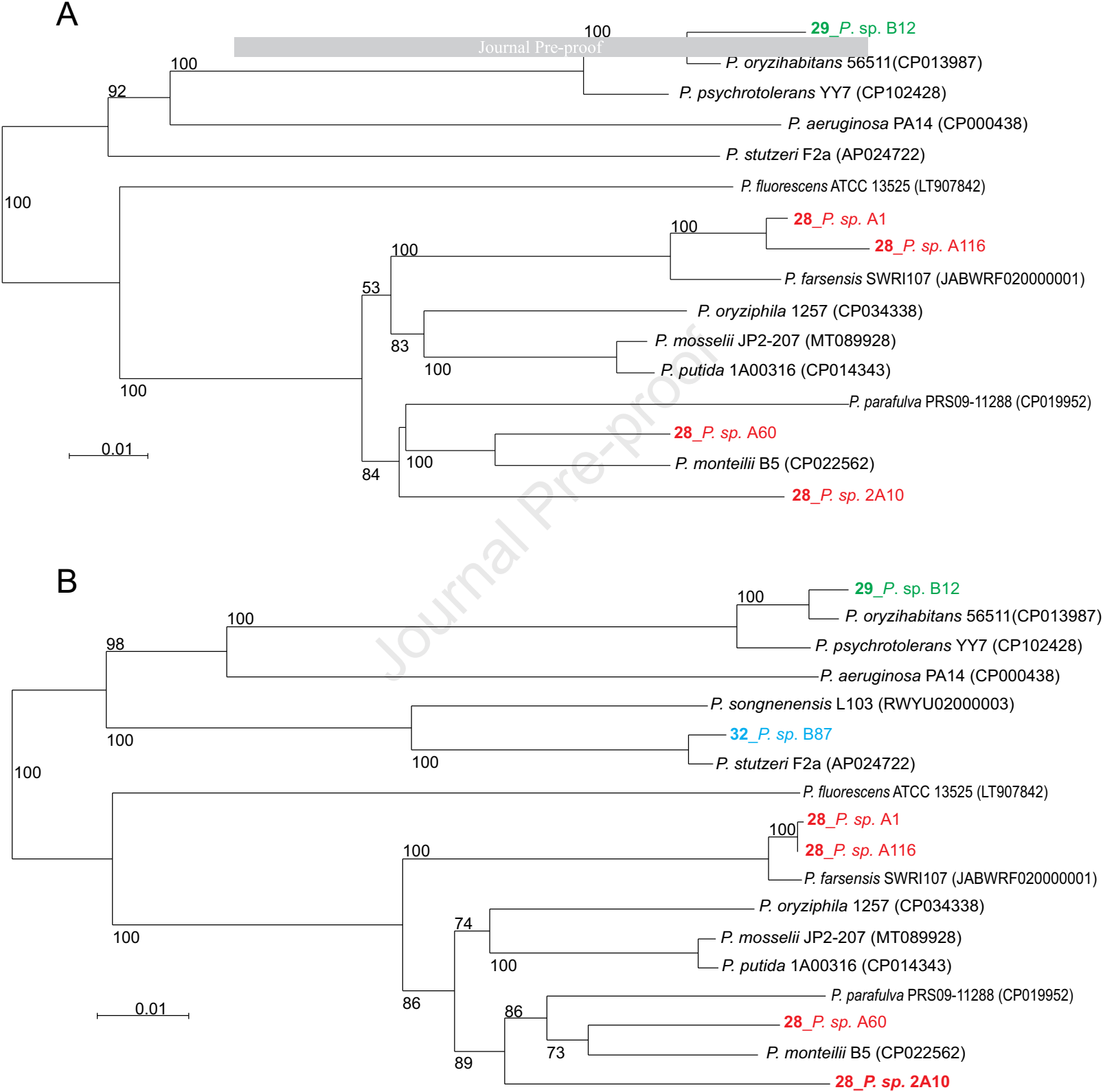
Fig. 5. Bacterial growth promoting-activity on *Melia azedarach* plants. (A) Leaf, stem, root, and total dry weight, (**B**) leaf area (cm^2) and specific leaf area (SLA, cm^2/g), (**C**) chlorophyll content (Chl-*a*, Chl-*a/b*, and total Chl) (mg/g), (**D**) leaf N and Mg content (%) for inoculated (*Bacillus* sp. A101, *Burkholderia* sp. M55, *Pseudomonas farsensis* A116, *Pseudomonas monteilii* A60 or *Cupriavidus* sp. N1) and non-inoculated (NI) plants growing under gnotobiotic conditions. Different letters indicate significant differences among treatments according to Tukey test ($p < 0.05$). (**E)** Principal component analysis (PCA) of plant growth parameters according to inoculation treatments.

