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Peanut Endophytic Phosphate Solubilizing Bacteria Increase Growth and P Content of Soybean and Maize Plants

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Abstract

Phosphorus (P) is a limiting factor of plant development due to its low availability in the soil. The use of endophytic phosphate solubilizing bacteria as a more sustainable alternative to the use of chemical phosphorus fertilizers is proposed in this study. The objectives were to analyze the effect of simple inoculations of native peanut endophytic phosphate solubilizing bacteria on plant growth promotion and P content of soybean and maize and to evaluate their survival and endophytic colonization capacity on these plants. In addition, bacterial plant cell wall degrading enzymes activities in presence or absence of root exudates was determined. Soybean, maize and peanut plants were grown on a microcosm scale and inoculated with *Enterobacter* sp. J49 or *Serratia* sp. S119. It was observed that phosphate solubilizing strains promoted the growth of maize and soybean plants and contributed significantly P to their tissues. A significant increase in the phosphate solubilizing capacity of the plant rhizosphere after the end of the assay was observed. The strains showed to survive in plant's growth substrate and in the case of *Enterobacter* sp. J49, it showed also to colonize endophytically maize and soybean. Root exudates of the three plants showed to produce changes in pectinase and cellulase activities of the strains. The bacterial strains analyzed in this study constitutes potential sources for the formulation of biofertilizers for their application for several crops in agricultural soils with low P content.

Introduction

Phosphorus (P) after nitrogen (N) is a macronutrient required by plants for their nutrition. Most agricultural soils contain large reserves of inorganic P, a considerable part of which has accumulated because of regular applications of phosphate fertilizers. However, only a small fraction of the entire P is immediately available for plants in most of the world's soils [1]. This is due to the fact that phosphate anions react with cations of Ca^{2+}, $Fe^{2+/3+}$ or Al^{3+}, which cause their precipitation or fixation by adsorption to soil particles [2]. In particular, there is a great extension of agricultural soils in the world corresponding to semiarid zones, that are characterized by low values of available P (< 15 mg kg^{-1}) [3]. If it is assumed that the critical thresholds of P for soybeans and maize, crops of greater relevance in agricultural production are from 12 to 13–16 mg kg^{-1}, respectively [4] levels of P found in the soils of semiarid regions could be limiting for crop production. Considering the importance of P for crops and that low values of this nutrient have been reported in the agricultural soils, need arises to look for alternative ways to excessive use of phosphorus fertilizers. Among them is the use of bacteria with phosphate solubilizing capacity as P-biofertilizers, which is a more economical option, friendlier to environment and more efficient in the contribution of P to the rhizosphere environment [5]. Bacteria that promote growth of plants are known as "Plant Growth-Promoting Bacteria" (PGPB) and they are defined as those that, in free soil, rhizosphere, rhizoplane,

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phyllosphere and/or within plant tissue, under certain conditions, are beneficial for plants [6]. In this regard, it is important to consider that successful colonization of a strain used as an inoculant is an important requirement to promote plant growth and health [7]. It is for this reason that not only must mechanisms responsible for promoting growth be studied, but also the steps involved in colonization of plants by these bacteria. Within these beneficial microorganisms, endophytic bacteria are of great interest since it is considered that the ability to colonize internal tissues of plants give them an adaptive advantage, and thus being a trait of interest when considering a bioformulate. Endophytic spread of bacteria inside the host may be an active process involving enzymes that degrade plant polymers [8]. Among them, due to main components of plants, pectinases and cellulases have been identified. These enzymes have been implicated in mechanisms of entry of microorganisms to internal tissues of the plants [9]. Endophytic colonization by bacteria used as potential biofertilizers has been an interesting object of study in recent years, since the benefits are transferred directly to the host in a closed-loop system [10]. Some authors consider that endophytic bacteria are more efficient to exert their PGP mechanism than rhizospheric or soil bacteria. The ability to establish in the internal tissues of the plat host constitutes not only a survival advantage for de PGPB but also an advantage to plants [11].

Serratia sp. S119 and *Enterobacter* sp. J49 belongs to a bacterial collection that was isolated from peanut root nodules grown in Córdoba (Argentina) production area [12]. These strains were selected for further studies due their efficient phosphate solubilizing ability and peanut plant growth promotion [13]. Considering that great part of the agricultural production area in the world corresponds to semiarid zones (15.2% of world land surface) [14] in which soybean and maize are some of the most important crops, the main objective of this study was to evaluate the effect of the inoculation of efficient phosphate solubilizing native peanut bacteria on the growth of these plants as well as their survival and phosphate solubilizing ability in plant growth substrate. In addition and considering that, endophytic property of the strains is a desirable trait, the ability to colonize internal tissues of soybean and maize was determined. In this regard and in order to establish relationship with endophytic colonization ability, plant cell wall degrading enzymes activities were evaluated in presence or absence of root exudates of maize and soybean.

Materials and Methods

Bacterial Strains, Media and Growth Conditions

Native peanut phosphate solubilizing bacteria, *Serratia* sp. S119 and *Enterobacter* sp. J49, isolated from peanut plants cultivated in the central and southern region of Córdoba, Argentina (latitude, 32°–34°, longitude, 63°–65°) were used [12]. *Pseudomonas fluorescens* P3 was used as a reference strain. All bacteria were cultured in LB (Luria–Bertani) media (Tryptone 10 g l^{-1}, yeast extract 5 g l^{-1}, NaCl 5 g l^{-1}) at 28 °C. Bacteria were maintained in 20% glycerol (v/v) at – 80 °C.

Plant Material, Surface Seed Disinfection and Germination

Seeds of *Zea mays* SYN860 TD/TG (Syngenta), *Glycine max* NS4611 STS (Nidera) and *Arachis hypogaea* L. (var. Granoleico), were surface disinfected. They were immersed in 96° ethanol for 30 s, alcohol was discarded and hydrogen peroxide (H_2O_2) 15% was added during 15 min. Finally, six washes were performed with sterile distilled water. Seeds were then placed in sterile petri dishes containing moist cotton and Whatman N°1 filter paper and incubated at 28 °C in dark until the radicle reached a length of approximately 2 cm.

Collection of Root Exudates from Maize, Soybean and Peanut

Disinfected and pregerminated seeds were transferred to Jensen tubes (1 per tube) containing 15 ml of Hoagland liquid medium (diluted 1:4 with water) [15] incubated in a growth chamber (16 h day/8 h night cycle, at a constant temperature of 28 °C). After 7 days of incubation, liquid medium containing root exudates (RE) was collected and concentrated by lyophilisation. Dried root exudates (RE) were suspended in sterile milli-Q water in a volume necessary to achieve a 20× concentration of each plant. REs were sterilized by filtration with a cellulose acetate membrane of 0.22 µm in pore diameter.

Plant Inoculation Microcosm Assay

Pregerminated seeds were transferred to pots of 30 cm diameter and 25 cm high, containing a sterile soil and vermiculite mix (2:1). Soil used had a low content of assimilable phosphorus (9 mg P kg^{-1} of soil, pH 7.4) and was supplemented with 0.2% $Ca_3(PO_4)_2$ [16]. It was obtained from an agricultural soil of La Pampa, Argentina, which is of Mollisols type, developed in medany plains, with lithological

composition of sandy–sandy loam structure on the surface, and loam–loam sandy below [17]. Plantlets were inoculated with 4 ml of each bacterial culture (~ 10^{10} CFU ml^{-1}) in the crown of the root of soybean and maize seedlings. Control treatments were plants (i) uninoculated, fertilized with soluble P (20 mM KH_2PO_4), (ii) uninoculated and unfertilized, (iii) inoculated with reference strain *Pseudomonas fluorescens* P3 in the same conditions as peanut native strains.

Plants were grown in a microcosm with temperature conditions that ranged between 21 and 29 °C and light conditions that corresponded to natural photoperiod of the time of assay. They were watered regularly with water and alternating with nutrient solution Hoagland diluted 1:4 with rain water without phosphorus [15].

Two independent trials were conducted at different times of year: The first assay (E1) was carried out in 2016 from September 12 to November 12, while the second (E2) was carried out in 2017 from March 10 to May 10. Experiments were performed with eight replicates for each treatment.

Effect of Bacterial Inoculation on the Growth of Plants

Maize plants were harvested at 45 days (V7), soybean at 50 days (R4) and peanut at 60 days (R3). Plant growth parameters measured were the following: aerial length (cm), aerial and radical dry weight (g) and phosphorus content (P) of aerial part (mg P/g dry plant). P content was determined following methodology described by Jackson [18]. For this, 0.5 g of aerial dry matter were digested overnight with 5 ml of HNO_3. After that, 10 ml of more HNO_3 were added and left one more night. Digestion volume was brought to a final volume of 50 ml with distilled water. A 5 ml aliquot was taken and transferred to 30 ml of distilled water. A volume of 2.5 ml of Barton Reagent (25 g l^{-1} ammonium molybdate; 1.25 g l^{-1} ammonium metavanadate; 250 ml l^{-1} nitric acid) and distilled H_2O was added to reach a volume of 50 ml. For the quantification, a P standard curve was made with known concentrations of KH_2PO_4, ranging from 2 to 16 ppm of P. Like the samples, 2.5 ml of Barton's reagent was added to each one and it was brought to a final volume of 50 ml in Nessler tubes. After 10 min, the absorbance was measured at 430 nm.

Determination of Bacterial Survival in Plant Growth Substrate

Survival of bacteria was determined by counting CFU of bacteria isolated from the rhizospheric soil zone. For this, at plant harvest, 1 g of rhizospheric soil sample of substrate used for growth of plants was obtained. Serial dilutions of soil samples in physiological solution were plated on Petri dishes containing NBRIP-BPB medium (glucose 10 g l^{-1}, $Ca_3(PO_4)_2$ 5 g l^{-1}, $MgCl_2 \cdot 6H_20$ 5 g l^{-1}, $MgSO_4 \cdot 7H_20$ 0.25 g l^{-1}, KCl 0.2 g l^{-1}, $(NH_4)_2SO_4$ 0.1 g l^{-1}, bromophenol blue 2.5 mg l^{-1}, pH 7.0) [19]. After incubation of plates at 28 °C during 24 h, CFU g^{-1} dry weight of rhizospheric soil was determined.

Phosphate Solubilizing Capacity of Plant Growth Substrate

Phosphate solubilization capacity of plant growth substrate was determined at end of trial using Das and Debnath technique [20]. For this, 1 g of rhizospheric soil sample was placed in an Erlenmeyer flask containing 15 ml of minimal NBRIP medium without bromophenol blue and incubated for 15 days at 28 °C with shaking (250 rpm and 2.5 cm eccentricity). At the end of this time, an aliquot of 1.5 ml was centrifuged at 10,000 rpm for 12 min to settle insoluble P. Soluble P in supernatant (SN) was determined by Fiske and Subbarow method [21].

Isolation of Endophytic Bacteria

Plant endophytic colonization of the two strains was analyzed by isolating bacterial cells from internal tissues of leaves, stem and roots of soybean, maize and peanut plants from the microcosm assay. For this eight plants of each plant species were sampled and three 3 g of fresh tissue was used. Initially, epiphytic bacteria were removed by surface disinfection. This was done by successive washes were carried out with 70% ethanol for 1 min, 3% sodium hypochlorite for 5 min, 70% ethanol for 30 s and finally 4 washes in sterile distilled water. To control the disinfection process, aliquots of sterile distilled water used in the final wash were plated on plates with LB medium. After surface disinfection, the tissue was macerated with 10 ml of physiological solution and 1 ml was transferred to 50 ml conical tubes containing 9 ml of physiological solution. Serial dilutions were seeded in plates containing LB medium to determine the number of endophytic bacteria expressed as CFU g^{-1} of plant tissue.

Genomic Fingerprints of Isolated Bacteria from Plant Tissues

To confirm that colonies isolated from plant tissues corresponded to inoculated strain, fingerprint analysis was performed by ERIC-PCR according to de Bruijn methodology [22]. Obtaining bacterial genomic DNA was performed following methodology described by Walsh et al. [23]. Bacterial colonies grown on plates with LB medium were resuspended in 200 µl of 1 M NaCl, shaking vigorously for 1 min

and centrifuged at 12,000 rpm for 6 min. Supernatant was discarded, and pellet was resuspended in 200 µl of sterile milli-Q water, and then centrifuged under the same conditions described above. Pellet obtained was resuspended in 150 µl of 6% Chelex 100 resin (BioRad, USA) and a heat treatment was carried out consisting of a first incubation at 56 °C for 20 min and then incubated at 99 °C for 8 min, shaking vigorous at end of each cycle. DNA belonging to strain inoculated at the beginning of assay was used as a control. For ERIC-PCR, primers E1 (5′-ATGTAAGCTCCT GGGGATTCAC-3′) and E2 (5′-AAGTAAGTGACTGGG GTGAGCG-3′) were used. Reaction mixture, with a final volume of 12 µl contained 0.62 µM of each primer, 1X PCR buffer, 200 µM of each nucleotide, 6 mM $MgCl_2$, 0.65 U of Taq DNA polymerase (Promega, USA) and 3.5 µl of template DNA solution. Amplifications were performed with the following cycle of temperatures/times: 95 °C for 1 min, 35 repetitions of 94 °C for 1 min, 52 °C for 1 min and 65 °C for 8 min, finally 68 °C for 16 min. PCRs were performed in a Mastercycler gradient block (Eppendorf, Germany). Amplification products were separated by horizontal electrophoresis at 80 V on 1.8% agarose gels, using 0.5× TBE as run buffer and seed buffer used was Blue.X (Genbiotech).