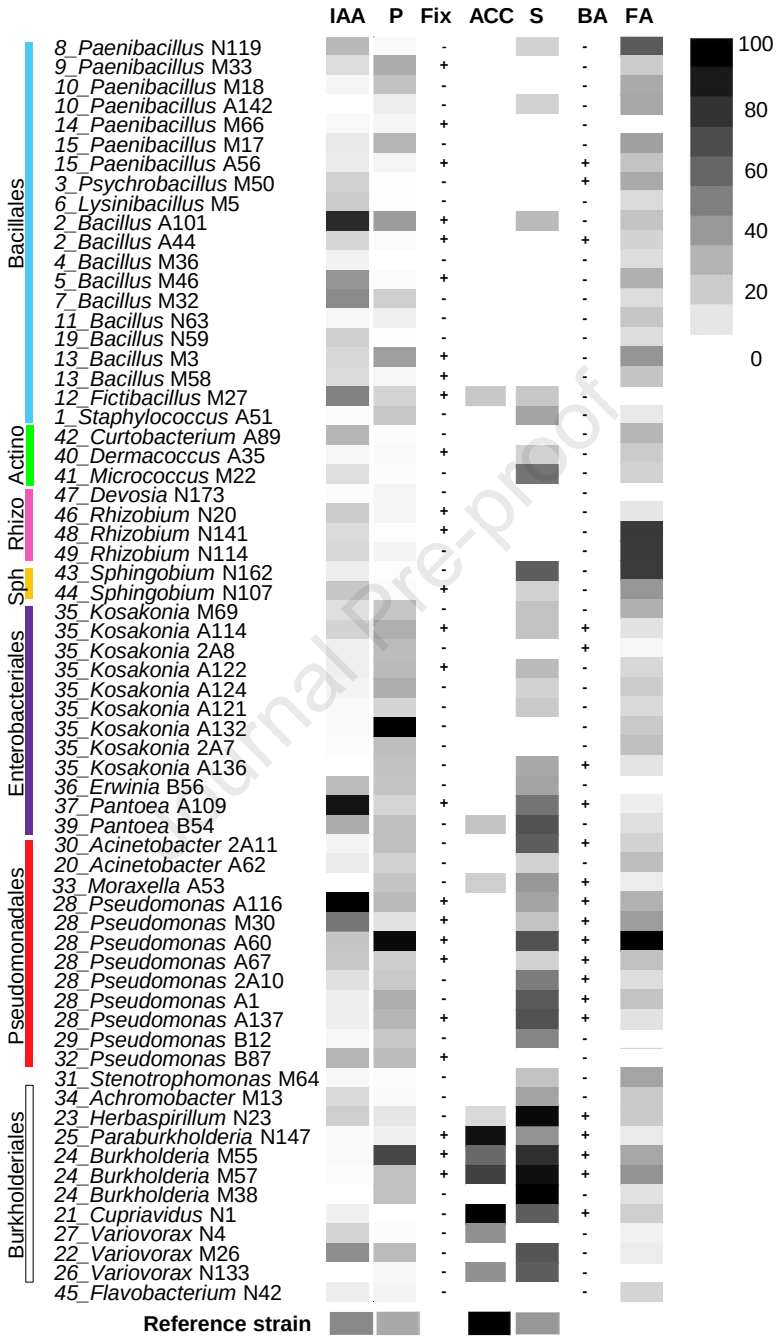
Fig. 6. Correlation of growth parameters. Pearson's correlation matrix of growth parameters, plant height, leaf (LDW), stem (STW), root (RDW), and total dry weight (TDW), leaf area, specific leaf area (SLA), chlorophyll *a* (Chl-*a*), chlorophyll *a/b* ratio (Chl-*a/b*), and total chlorophyll (total Chl), leaf N, P, K, Ca and Mg content, measured in *Melia azedarach* plants inoculated with endophytic bacteria (*Bacillus* sp. A101, *Burkholderia* sp. M55, *Pseudomonas farsensis* A116, *Pseudomonas monteilii* A60 or *Cupriavidus* sp. N1) under gnotobiotic conditions.

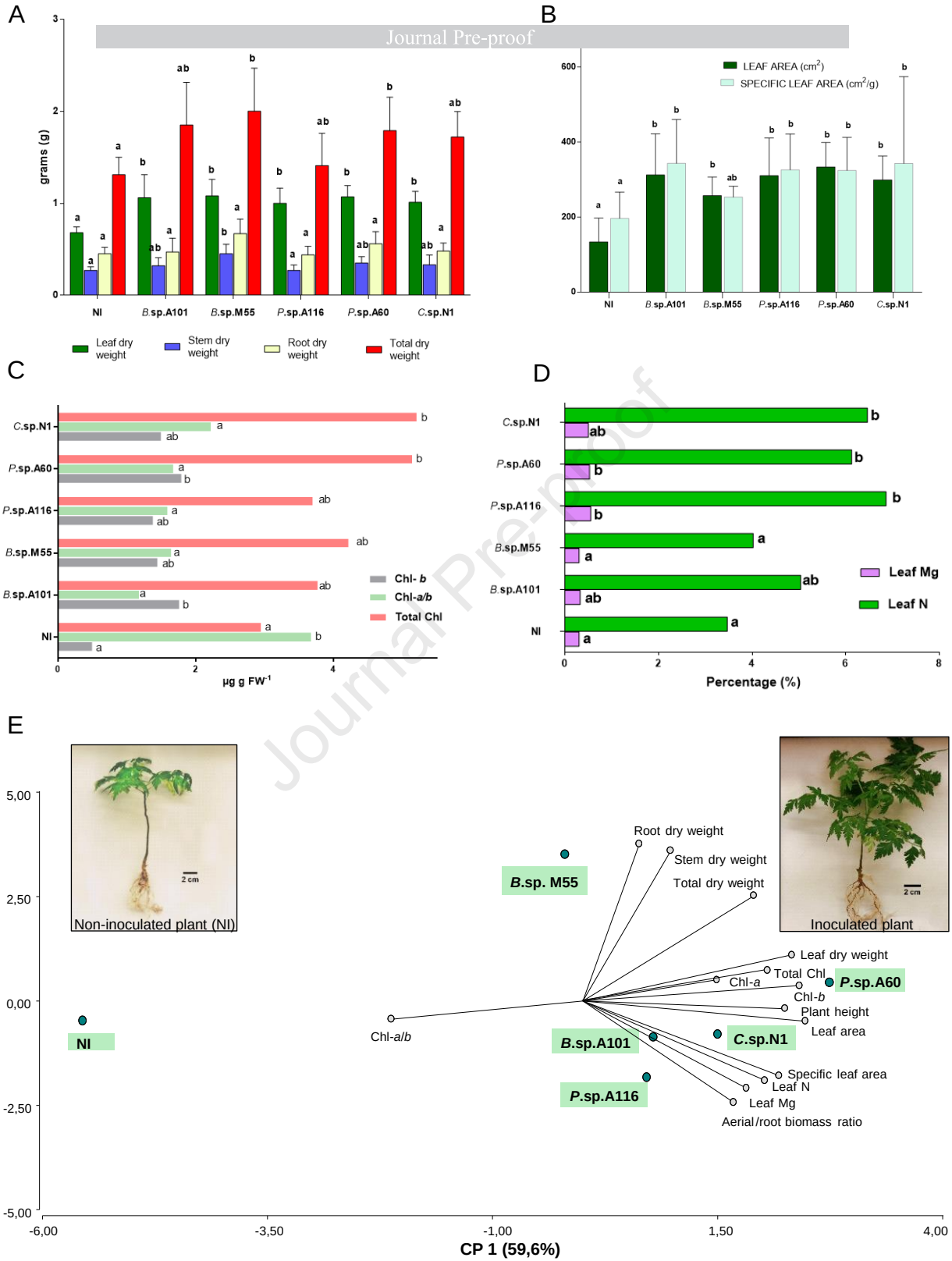
Fig. 7. Gnotobiotic root elongation assay in tomato seedlings. ACC deaminase-producing bacteria, *Burkholderia* sp. M55 (*B.* M55), *Burkholderia* sp. M57 (*B*. M57), *Paraburkholderia* sp. N147 (*P*. N147), *Cupriavidus* sp. N1 (*C.* N1), *Variovorax* sp. N4 (*V*. N4) and *Variovorax* sp. N133 (*V.* N133), were analyzed for their effect on shoot length (A) and root length (B) of tomato seedlings growing under saline conditions (100 mM NaCl). Non-inoculated plants were tested under saline (NI stressed) and normal conditions (NI unstressed). Different letters indicate significant differences among treatments according to Kruskal Wallis test ($p<0.0001$).