Pectinases and Cellulases Activities Analysis

For quantitative determination of extracellular constitutive pectinase and cellulose activities, both strains were grown in TY culture medium in absence or presence of root exudates (1×) at 28 °C in agitation (150 rpm). Samples were taken at 7 and 24 h and centrifuged at 10,000 rpm at 4 °C for 10 min in a HITACHI CR 22G refrigerated centrifuge and cells were discarded. Supernatant was stored at − 20 °C until it was used. Pectinase was analyzed by measuring polygalacturonase (PG) and pectin lyase (PL) activities. For the former methodology described by Sunnotel and Nigam [24] with some modifications was followed. For this, 100 µl of 1% polygalacturonic acid solution (PGA) in 0.05 M acetate buffer (pH 4.5) were added to 500 µl of supernatant. Mixture was incubated at 40 °C for 10 min and 400 µl of DNS solution (3,5-dinitrosalicylic acid 2%; NaOH 2.8%; Na–K tartrate 13.3%) were added to it and incubated at 100 °C for 15 min and then brought to a final volume of 5 ml with milli-Q water. Absorbance was measured at 530 nm and a standard curve for galacturonic acid was performed in a concentration range of 5–100 µM. A unit of enzyme activity was defined as the amount of enzyme that catalyzes the formation of 1 µmol of PGA per minute.

Pectin lyase activity was analyzed by following the methodology described by Sunnotel and Nigam [24] with modifications. For this, 5 ml of 1% pectin solution in 0.05 M Tris–HCl buffer (pH 8.5) was added to 1 ml of supernatant and it was brought to final volume of 10 ml with distilled H_2O and incubated at 40 °C for 2 h. Further 0.6 ml of 9% $ZnSO_4$ and 0.6 ml of 0.5 M NaOH were added to the mixture and subsequently, it was centrifuged at 3000 rpm for 10 min. Then, 5 ml of supernatant obtained were taken, to which 3 ml of 0.04 M thiobarbituric acid and 2.5 ml of 0.1 N HCl and 0.5 ml of distilled H_2O were added. Mixture obtained was placed in a bath at 100 °C for 30 min, cooled at room temperature, and measured at 550 nm in a Spectrum SP-1102 spectrophotometer. One unit of PL activity was defined as the amount of enzyme that caused a change in absorbance of 0.01 under this condition.

Cellulase (CL) activity was measured following the methodology described by Ariffin et al. [25] with modifications. For this in 2 ml of supernatant 2 ml of 1% CMC solution in citrate buffer sodium citrate/citric acid 0.05 M (pH 4.8) were added and incubated at 40 °C for 30 min. Further, 3 ml of DNS solution (3,5-dinitrosalicylic acid 2%; NaOH 2.8%; Na–K tartrate 13.3%) was added and incubated at 100 °C for 15 min. After that time, absorbance at 575 nm was measured. A glucose standard curve was performed in a concentration range of 20–400 µM. One unit of cellulase activity was expressed as 1 µmol of glucose released per ml of enzyme per minute. Quantification of inducible extracellular cellulase activity was also performed. For this, both strains were grown in CMC medium (KH_2PO_4 1 g l^{-1}, K_2HPO_4 1.145 g l^{-1}, $MgSO_4{\cdot}7H_2O$ 0.4 g l^{-1}, $(NH_4)_2SO_4$ 5 g l^{-1}, $CaCl_2{\cdot}2H_2O$ 0.05 g l^{-1}, $FeSO_4{\cdot}7H_2O$ 1.25 µg l^{-1}, carboxymethyl cellulose 10 g l^{-1}, pH 7) at 28 °C with agitation. Samples were taken at 15 and 39 h and procedure was the same as described for constitutive cellulose activity determination.

PG, PL and CL activities were determined when bacteria grow in presence of root exudates by adding RE (1×). Specific enzymatic activity was obtained by dividing enzymatic activity with cells obtained by cell count.

Statistical Analysis

Data obtained from proposed trials were analyzed with INFOSTAT statistical software [26]. Data were analyzed using ANOVA and comparison of means was made using protected Fisher's test (LSD), with a significance level of 0.05. Prior to statistical analyses, the data were tested for homoscedasticity (Levene's test) and normality (modified Shapiro–Wilk's test) (data show in supplementary material S2) [27]. Results of specific enzymatic activities (U_{PG}/cell; U_{PL}/cell and U_{CL}/cell) were transformed with $3\sqrt{x}$ in order to carry out assumptions aforementioned. Figures and tables present untransformed values.

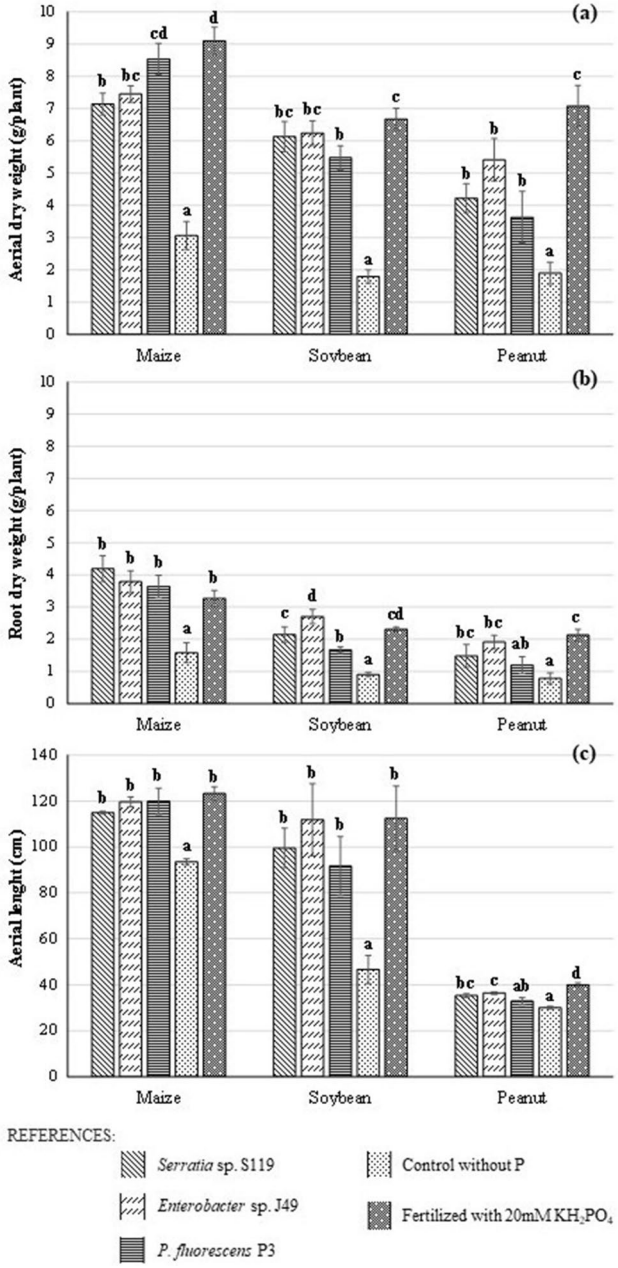

Fig. 1 Aerial dry weight (**a**), root dry weight (**b**), aerial length (**c**) of maize, soybean and peanut plants inoculated with peanut native phosphate solubilizing strains *Serratia* sp. S119 and *Enterobacter* sp. J49. Data represent mean ± SE ($n = 8$). Different letters indicate statistically significant differences ($P < 0.05$) between treatments within the same plant species

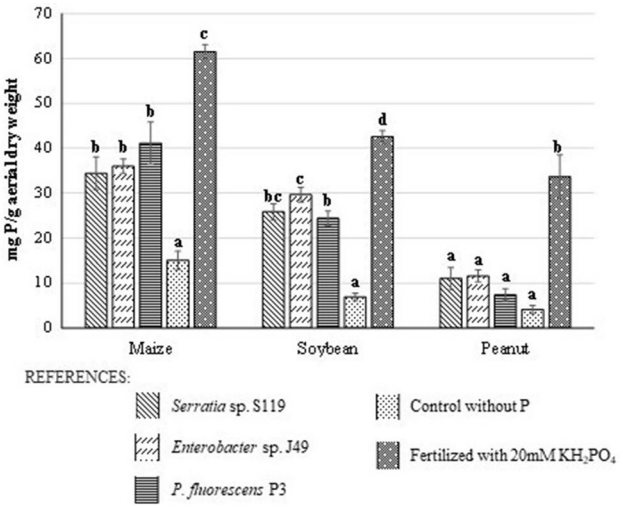

Fig. 2 Phosphorus content in aerial tissues of maize, soybean and peanut plants inoculated with peanut native phosphate solubilizing strains *Serratia* sp. S119 and *Enterobacter* sp. J49, expressed as mg P/g dry plant. Data represent mean ($n = 8$). Different letters indicate statistically significant difference ($P < 0.05$). Treatments: S119: plants inoculated with *Serratia* sp. S119; J49: plants inoculated with *Enterobacter* sp. J49; P3 = plants inoculated with *P. fluorescens* P3; Control: uninoculated and unfertilized plants; Fertilized: plants fertilized with 20 mM KH_2PO_4

Results

Effect of Inoculation of *Serratia* sp. S119 and *Enterobacter* sp. J49 on Growth and Aerial P Content of Soybean and Maize Plants

Results obtained showed that inoculation with peanut native strains on soybean and maize produced significant increases in all plant growth parameters analyzed and in aerial P content respect to uninoculated plants (Figs. 1, 2). For aerial dry weight, it was possible to observe that there was a significant increase in plants inoculated with both strains respect to control plants (uninoculated and unfertilized) (Fig. 1a). Plants inoculated with either *Serratia* sp. S119 or *Enterobacter* sp. J49 showed similar values to those observed with plants inoculated with reference strain with the exception of maize plants treated with *Serratia* strain (Fig. 1a). In the case of maize plants, only inoculation with reference strain *P. fluorescens* P3 reached similar values to that observed in plants treated with P fertilizer in this plant growth parameter. Treatment with strain *Enterobacter* sp. J49 indicated that plants had an aerial dry weight similar to those treated with *Pseudomonas* strain, although values were significantly lower than fertilized plants. In the case of root dry weight, an increase was also observed with respect to the control treatment (uninoculated and unfertilized), added to the fact that in the three plants the treatments inoculated with the native peanut strains did not show significant differences with the fertilized treatment (Fig. 1b). Root dry weight values of bacterial inoculated plants with peanut native strains showed increases similar to fertilized plants and, in soybean plants, higher than plants treated with the reference strain. For aerial length, inoculation with both strains showed increases similar to that obtained with P fertilized

plants and to those inoculated with reference strain, this being more marked in soybean and maize (Fig. 1c). Furthermore, results indicated a significant increase of P in soybean and maize plants inoculated with bacterial strains with respect to control plants (uninoculated and unfertilized) (Fig. 2). Similarly, aerial P content of maize plants showed similar values to those treated with reference strain, while soybean plants inoculated with *Enterobacter* sp. J49 showed significant higher values with respect to this treatment. In peanut plants, although an increase in *P* values was observed in aerial tissues, they do not show significant differences with the control treatment (uninoculated and unfertilized) (Fig. 2).

Survival of Inoculated Bacteria on Soybean and Maize Rhizosphere

Both bacteria survived in maize, soybean and peanut rhizosphere in a range of 10^3–10^5 CFU/g soil (Table 1). It was possible to observe that, for the three plant species under study, the strain *Enterobacter* sp. J49 was the one that reached the highest survival in the rhizosphere during time of assay. Through genetic profiles obtained by ERIC-PCR it was possible to corroborate that the data reported regarding count of surviving cells in the substrate, corresponded to strains inoculated at the beginning of assay (Fig. 3).

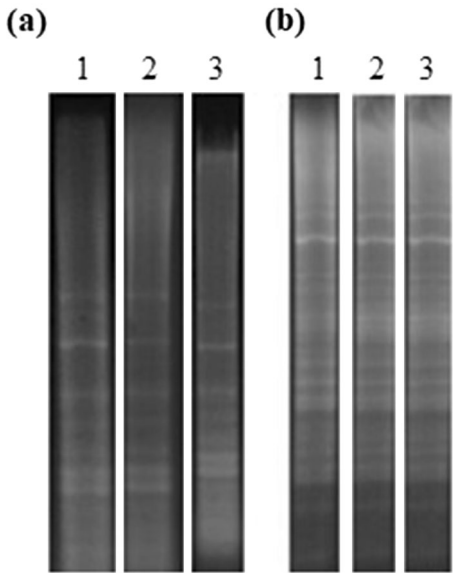

Fig. 3 ERIC-PCR profiles obtained from colonies recovered from the survival assay of *Serratia* sp. S119 (**a**) and *Enterobacter* sp. J49 (**b**). **a** Lane 1: DNA from culture of inoculated bacteria; lane 2: DNA from strains recovered from soil substrate of peanut; lane 3: DNA from strains recovered from soil substrate of soybean; **b** Lane 1: DNA from culture of inoculated bacteria; lane 2: DNA from strains recovered from soil substrate of peanut lanes 3: DNA from strains recovered from soil substrate of maize

Phosphate Solubilizing Capacity of Substrate at End of Assay

For the three plants, it was possible to observe that substrates of treatments inoculated with native phosphate solubilizing strains showed a significant increase in the ability to solubilize phosphate compared to the P fertilized treatment and to those treated with reference strain ($P < 0.05$) (Fig. 4). Treatments inoculated with the strains *Serratia* sp. S119 and *Enterobacter* sp. J49 produced an increase in phosphate solubilizing ability of substrate higher than 350% respect control plants (uninoculated and unfertilized). Substrates of plants treated with the reference strain *P. fluorescens* P3, showed an increase in this parameter of approximately 150% with respect to control plants (uninoculated and unfertilized).

Endophytic Colonization by Native Peanut Phosphate Solubilizing Bacteria

Peanut plants, their native host, were included as positive control of endophytic colonization capacity. It was possible to isolate bacteria in all organs of three plants under study. To confirm presence of inoculated bacteria inside the plant tissues, 137 colonies were selected for ERIC-PCR. Genomic fingerprints obtained indicated that it was

Table 1 Number of rhizospheric bacteria per gram of soil substrate of maize, soybean and peanut plants inoculated with the strains *Serratia* sp. S119 or *Enterobacter* sp. J49

	To (CFU/g substrate)	Tf (CFU/g substrate)		
		Peanut	Soybean	Maize
Serratia sp. S119	$4.8 \times 10^6 \pm 4.5 \times 10^5$; **a**,**d**	$5.4 \times 10^3 \pm 7.7 \times 10^2$; **a**,**a**	$1.8 \times 10^4 \pm 3.9 \times 10^3$; **a**,**b**	$3.1 \times 10^4 \pm 4.6 \times 10^3$; **a**,**c**
Enterobacter sp. J49	$5.4 \times 10^6 \pm 4.1 \times 10^5$; **a**,**d**	$8.0 \times 10^4 \pm 3.9 \times 10^3$; **b**,**a**	$1.9 \times 10^5 \pm 7.0 \times 10^4$; **b**,**b**	$2.3 \times 10^5 \pm 2.9 \times 10^4$; **b**,**c**

The data represent the mean ± SE ($n = 8$)

To: number of bacterial cells at the beginning of the assay, Tf: number of bacterial cells at the end of the assay

Different letters before the comma represent significant differences ($P < 0.05$) between bacterial strains within the same plant species, while different letters after the comma represent significant differences ($P < 0.05$) between time of inoculation and harvest for the same bacterial strain

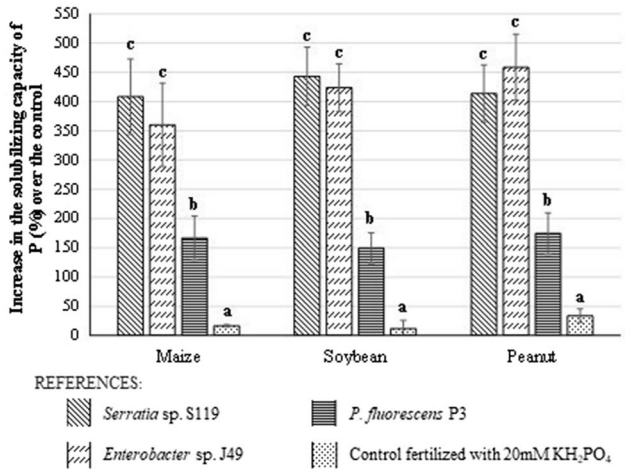

Fig. 4 Phosphate solubilizing activity of PSB strain present in substrate at end of assay, with respect to control, in plants of maize, soybean and peanut expressed as percentage (%). Data represent mean ($n=8$) Different letters indicate a statistically significant difference ($P<0.05$) Treatments: S119: *Serratia* sp. S119; J49: *Enterobacter* sp. J49; P3 = *P. fluorescens* P3; Fertilized: with 20 mM KH_2PO_4

possible to observe endophytic presence of the *Enterobacter* sp. J49 in maize leaves, soybean stem and in the three tissues of peanut (supplementary material Fig. S1). On the other hand, *Serratia* sp. S119 was isolated only from peanut stems.

Production of Pectinase and Cellulase Enzymes by *Serratia* sp. S119 and *Enterobacter* sp. J49 in Presence of Soybean, Maize and Peanut Root Exudates

To evaluate the effect of RE on production of pectinase enzymes, strains were grown in TY medium supplemented or not with RE. From the determination of polygalacturonase activity, it was possible to observe that both strains were capable of producing this type of enzyme (Table 2, Fig. 5). It was possible to observe that specific PG enzymatic activity was higher in *Serratia* sp. S119 than in *Enterobacter* sp. J49 (Fig. 5a, b). Results obtained for *Serratia* sp. S119 indicated that production of these enzymes would be related to growth stage in which cells are found (Fig. 5a). *Enterobacter* sp. J49, showed PG activity only at 24 h of growth (Table 2, Fig. 5b). The addition of RE showed no significant effect on enzymatic activity of the bacteria.

Regarding constitutive pectin lyase (PL) activity, both bacteria showed constitutive activity of these enzymes (Table 2). Specific PL enzymatic activity was significantly higher in *Serratia* sp. S119 than in *Enterobacter* sp. J49 at 24 h (Fig. 5c, d). Analysis of the effect of RE on PL activity of *Serratia* sp. S119 indicated that maize and peanut RE generated a significant decrease in pectinase activity with respect to control (without RE) and treatment with soybean RE (Fig. 5c). In the case of *Enterobacter* sp. J49, effect of RE on activity of this strain did not produce significant differences in most treatments, except for 7 h time sample in

Table 2 Polygalacturonase (PG) and pectin lyase (PL) activity produced by the *Serratia* sp. S119 and *Enterobacter* sp. J49 at 7 and 24 h of growth in presence or absence of root exudates (RE) of peanut, maize and soybean

Treatments	7 h			24 h		
	Cell concentration in TY medium (CFU ml^{-1})	Enzymatic activity PG (U_{PG} ml^{-1})	Enzymatic activity PL (U_{PL} ml^{-1})	Cell concentration in TY medium (CFU ml^{-1})	Enzymatic activity PG (U_{PG} ml^{-1})	Enzymatic activity PL (U_{PL} ml^{-1})
S119	$7.9\times10^{+9}\pm3.5\times10^{+8}$; **a**	$8.3\times10^{-3}\pm1.6\times10^{-3}$; **a**	ND	$1.0\times10^{+10}\pm6.8\times10^{+8}$; **a**	$9.9\times10^{-3}\pm9.2\times10^{-4}$; **a**	$1.1\times10^{-2}\pm5.6\times10^{-4}$; **c**
S119 + RE soybean	$7.9\times10^{+9}\pm5.4\times10^{+8}$; **a**	$6.7\times10^{-3}\pm6.7\times10^{-4}$; **a**	ND	$1.0\times10^{+10}\pm5.5\times10^{+8}$; **a**	$1.0\times10^{-2}\pm6.3\times10^{-4}$; **a**	$1.1\times10^{-2}\pm7.5\times10^{-4}$; **c**
S119 + RE maize	$7.9\times10^{+9}\pm5.3\times10^{+8}$; **a**	$6.6\times10^{-3}\pm9.5\times10^{-4}$; **a**	ND	$9.5\times10^{+9}\pm2.6\times10^{+8}$; **a**	$1.1\times10^{-2}\pm4.2\times10^{-4}$; **a**	$6.7\times10^{-3}\pm2.1\times10^{-4}$; **b**
S119 + RE peanut	$8.2\times10^{+9}\pm9.5\times10^{+8}$; **a**	$7.4\times10^{-3}\pm1.2\times10^{-3}$; **a**	ND	$1.0\times10^{+10}\pm9.3\times10^{+8}$; **a**	$9.2\times10^{-3}\pm8.6\times10^{-4}$; **a**	$4.7\times10^{-3}\pm4.9\times10^{-4}$; **a**
J49	$1.3\times10^{+10}\pm9.8\times10^{+8}$; **a**	ND	$3.3\times10^{-3}\pm2.1\times10^{-4}$; **a**	$1.6\times10^{+10}\pm1.5\times10^{+9}$; **a**	$5.0\times10^{-3}\pm3.8\times10^{-4}$; **a**	$4.7\times10^{-3}\pm2.1\times10^{-4}$; **a**
J49 + RE soybean	$1.4\times10^{+10}\pm1.6\times10^{+9}$; **a**	ND	$3.7\times10^{-3}\pm3.3\times10^{-4}$; **a**	$1.8\times10^{+10}\pm1.9\times10^{+9}$; **a**	$4.5\times10^{-3}\pm2.8\times10^{-4}$; **a**	$4.5\times10^{-3}\pm3.4\times10^{-4}$; **a**
J49 + RE maize	$1.3\times10^{+10}\pm1.5\times10^{+9}$; **a**	ND	$4.8\times10^{-3}\pm3.1\times10^{-4}$; **b**	$1.7\times10^{+10}\pm1.6\times10^{+9}$; **a**	$4.8\times10^{-3}\pm2.9\times10^{-4}$; **a**	$5.3\times10^{-3}\pm4.2\times10^{-4}$; **a**
J49 + RE peanut	$1.3\times10^{+10}\pm1.3\times10^{+9}$; **a**	ND	$4.0\times10^{-3}\pm2.6\times10^{-4}$; **ab**	$1.9\times10^{+10}\pm1.7\times10^{+9}$; **a**	$4.9\times10^{-3}\pm2.8\times10^{-4}$; **a**	$3.8\times10^{-3}\pm4.0\times10^{-4}$; **a**

Data represent mean ± S.E. ($n=8$)

ND not detected

Different letters indicate significant differences ($P<0.05$) between treatments for each of the variables measured at a given time and for each strain separately

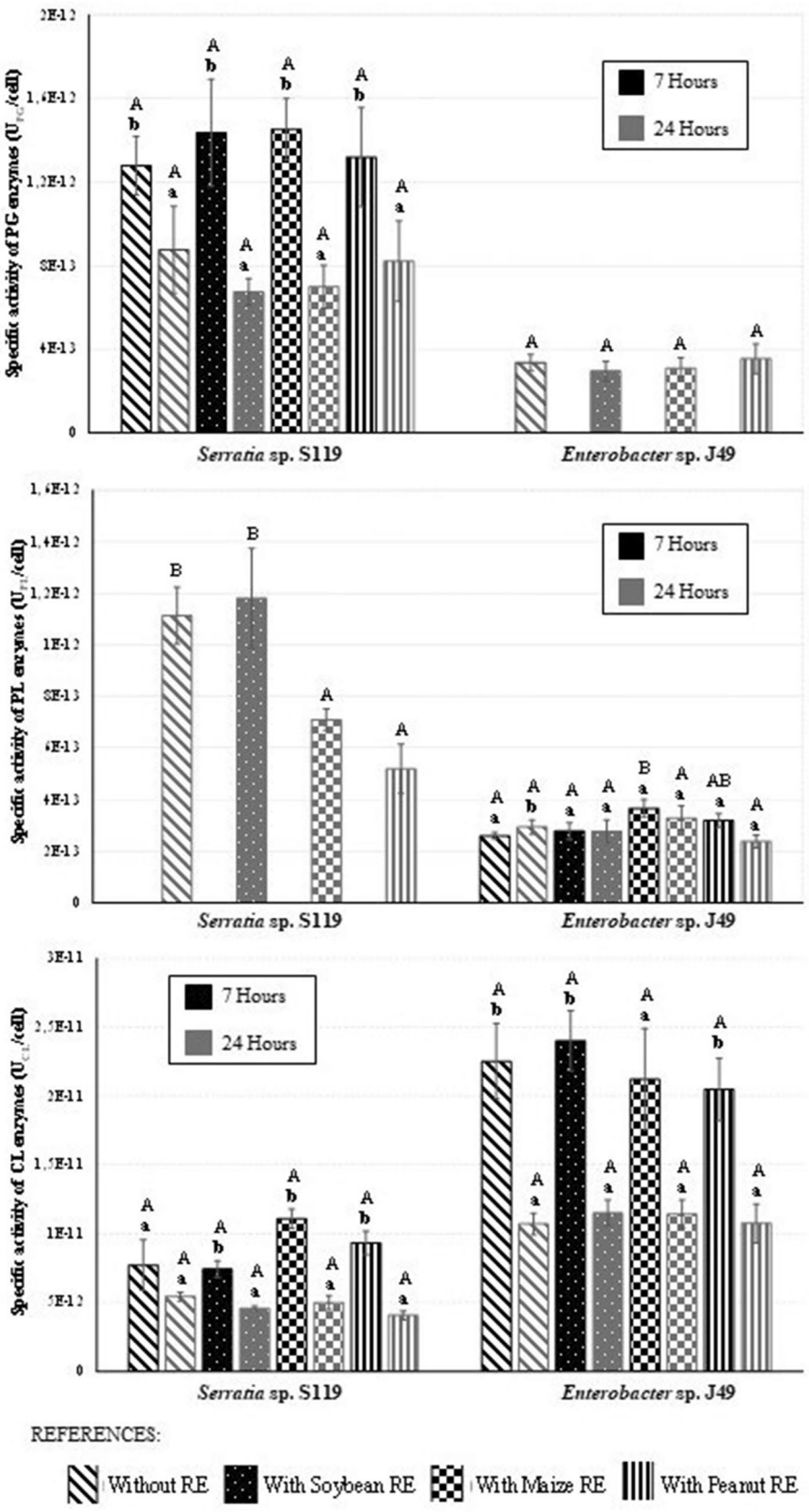

Fig. 5 Specific polygalacturonase (PG) activity (**a**), pectin lyase (PL) activity (**b**), and cellulase (CL) activity (**c**) of strains *Serratia* sp. S119 and *Enterobacter* sp. J49 at different times of growth. Data represent mean ± S.E. ($n = 8$). Different capital letters indicate significant differences ($P < 0.05$) between treatments for each strain at the same time. Different lowercase letters below indicate significant differences ($P < 0.05$) between two times for the same treatment for each strain

Table 3 Cellulase (CL) activity produced by the *Serratia* sp. S119 and *Enterobacter* sp. J49 at 15 and 39 h of growth in presence or absence of root exudates (RE) of peanut, maize and soybean

Treatments	15 h		39 h	
	Cell concentration in CMC medium (CFU ml^{-1})	Enzymatic activity CL (U_{CL} ml^{-1})	Cell concentration in CMC medium (CFU ml^{-1})	Enzymatic activity CL (U_{CL} ml^{-1})
S119	$1.60 \times 10^{+9} \pm 2.52 \times 10^{+8}$; **a**	$1.15 \times 10^{-2} \pm 8.41 \times 10^{-4}$; **b**	$2.30 \times 10^{+9} \pm 1.07 \times 10^{+8}$; **a**	$1.24 \times 10^{-2} \pm 4.86 \times 10^{-4}$; **a**
S119 + RE soybean	$1.09 \times 10^{+9} \pm 2{,}22.10^{+7}$; **a**	$8.06 \times 10^{-3} \pm 4.86 \times 10^{-4}$; **a**	$1.98 \times 10^{+9} \pm 1.25 \times 10^{+8}$; **a**	$9.03 \times 10^{-3} \pm 4.86 \times 10^{-4}$; **a**
S119 + RE maize	$1.40 \times 10^{+9} \pm 1.26 \times 10^{+8}$; **a**	$1.53 \times 10^{-2} \pm 4.86 \times 10^{-4}$; **c**	$2.48 \times 10^{+9} \pm 1.09 \times 10^{+8}$; **a**	$1.24 \times 10^{-2} \pm 1.75 \times 10^{-3}$; **a**
S119 + RE peanut	$1.44 \times 10^{+9} \pm 5.88 \times 10^{+7}$; **a**	$1.34 \times 10^{-2} \pm 9.72 \times 10^{-4}$; **bc**	$2.74 \times 10^{+9} \pm 3.11 \times 10^{+8}$; **a**	$1.10 \times 10^{-2} \pm 4.86 \times 10^{-4}$; **a**
J49	$9.56 \times 10^{+8} \pm 1.57 \times 10^{+8}$; **a**	$2.07 \times 10^{-2} \pm 9.72 \times 10^{-4}$; **a**	$2.03 \times 10^{+9} \pm 6.94 \times 10^{+7}$; **a**	$2.17 \times 10^{-2} \pm 8.41 \times 10^{-4}$; **ab**
J49 + RE soybean	$8.11 \times 10^{+8} \pm 7.78 \times 10^{+7}$; **a**	$1.92 \times 10^{-2} \pm 1.29 \times 10^{-3}$; **a**	$1.77 \times 10^{+9} \pm 8.39 \times 10^{+7}$; **a**	$2.02 \times 10^{-2} \pm 8.41 \times 10^{-4}$; **a**
J49 + RE maize	$9.56 \times 10^{+8} \pm 2.12 \times 10^{+8}$; **a**	$1.87 \times 10^{-2} \pm 1.68 \times 10^{-3}$; **a**	$2.37 \times 10^{+9} \pm 2.69 \times 10^{+8}$; **a**	$2.65 \times 10^{-2} \pm 4.86 \times 10^{-4}$; **c**
J49 + RE peanut	$9.89 \times 10^{+8} \pm 1.24 \times 10^{+8}$; **a**	$1.97 \times 10^{-2} \pm 4.86 \times 10^{-4}$; **a**	$2.21 \times 10^{+9} \pm 2.33 \times 10^{+8}$; **a**	$2.31 \times 10^{-2} \pm 8.41 \times 10^{-4}$; **b**

Data represent mean ± S.E. ($n = 8$)

Different letters indicate significant differences ($P < 0.05$) between treatments for each of the variables measured at a given time and for each strain separately

which an increase in PL activity was observed compared to control treatment (without RE) (Fig. 5d).

Both strains studied produced cellulase enzymes when they were induced with CMC (Table 3). Strain *Enterobacter* sp. J49 presented a higher specific enzymatic activity with respect to *Serratia* sp. S119 (Fig. 5e, f). Enzymatic activity of the *Serratia* sp. S119 was modified by addition of RE at 15 h (Table 3). Addition of maize RE produced a significant increase in activity, whereas soybean RE, on the contrary, decreased cellulase activity of the strain. Cellulase activity of *Enterobacter* sp. J49 was modified by addition of RE at 39 h (Table 3). Like what happened with *Serratia* sp. S119, maize RE increased activity and soybean RE decreased significantly this enzymatic activity. In both cases, when expressing activity as a function of cell number, this effect of RE was no longer evident. The activity was greater at 15 h when they are metabolically more active than at 39 h, indicating that the enzymatic activity is associated with bacterial growth.

Discussion

The use of phosphate solubilizing bacteria is an interesting strategy to make phosphorus available in soil. This group of bacteria constitutes a biotechnological tool, supplanting use of chemical fertilizers, which, in addition to their polluting effect on soil, are not very efficient due to their fixation in soil [28].

In this study, the plant growth-promoting effect of two efficient native peanut phosphate solubilizing bacteria on soybean and maize growth was evaluated. Peanut plant was included in the assays as a control and in order to compare the effect of the inoculated bacteria on plant´s growth and P acquisition of other crops of agricultural interest. In addition, the ability of the strains to endophytically colonize peanut led us to analyze whether it was exclusive to its host plant or whether the bacteria were capable of infecting tissues of other plants.

Strains used in this study previously demonstrated to increase significantly growth and yield of peanut in microcosm and field assays, respectively [12, 13]. In this study, the ability of these strains to promote plant growth and supply P to other crops of great economic importance was evaluated. Time of harvest selected for each plant was considering the moment of more P requirement for each plant. Both maize and soybean plants inoculated with native peanut strains *Serratia* sp. S119 and *Enterobacter* sp. J49 showed an increase in all plant's growth parameters analyzed. In previous studies, capacity of the peanut native strain *Serratia* sp. S119 to promote maize growth was studied since this crop is used in rotation with this legume [29]. In the present study it was possible to confirm that *Serratia* sp. S119 promoted growth of maize, overcoming the fertilized treatment with P. There are numerous reports showing the growth-promoting effect of phosphate solubilizing strains on maize crops or other crops like chickpea and barley [1, 30]. Other authors have also demonstrated the ability of Enterobacteria to promote the growth of plants used in this study. Chabot et al. [31] demonstrated that bacterial isolates obtained from a Quebec soil, *Serratia* sp. 22b and *Enterobacter* sp. 22a, both phosphate solubilizers, presented a growth-promoting effect on maize and lettuce plants. Studies that show beneficial effects of the PSB are not restricted to productive crops only. In relation to this, Castagno et al. [32], found that inoculation with phosphate solubilizing strain *Pantoea eucalypti* M91 promoted growth of *Lotus tenuis* grassland in first phenological stages, favoring its implantation in that environment.

Beneficial effect of PGPB is usually not evident when inoculated into fields, which in many cases may be due to poor colonization. Successful colonization of a strain used as an inoculant is a requirement to promote plant growth and health [7]. According to Bashan et al. [33], a desirable characteristic of a bacteria to be considered as a potential bioinoculant is its ability to survive in the soil, maintaining its plant growth-promoting abilities. In this study, both strains, *Serratia* sp. S119 and *Enterobacter* sp. J49, demonstrated to be present in substrate at end of assay and to maintain the ability to solubilize phosphate. Numerous studies revealed that availability of N and P are positively correlated with the proliferation and activities of microorganisms in soil [34, 35]. It has been shown that an increase in the number of phosphate solubilizing microorganisms in soil produces a greater release of available P in rice rhizosphere soil [20]. This highlights the advantage of inoculating the soil or plants with phosphate solubilizing bacteria, since colonization is favored and its plant beneficial effect is reflected in an increase of the yield of the crops. In addition to plant growth-promoting effect on maize, soybean and peanut these strains, showed to survive in the soil substrate and to maintain their phosphate solubilizing ability. Moreover, in previous results it has been described that in peanut growing zone of Córdoba, bacteria belonging to *Enterobacteriaceae* family predominate [36], so their application to soil would not generate an unbalance in structure or functionality of bacterial communities of soil.

Results obtained demonstrate the ability of strains under study to establish themselves in the rhizosphere, maintaining their solubilizing capacity, and colonizing plant tissues endophytically. Endophytic bacteria are widely present in agricultural crops, as determined in this work, where both strains endophytically colonized some or all of the studied plants. It was possible to detect that, like what happened in the rhizosphere, plants inoculated with *Enterobacter* sp. J49 showed a higher number of endophytic bacteria. There are previous reports that describe endophytic isolates of the genus *Serratia* spp. [37] and *Enterobacter* spp. [38, 39] from agricultural important plants such as rice, maize and soybean. The ability to endophytically colonize plants gives to a potential bacterial biofertilizer a survival advantage. Inside plant, microorganisms obtain nutrients and a safe niche to reside, while the host benefits from bacterial activities that result in promotion of plant growth [11].

For successful interactions with plants, many bacterial traits are required that help respond to environmental stimuli, communication, niche adaptation, adherence, and colonization of plants [40]. These characteristics allow them to successfully colonize their host plants. Microorganisms possess physiological capacities, such as competence of rhizosphere, motility to reach host plant, mechanisms of entry and propagation within the plant and ability to overcome its immunity [41]. Production of hydrolytic enzymes can help in intracellular colonization and development of endophytic colonization [42]. Studies describe that endophytes bacteria can actively penetrate plant cells, thanks to production of cellulolytic and pectinolytic enzymes [43]. Walitang et al. [8] found that almost all endophytic rice isolates presented catalase, pectinase and cellulase activities, highlighting the importance of these characteristics for survival and colonization. In this study, preliminary results indicated that strains under study, although differentially between them, presented capacity to produce the two types of pectinases analyzed. Both strains under study showed a constitutive production of pectinase enzymes (PG and PL), which were not significantly modified with root exudates concentrations used. Only PL enzymatic activity of the *Serratia* sp. S119 was modified by addition of RE at 15 h, in a metabolically active phase. On the other hand, results of PG activity suggests that production of these enzymes would not be associated with cell growth. Since pectin is a high molecular weight polysaccharide, there are studies that suggest that some microorganisms can produce, in a constitutive way, low levels of basal enzymatic activities that begin to act on pectin, releasing reducing sugars to medium [44].

Furthermore, cellulose is the main component of the cell wall of most land plants, where it is found as microfibrils, forming a structurally strong framework in cell walls [45]. Our results demonstrated that cellulase enzymes are inducible in both strains. Other authors have reported that cellulase production depends on the presence of the substrate that acts as its inducer [46]. Cellulolytic activity has been reported for both *Serratia* [47], and *Enterobacter* genus [48]. Also, it has been determined that bacterial growth time is an important parameter to optimize the production of cellulases [49]. The maximum values of enzymatic activity of the studied strains were reached during the exponential growth phase of the bacteria. These results coincide with those reported for *S. marcescens* strain where cellulase production acts as primary metabolite, since it occurs from cell growth stage to the end of exponential stage or beginning of stationary stage [50]. Regarding bacteria of genus *Enterobacter*, the bibliography indicates that this relationship is strain dependent [51, 52].

Conclusion

Strains *Serratia* sp. S119 and *Enterobacter* sp. J49 would allow optimizing the production of crops of agricultural interest. This would be achieved through its growth-promoting effect in plants, survival capacity and maintaining phosphate solubilizing capacity in the growth substrate. The fact of endophytic colonization of these bacteria in plant

tissues gives them adaptive and survival advantages over other rhizospheric microorganisms. The changes produced in pectinase and cellulase activities in the presence of root exudates suggests an involvement of these bacterial enzymes and plants compounds in the molecular dialogue that is established between these partners.

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Author Contributions CTL, GSL and TT designed the research work. CTL, LML and MSA conducted laboratory experiments. CTL, GSL and TT analyzed data and wrote the manuscript. All authors read and approved the manuscript.

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Declarations

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

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