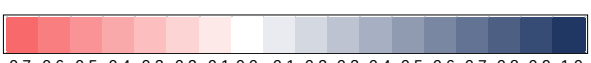


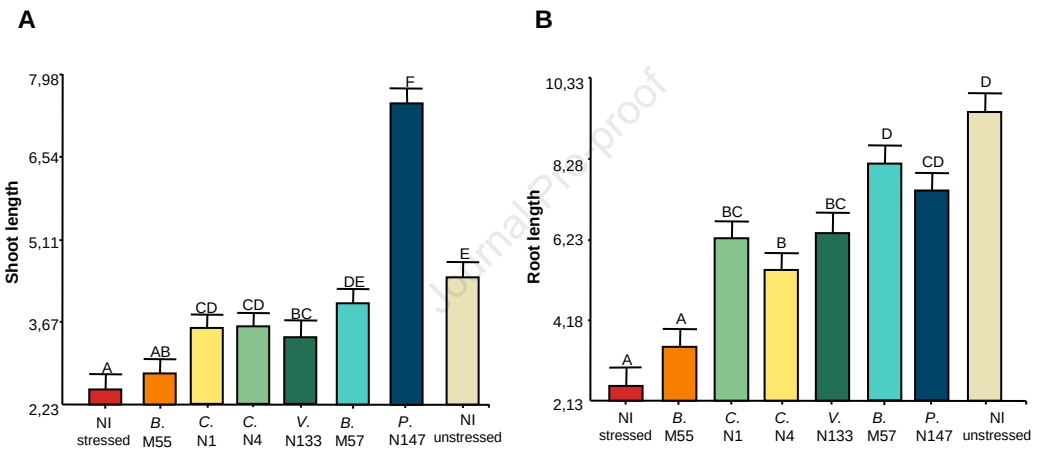






	Plant height	Leaf area	LDW	STW	RDW	TDW	SLA	Chl-*a*	Chl-*b*	Total Chl	Leaf P	Leaf K	Leaf Ca	Leaf Mg	Leaf N
Plant height	1	0.363	0.394	0.344	0.188	0.368	0.014	0.003	-0.024	-0.018	-0.210	0.148	-0.286	0.416	-0.192
Leaf area	0.363	1	0.708***	0.238*	0.270*	0.532***	0.233*	0.301	0.705**	0.551*	-0.078	0.277	-0.348	0.427*	0.365
LDW	0.394	0.708***	1	0.544 ***	0.679***	0.910***	-0.294	0.172	0.699**	0.467*	-0.577**	-0.058	0.054	0.013	0.004
SDW	0.344	0.238*	0.544***	1	0.519***	0.734***	-0.301**	0.055	0.292	0.178	-0,652**	-0.053	-0.035	0.104	-0.351
RDW	0.188	0.270*	0.678***	0.519***	1	0.857***	-0.322**	-0.038	0.415*	0.227	-0.608*	-0.449*	0.320	-0.278	-0.202
TDW	0.368	0.532***	0.910***	0.734***	0.856 ***	1	-0.347**	0.052	0.585*	0.346	-0.677**	-0.219	0.147	-0.081	-0.135
SLA	0.014	0.233*	-0.294	-0.301**	-0.322**	-0.347**	1	0.222	0.163	0.205	0.484*	0.459*	-0.417*	0.482*	0.503*
Chl-*a*	0.003	0.301	0.172	0.055	-0.038	0.052	0.222	1	0.211	0.822***	0.522*	0.441	-0.275	0.230	0.470
Chl-*b*	-0.024	0.705**	0.698**	0.292	0.415*	0.585*	0.163	0.211	1	0.684**	-0.071	0.622**	-0.173	0.511*	0.431
Total Chl	-0.018	0.551*	0.467*	0.178	0.227	0.346	0.205	0.822***	0.684**	1	0.300	0.622**	-0.318	0.420	0.492*
Leaf P	-0.210	-0.078	-0.577**	-0,652**	-0.608*	-0.677**	0.484*	0.522*	-0.071	0.300	1	0.365	-0.204	0.046	0.265
Leaf K	0.148	0.277	-0.058	-0.053	-0.448*	-0.219	0.459*	0.441	0.622**	0.622**	0.365	1	-0.363	0.437	0.711*
Leaf Ca	-0.286	-0.348	0.054	-0.035	0.320	0.147	-0.417*	-0.275	-0.173	-0.318	-0.204	-0.363	1	-0.364	-0.671*
Leaf Mg	0.416	0.427*	0.013	0.104	-0.278	-0.081	0.482*	0.230	0.511*	0.420	0.046	0.437*	-0.364	1	0.768*
Leaf N	-0.192	0.365	0.004	-0.351	-0.202	-0.135	0.503*	0.470	0.431	0.492*	0.265	0.711*	-0.671*	0.768*	1





Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: