Title: Impact of phosphorus deficiency on the interaction between the biofertilizer strain Serratia sp. S119 with peanut (Arachis hypogaeae L.) and maize (Zea mays L.) plants

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Title page

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Names of the authors: Liliana Mercedes Ludueña[1,2], Pilar Fernández Valdés[1,2], María Soledad Anzuay[1,2], Romina Dalmasso[1,2], Jorge Guillermo Angelini[1,2], Gonzalo Torres Tejerizo[3], Tania Taurian[1,2].

Affiliation and addres(es) of the author(s): 1. Instituto de Investigaciones Agrobiotecnológicas (INIAB, UNRC-CONICET) Río Cuarto, Córdoba, Argentina. 2. Departamento de Ciencias Naturales, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto, Agencia Postal 3, 5800 Rio Cuarto, Córdoba, Argentina. 3. Instituto de Biotecnología y Biología Molecular (IBBM-CONICET), Universidad Nacional de La Plata, Buenos Aires, Argentina.

Corresponding author: Tania Taurian. Instituto de Investigaciones Agrobiotecnológicas (INIAB, CONICET-UNRC). Departamento de Ciencias Naturales, Facultad de Ciencias Exactas, Físico-Químicas y Naturales, Universidad Nacional de Río Cuarto, Agencia Postal 3, 5800 Río Cuarto, Córdoba, Argentina. TEL number: 54-358-4676438, Fax number: 54-358-4676230, E-mail: **ttaurian@exa.unrc.edu.ar**

Abstract

The objective of this study was to evaluate the impact of phosphorus deficiency in the interaction between the biofertilizer *Serratia* sp. S119 strain and peanut and maize plants. Plant assays were performed under P deficiency and several parameters of plant-bacterial interaction were measured. Bacterial chemotaxis, root colonization and biofilm production were measured as the first steps of the plant-bacteria interaction. Also, the effect of root exudates of plants grown in P deficiency on the ability to solubilize insoluble phosphate of *Serratia* sp S119 was analyzed. Results were further correlated with analyzes of the *pqq*E gene expression and the measurement of the activity of *pqq* promoter. Finally, the effect of the root exudates on the ACC deaminase activity of S119 strain was measured. Results indicated that plant root exudates obtained under P-deficient conditions positively attract *Serratia* sp. S119 and stimulate its colonization and biofilm formation. These plant root compounds showed also to produce a stimulating effect of the phosphate solubilization ability and ACC deaminase activity of S119 strain. Finally, root exudates obtained

under P-deficient conditions induce *pqq*E gene expression and the activity of *pqq* promoter of S119 strain. In conclusion, root exudates contain specific signal molecules that attract phosphate solubilizing bacteria to the rhizosphere of peanut and maize plants growing in P deficient condition. Signal molecules would favor the colonization and establishment of the PSB strain in the surface tissues as well as in inner tissues, stimulate phosphate solubilization ability and enhance ACC deaminase activity to decrease plant's nutritional stress response.

Keywords: *Serratia* sp. S119, biofertilizer, root exudates, peanut, maize

Introduction

In the last decades, according to the exponential growth of the human population, there has come up the need to increase food manufacture. To achieve this goal, the first step is to enhance agricultural production. For this purpose, the strategies to be used should consider environmental health intending to avoid or diminish the usage of agrochemicals. In this sense, it is necessary to replace old methods by the development of new eco-friendly and sustainable ones. The use of plant beneficial bacteria, called *plant growth promoting bacteria* (PGPB) is one of the main eco-friendly options to perform an increase in crop production and one of the most extensively studied (Wang et al., 2021). PGPB is a group of microorganisms associated with plants that can stimulate their growth and health (Backer et al. 2018; Orozco-Mosqueda et al. 2021). PGPB can promote plant growth indirectly, by their antagonistic activity towards pathogens or by increasing plant immune system. PGPB can also promote plant growth directly, by facilitating nutrient acquisition, such as phosphorus (P), nitrogen (N) and essential minerals, and by producing phytohormones and enzymes (Backer et al. 2018; Valetti et al., 2018; Orozco-Mosqueda et al. 2021).

Phosphorus is an essential macronutrient required for plant growth and development but its availability in soils is limited (Weissert and Kehr, 2017; Wu et al., 2019). Plants absorb P from soil as $H_2PO_4^-$ and HPO_4^{-2} ions. Nevertheless, these phosphate ions are poorly available due to their fixation with other components, which causes a P deficient environment (Suleman et al., 2018; Mei et al., 2021). Most cultivated soils have insufficient amounts of available P, being a worldwide limiting nutrient for agricultural production (Sulieman and Tran, 2015). Phosphate solubilizing bacteria (PSB) are a group of PGPB that have the ability to solubilize insoluble phosphate, and by this trait they are considered one of the most effective strategy to

supply phosphorus (P) to plants (Anzuay et al., 2015; Solanki et al. 2018; Wu et al., 2019). PSB plays an important role in the transformation and biogeochemical cycling of insoluble and soluble P in agronomic soils (Li F. et al., 2017; Yu et al., 2019). The most efficient BSPs are Gram negative and their main mechanism of phosphate solubilization is the production of gluconic acid (GA) which is obtained through the direct glucose oxidation pathway by the action of the quinoprotein (Pyrroloquinoline-Quinone-Containing) Glucose Dehydrogenase (GDH-PQQ) (Anzuay et al., 2013; Ben Farhat et al., 2013). PQQ is a cofactor commonly reported as essential for bacterial phosphate solubilizing phenotype (Choi et al., 2008; Shen et al., 2012; Ludueña et al., 2017). The enzymes necessary for the synthesis of this cofactor are encoded in an operon called *pqq* that contains at least five genes: *pqqABCDE* with its corresponding upstream promoter region (Krishnaraj and Dahale, 2014; Ludueña et al., 2017).

Plant-PGPB interaction begins in the rhizosphere through the exchange of signal molecules between both organisms. In fact, plants recruit PGPB to the rhizosphere through the release of specific signal molecules and compounds exuded across the roots (Bardy et al., 2017). This first event (recognition phase) consists in a bacterial chemotactic response to specific molecules present in root exudates (RE) (Feng et al., 2021). These RE are a mixture of a wide variety of compounds, including primary and secondary metabolites. Primary metabolites are secreted in larger quantities than secondary metabolites and include carbohydrates, amino acids and organic acids. Secondary metabolites comprise flavonoids, glucosinolates, auxins, etc (Badri and Vivanco 2009; Vives-Peris et al., 2020). So far, most of the identified chemo-attractants of root exudates for PGPB were low-molecular-weight compounds. After this first dialog, the next step is bacterial colonization of plant tissues. Plant colonization is crucial to finally observe a beneficial effect of PGPB, and for this, it will be necessary to accomplish a series of concatenated events that will occur in the rhizosphere. Thus, after chemotactic response, bacteria will move toward the root surface (rhizoplane) to begin with the attachment which is the next event. Attachment of bacteria to the rhizoplane is the first physical step in many PGPBs-plant interactions (Knights et al., 2021). Following attachment, bacterial colonies can grow and develop a structure called biofilm. The formation of this biofilm is a key phase of successful root colonization and is a common strategy employed by many soil bacteria. Biofilm provides a physical barrier against environmental stresses including changes in pH, osmotic oscillations and UV radiation (Davey and O'Toole, 2000; Knights et al., 2021). Finally, the last event consists in bacterial movement to other sites in root tissues or to other plant organs like stems and leaves to continue with colonization. Understanding rhizosphere colonization mechanisms by PSB is essential to develop

inoculants able to compete and efficiently colonize the rhizosphere of plant crops, and therefore, to have a great impact on crop production under environments with P deficiency (Santoyo et al., 2021).

Once bacteria have colonized plant tissues, they can settle in different locations and according to this, being classified as epiphytes or endophytes. Epiphytic bacteria reside the surface of root tissues (rhizoplane) and/or the surface of stems and leaves (phylloplane). Endophytes are those bacteria that invade internal tissues of plants without developing disease symptoms (Hartlova et al. 2011; Taule et al., 2021). Endophytic bacteria present an advantage over other soil microorganisms since the inside plant tissues constitutes a protected environment that favor bacterial nutrient acquisition and protect them against environmental stresses (Badri et al. 2009; Downie 2010; Berendsen et al. 2012; Compant et al. 2020). These endophytic bacteria, on the other hand, benefit plant growth and development by different traits in response to the environmental conditions. For example, when plants grow under biotic or abiotic stress they increase ethylene levels which is a desired effect since high levels of this phytohormone cause deleterious plant responses such as leaf abscission, loss of chlorophyll pigments, inhibition of root growth and nodulation (Kudoyarova et al., 2019). Endophytic bacteria equipped with ACC deaminase activity confer a stress-protective response by cleaving the precursor of ethylene (ACC), and therefore, reducing its plant synthesis (Chen et al., 2013). Besides, endophytic bacteria have been reported to be involved in the plant phytohormone balance (producing indole acetic acid, gibberellin, cytokinins) and in the improvement of nutrient acquisition (biological N_2 fixation) (Iniguez et al., 2004; Turner et al., 2013; Etalo et al., 2018).

Peanut is one of the five most important oilseeds produced in the world (Stanley and Shi, 2016) and generally, its production is intercropped with maize, America's most widely grown cereal (Pedelini y Monetti, 2022). In Argentina, peanut (*Arachis hypogaea* L.) crop production represents a very important economic activity and almost 90 % of agricultural activities associated with this crop is concentrated in Córdoba province. Recently, the intensification of agricultural practices has decreased the nutritional quality of soils in the province of Córdoba causing deficiency on the levels of available P to plants (Anzuay et al., 2015; 2017). Thus, the concentration of this nutrient would be limiting for the growth and development of the aforementioned crops. Traditionally, soil P deficiency has been amended by applying chemical fertilizers; however, this strategy is not efficient since only 10-20% of the added P remains available for plants and their utilization can produce a severe impact in soil environment (Roberts and Johnston, 2015). Therefore, the employment of native PSB is an alternative to chemical fertilizers to solve P deficiency in agricultural soils. *Serratia* sp. S119 is a native peanut endophyte PSB that improves the

growth of peanut and maize by solubilizing high amounts of insoluble P (Taurian et al. 2010; Anzuay et al. 2015; 2017; Ludueña et al. 2017a,b). Understanding the interactions between PSB and agronomic important crops like peanut and maize is crucial to develop new strategies for optimizing the yield in a more sustainable way. The aims of this work were (1) to analyze the epiphytic and endophytic colonization of peanut and maize roots by *Serratia* sp. S119 under P deficiency plant growth conditions and, (2) to analyze the effect of peanut and maize root exudates obtained from plants grown under P deficiency conditions on several bacterial traits associated to its interaction with plants such as chemotaxis, biofilm production, phosphate solubilization and ACC deaminase activities and on the expression of *pqq*E gene and the activity of *pqq* operon of the PSB *Serratia* sp. S119 strain.

Material and Methods

PSB strains used in this study

Native peanut biofertilizer strain *Serratia* sp. S119 and *Serratia* sp. S119-466 carrying the pK18mobII-mCherry-470bp construct (Ludueña et al., 2017) were grown in Luria Bertani medium (LB, Miller 1972). When required, filter sterilized antibiotic kanamycin (Km), 50 μg ml^{-1} and chloramphenicol (Cm), 30 μg ml^{-1}, were added. For long-term storage, bacteria were maintained in LB broth with 15% glycerol at -80 ºC.

Seed material and plant growth conditions

Seeds of peanut (*Arachis hypogaea* L.) cv. MAX757 and maize (*Zea mays* L.) Hybrid NK900 TD MAX were used in this study. Peanut seeds were surface disinfected in 96 % ethanol for 30 s, rinsed in sterile water, dipped in H_2O_2 30 % during 20 min, and washed 5 times in sterile water modified from Taurian et al. (2002). Maize seeds were surface disinfected in 96 % ethanol for 30 s, rinsed in sterile water, dipped in 6 % sodium hypochlorite for 10 min, and washed 6 times in sterile water (Pereira et al. 2011). Seeds were further germinated at 28 °C in sterilized Petri dishes with one layer of Whatman N°1 filter paper and moist cotton, until the radicle reached approximately 2 cm length. Seedlings were then transferred to sterilized plastic pots containing a mixture of sterile vermiculite and sand (2:1) and supplemented with $Ca_3(PO_4)_2$ (2 g l^{-1}) as the unique source of insoluble P (P-deficient growth condition). As a control, peanut and maize plants were grown in pots with KH_2PO_4 (20 mM) as a soluble source of P (P-available growth conditions). Peanut and maize plants were maintained in a grow chamber with light intensity of 200 μR/$m^{-2}sec^{-1}$,16-h day/8-h night cycle, at a constant temperature of 28 °C and a relative humidity of 50 %, watered twice a

week with sterilized tap water and once a week with the nutrient solution described by Hoagland and Arnon (1950) but devoid of soluble phosphate.

Early root colonization assay

To determine early root colonization, epiphytic and endophytic populations of inoculated bacterium were evaluated during the first 7 days of plant-bacteria interaction. For this purpose, peanut and maize plantlets obtained as previously described were inoculated with *Serratia* sp. S119 strain. Bacterial inoculum was obtained from an overnight LB broth of S119 strain incubated at 28 °C at 120 rpm and 3 ml of approximately 10^9 CFU.ml^{-1} were deposited in the crown of the plantlet root. Isolation of epiphytic and endophytic bacteria from peanut and maize plant roots were performed as described by Kuklinsky-Sobral et al. (2004) with modifications. Briefly, for the isolation of epiphytic bacteria, complete root tissue of either peanut or maize plants were placed in a 200 ml Erlenmeyer flask containing 6 g of previously disinfected 0.1 cm diameter glass beads and 50 ml of NaCl 0.9%. Then, flasks were agitated at 150 rpm for 1 h at room temperature and further serial dilutions of this suspension were streaked into Petri dishes containing 25 ml of LB medium. To isolate endophytic bacteria, the same root tissues were further surface disinfected by serial washing in 70 % ethanol for 1 min, 2 % sodium hypochlorite for 3 min, 70 % ethanol for 30s and two rinses in sterilized distilled water. The disinfection process was checked by plating aliquots of the water used in the final rinse onto LB and incubating the plates at 28 °C. After disinfection process, root tissues were macerated individually in mortars containing 10 ml NaCl 0.9%. Next, macerated tissues were transferred to flasks with glass beads as was described above and agitated at 150 rpm for 1 h. Appropriate dilutions (10^{-1}, 10^{-2}, 10^{-3} for endophytic bacteria and 10^{-3}, 10^{-4}, 10^{-5} for epiphytic bacteria) of each flask content were plated onto LB supplemented with chloramphenicol since S119 strain is naturally resistant to this antibiotic and incubated at 28 °C for 24 h. CFU ml^{-1} were determined by the microplate method (Somasegaran and Hoben 1994).

Peanut and maize root exudates collection

Root exudates (REs) were collected from peanut and maize plants grown in P-deficient conditions (named MN-RE-TCP and MZ-RE-TCP, respectively) or, in P-available growth conditions (named MN-RE-SOLP and MZ-RE-SOLP) as it was described previously in section 2.2. For this purpose, all plantlets were harvested at 7 and 9 days after emergence in pots, for maize and peanut, respectively. Next, plant roots were immersed in sterile flasks containing 100 ml of sterile water and covered in order to protect them from

light. Roots remained submerged in water for 24 h in the same growth chamber described in section 2.2. After this period, roots were removed and the water with the dissolved REs was lyophilized. The lyophilized REs were weighed, suspended in a mix of methanol:water (1:1) and finally, filtered using 0.22 mM pore size syringe filters and stored at -80°C. Stock solutions of REs were prepared in order to obtain a concentration of 10 mg ml^{-1}.

Bacterial growth in presence of peanut and maize root exudates

Fifty microliters of a pre-inoculum (10^8 cells ml^{-1}) of *Serratia* sp. S119 strain grown in LB media for 24 h (28 °C and 150 rpm) were washed with sterile saline solution and used to inoculate 15 ml of fresh NBRIP-PSOL medium supplemented with peanut and maize root exudates (RE-PTC and RE-PSOL). The concentrations of RE used were 50 or 100 µg ml^{-1} and bacterial cultures were incubated on an orbital shaker (150 rpm) at 28 °C until stationary growth phase (OD_{620nm}=2, 10^9-10^{10} cfu ml^{-1}). Bacterial growth was determined from to 2 until 24 h by counting bacterial CFU ml^{-1} in LB medium using the drop plate method (Somasegaran and Hoben, 1994).

Bacterial chemotaxis assay

Chemotaxis of *Serratia* sp. S119 towards RE was performed by the capillary assay described by Rudrappa et al. (2008) with modifications. Briefly, a 200 ml pipette tip was used as a chamber for holding 100 mL of bacterial suspension in chemotaxis buffer (KH_2PO_4/K_2HPO_4 buffer 10 mM pH 7, disodium EDTA 0.1 mM) to a final OD_{620nm} of 0.8-1 (10^6 - 10^8 cfu ml^{-1}). Bacterial suspension was prepared from an overnight LB broth grown at 28°C at 150 rpm. A 4-cm 25-gauge needle was used as the chemotaxis capillary and was attached to a 1 ml tuberculin syringe containing 100 ml of the peanut or maize REs (50 and 100 µg.ml^{-1} of each RE diluted from the stock solution in chemotaxis buffer). The needle tip and the bacterial suspension were maintained in contact during 45 min at room temperature. After this incubation period, the needle was removed and the syringe content diluted and streaked into plates containing LB medium. Accumulation of bacteria in the needle tip was calculated as the average from the CFUs obtained in triplicate plates and the results were expressed as the mean of at least three separate capillary assays for each determination. CFU ml^{-1} were measured by the drop plate method (Somasegaran and Hoben 1994).

Bacterial biofilm formation in presence of root exudates

The effect of REs on bacterial biofilm formation was performed according to Bais et al. (2004) with modifications. An overnight LB broth of *Serratia* sp. S119 was used to inoculate 15 ml of LB media with 50 or 100 µg ml^{-1} of respective RE to get an initial OD_{620} of 0.8 (10^6 cfu ml^{-1}). Two hundred µl of bacterial suspension were placed in 96 well microtiter plates and incubated at 28°C for 24 h. After this time, absorbance was recorded at 600 nm. Each plate was further washed with distilled water and the attached cells were stained with a 0.1% Cristal Violet solution during 15 min at room temperature. Staining solution was decanted and washed to remove excess stain. Finally, the formed biofilm was dissolved in 200 µl of ethanol and absorbance was recorded at 560 nm. Biofilm formation index (BI) was calculated using the formula: $BI=OD_{560}/OD_{600}$. As control, chemotaxis buffer was added in replacement of bacterial culture broth.

Bacterial phosphate solubilization determination in presence of root exudates

In vitro inorganic phosphate-solubilizing ability was determined in NBRIP-BPB broth medium (Mehta and Nautiyal 2001). The quantity of solubilized inorganic phosphate was determined following Fiske and Subbarow (1925) method. One hundred microlitres of an overnight inoculum (10^9 CFU.ml^{-1}) in LB medium was transferred to 15 ml of NBRIP-BPB medium (pH 7) supplemented with RE. After 24, 48, 72, 96, 120, 144 and 160 h of growth, 1.5 ml of bacterial cultures were sampled and centrifuged for 12 min at 10,000 rpm. The amount of soluble P released to the medium was quantified spectrophotometrically by measuring absorbance at 660 nm. At each incubation time, CFU ml^{-1} in LB medium by using the drop plate method (Somasegaran and Hoben 1994) were determined.

PQQ operon promoter activity

To evaluate the effect of peanut and maize REs on the activity of *pqq* operon, the *Serratia* sp. S119 strain carrying the pK18mobII-mCherry-470bp construct was used (Ludueña et al., 2017). This plasmid contains the *pqq* operon promoter region of S119 strain merged with the fluorescent protein m-Cherry. The bacterial strain containing this plasmid was named S119-p470 and was grown in NBRIP-SOLP medium supplemented with REs (50 or 100µg ml-1). NBRIP-SOLP is NBRIP medium in which tricalcium phosphate was replaced for 1.2 mM K_2HPO_4 in order to create a P available minimal medium. The fluorescence of the red protein m-Cherry (exitation: 587 and emmision: 610 nm) was measured in each

bacterial culture every 2 h (during 24 h) by using a fluorometer (Fluoromax). Bacterial growth was analyzed by determining optical density at 620 nm and the number of UFC ml^{-1} (Somasegaran and Hoben 1994).

pqqE gene expression by quantitative real-time PCR (qRT-PCR):

The quantification of *pqq*E gene expression was performed using the Brilliant III ultra-Fast SYBR Green QPCR Master Mix kit (Agilent Technologies) in a Stratagene Mx3005P equipment. Initially *Serratia* sp. S119 strain was grown in NBRIP-SOLP medium (with K_2HPO_4 as a source of soluble P) (Ludueña et al., 2017b) until exponential growth phase (6 h, 10^7 cfu/ml) with the addition of peanut and maize RE (50 and 100 µg/ml). Bacterial RNA extraction was carried out using Nucleospin RNA Kit (Macherey Nagel) following the instructions indicated by the manufacturer. The RNA was suspended in RNase-free PCR-quality water and then these samples were subjected to an additional treatment with the enzyme DNAse (Epicentre). Integrity of the RNA was checked in a 0.8% agarose gel with the addition of formamide in the loading buffer. Further, quantity and purity of RNA obtained was determined by spectrophotometry (A_{260}nm/A_{280}nm ratio) using Nanodrop (THERMOSCIENTIFIC). The cDNA was synthesized using the MMLV reverse transcriptase 1° Strand cDNA synthesis kit (Life Technologies) according to the manufacturer's instructions.

qRT-PCR was performed using 2 µl (1/10 dilution) of cDNA as template. The primers F1PQQEserratia and R1PQQEserratia were used for the amplification of a fragment of *pqq*E target gene and the primers rpoBFserratia and rpoBRserratia for the *rpo*B (bacterial RNA polymerase) housekeeping gene (Ludueña et al., 2017). The determination of transcript levels by qRT-PCR was performed following the methodology described by Livak and Schmittgen (2001) to evaluate gene expression in bacteria.

Bacterial ACC deaminase activity

Bacterial cell extracts preparation

Serratia sp S119 strain was cultured overnight in 15 ml LB medium at 28°C and 150 rpm. After this time, bacterial culture (10^9 ufc ml^{-1}) was transferred to sterile conical tubes and centrifuged at 9,000 rpm 10 min at 4°C. Bacterial pellet was conserved and suspended in 7.5 ml of NBRIP minimal medium (Mehta y Nautiyal, 2001) depleted of N and supplemented with 45 µl of ACC 0.5 M and 50 or 100 µg ml^{-1} of REs. Bacterial cultures were incubated overnight at 28°C in a shaker at 150 rpm. The accumulated biomass was harvested by centrifugation at 9000 rpm for 10 min at 4°C. Cellular pellets were washed by suspending

them in 5 ml of Tris-HCl 100 mM pH 7.6 and further a second centrifugation was repeated. Wash step was repeated twice and the pellets obtained were stored to -20°C until their use.

ACC deaminase quantification

For the enzyme activity quantification, the stored pellets were suspended with 1 ml of Tris-HCl (pH 7.6) and then centrifuged at 18000 rpm 5 min. The pellets were suspended in a solution of 1 ml of Tris-HCl (pH 8.5) supplemented with 30 µl of Toluene. Immediately all sample tubes were mixed vigorously with vortex. Next, tube content was divided into two new 1.5 ml sterile tubes. A first tube containing 200 µl of sample was supplemented with 20 µl of ACC (0.5M) (A) and a second tube containing 200 µl of sample was used as a control without the addition of ACC (B). Tubes A and B were mixed vigorously with vortex during 30 sec and then incubated 15 min at 30°C. At the end of incubation time 1 ml of HCl 0.56N was added into the tubes and mixed. Both tubes were centrifuged at 18000 rpm 5 min at room temperature and 1 ml of each supernatant was transferred to a glass tube containing 800 µl of HCl 0.56N. After mixing vigorously, 300 µl of 2,4-dinitrofenilhydrazine was added and all samples were incubated 30 min at 30°C. Finally, 2 ml of NaOH 2N were added to each tube and after mixing absorbance was recorded at 540 nm. The absorbance detected corresponded to the amount of α-ketobutyrate produced when the ACC deaminase enzyme degrades the ACC (ethylene precursor) into ammonia and α-ketobutyrate. The number of µmol of α-ketobutyrate was determined by using an α-ketobutyrate standard curve (0.1-1 µmol) as was described by Honma and Shimomura (1978). Finally, 100 µl of the initial sample was stored at 4°C for total protein determination.

Statistical analyses

Statistical analyses were performed using the Infostat software by ANOVA and differences among treatments were detected by LSD and Tukey test ($p < 0.05$). Three replicates were used for all bacteria growth assays.

Results

Under P deficient growth conditions, peanut and maize plants promote the root colonization by the BSP Serratia sp. S119 strain

Peanut and maize early root colonization by *Serratia* sp. S119 strain under P deficient growth conditions was analyzed. In both plants it was possible to observe a statistically significant higher number of bacteria detected on the root surface (epiphytic bacteria) with respect to those detected inside root tissues (endophytic bacteria) irrespective of the P source in which plants were grown (Figure 1A and B). In peanut plants, it was possible to observe that since 72 h post-inoculation, the number of epiphytic bacteria was significantly higher in root tissue of plants grown under P deficient conditions compared to the number of bacteria detected in plants grown with an available source of P (Figure 1A). A similar result was detected in the number of peanut endophytic bacteria, in which a statistically significant increase of CFU was observed between the 96 and 144 h after inoculation in those plants grown in P deficiency. In maize plants, a significant increase in the colonization of *Serratia* sp. S119 strain was observed on the surface of plants grown with P deficiency with respect to the colonization of plants grown with an available source of P at all measured time points with the exception of 48 and 144 h (Figure 1B). Regarding maize endophytic colonization of S119 strain, it was possible to observe at 48, 96 and 120 h of growth a significantly higher number of CFU inside root tissue of plants grown with P deficiency compared to that detected on plants grown with an available source of P.

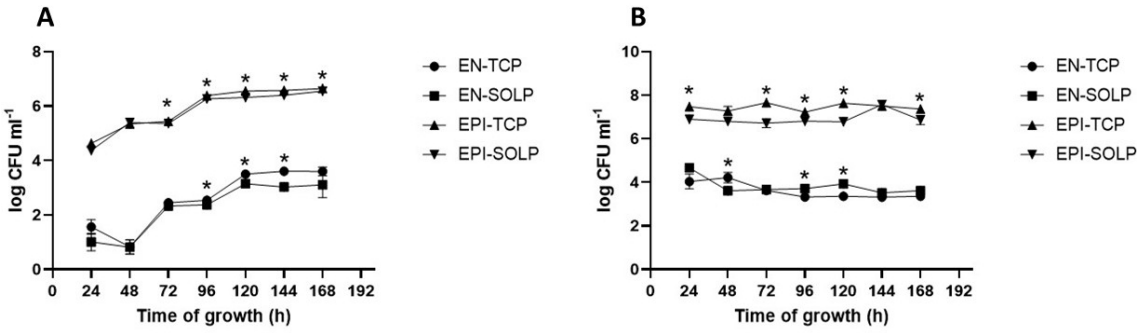


Figure 1. Peanut (A) and maize (B) colonization by *Serratia* sp. S119 strain during the first 7 days of contact (log CFU ml-1). EN: endophyte. EP: epiphyte. TCP: insoluble tricalcium phosphate P source. SOLP: soluble potassium phosphate P source. Data are means ± SE of 3 replicates (n=3). * indicate significant differences between treatments with the same bacterial location (endophyte or epiphyte). $p < 0.05$ according to the LSD test.

Root exudates of plants grown under P-deficient conditions positively attract Serratia sp. S119 strain and stimulate its biofilm formation

Initially, viability of *Serratia* sp. S119 growing in the presence of 50 and 100 µg ml^{-1} of RE of peanut and maize grown in P deficient conditions RE-TCP was assayed. Results obtained indicated that S119 strain

showed similar viability in the presence or absence of 50 or 100 µg ml^{-1} of peanut and maize RE obtained under P deficient and P available conditions (data not shown).

The ability of RE to attract bacteria was evaluated by chemotaxis assays. In presence of 100 µg ml^{-1} of peanut and maize RE a significantly higher number of bacteria were detected with RE-TCP than those observed with RE-SOLP (Figure 2A and 2B). When S119 strain grew in the presence of 50 µg ml^{-1} of either peanut or maize RE no differences were observed between those obtained from plants grown in P-deficient or P-available conditions.

Results obtained from biofilm assay indicated that S119 produced a statistically significant higher BI when it was exposed to 100 µg ml^{-1} of MN-RE-TPC with respect to the production when it grown in presence of 100 µg ml^{-1} of MN-RE-SOLP and the other treatments (Figure 2C). Fifty µg ml^{-1} of either MN-RE-TCP or MN-RE-SOLP did not produce effect on the BI of S119 with respect to control condition. When S119 was exposed to maize´s RE it was possible to observe that the BI significantly enhanced in the presence of 50 µg ml^{-1} of MZ-RE-TCP with respect to the same concentration of MZ-RE-SOLP and the other treatments and control condition (Figure 2D).

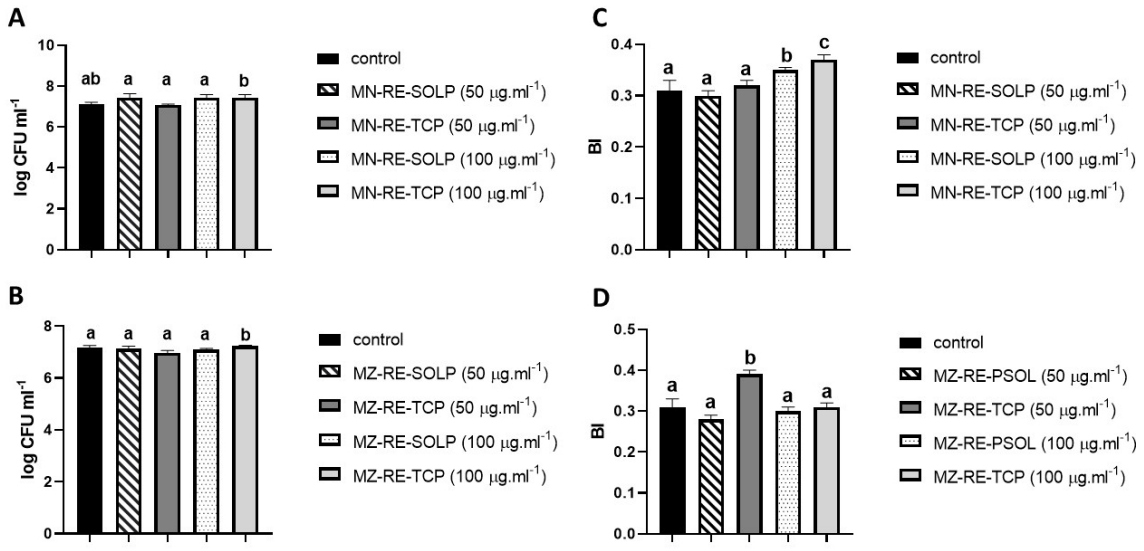
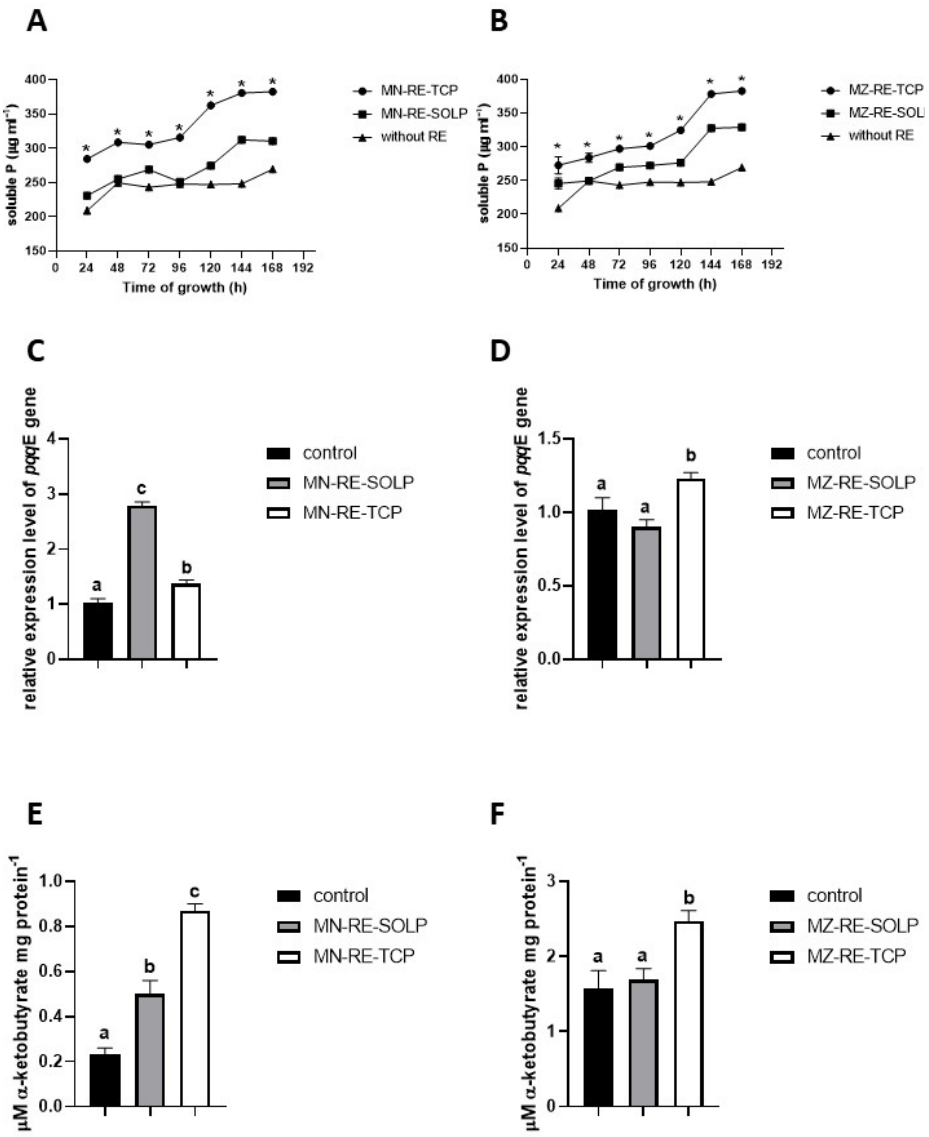


Figure 2. Chemotaxis of *Serratia* sp. S119 to peanut (A) or maize (B) root exudates (RE) expressed as log CFU ml-1. Biofilm Index (BI) of *Serratia* sp. S119 in presence of peanut (C) or maize (D) root exudates (RE). MN: peanut. MZ: maize. TCP: insoluble tricalcium phosphate P source. SOLP: soluble potassium phosphate P source. Data are means ± SE of 3 replicates (n=3). Different letters indicate significant differences between treatments with the same RE concentration and the control conditions. $p < 0.05$ according to the LSD test.

Phosphate solubilization activity and pqqE gene expression of Serratia sp. S119 are stimulated by root exudates of plants grown under P-deficient conditions

348 The addition of RE obtained under P deficient conditions (RE-TCP) in the medium of S119 strain indicated
349 an increase in the levels of soluble P released to the supernatant of the culture medium with respect to those
350 detected in the presence of RE-SOLP and in the absence of RE (Figure 3A and B).
351 It was possible to observe that the expression of *pqq*E gene was significantly increased in the presence of
352 MN-RE-SOLP and MN-RE-TCP with respect to the control without RE, being the highest expression
353 detected with MN-RE-SOLP (Figure 3C). When S119 grew in the presence of MZ-RE-TCP the *pqq*E gene
354 expression detected was statistically higher with respect to levels observed when it was exposed to MZ-
355 RE-SOLP and in absence of REs (Figure 3D).
356



357

358 **Figure 3.** Levels of soluble P released by strain *Serratia* sp. S119 growing in NBRIP-SOLP medium supplemented
359 with peanut (A) or maize (B) root exudates (RE). Relative expression level of *pqq*E gene of *Serratia* sp. S119 growing
360 in presence of peanut (C) or maize (D) root exudates (RE). ACC activity (µM α-ketobutyrate mg protein^{-1}) of *Serratia*
361 sp. S119 growing in presence of peanut (E) or maize (F) root exudates (RE). MN: peanut. MZ: maize. TCP: insoluble

tricalcium phosphate P source. SOLP: soluble potassium phosphate P source. Data are means ± SE of 3 replicates (n=3). * and different letters indicate significant differences between treatments, $p < 0.05$ according to the LSD test.

The activity of pqq promoter region of Serratia sp S119 is induced by root exudates of peanut and maize plants grown under P deficient conditions

Results obtained when bacteria grew in the presence of 50 µg ml^{-1} of peanut RE indicated no differences in the activity of the *pqq* promoter regardless of the availability of P present during its recollection in all time points analyzed (Table 1). When S119 was exposed to 100 µg ml^{-1} of MN-RE-SOLP, a higher *pqq* promoter activity was recorded at 8 h of growth with respect to the MN-RE-TCP treatment. At 4 and 6 h of growth, irrespective of P source used, a higher *pqq* promoter activity was detected with respect to the control conditions without RE. In the presence of 50 µg ml^{-1} of maize RE no differences were detected between RE-PTC and RE-SOLP treatments. An induction of *pqq* promoter activity at 4 and 6 h of growth with these both REs was detected, compared to values detected when S119 strain grew without the addition of RE. With 100 µg ml^{-1} maize RE, results obtained indicated a significantly higher *pqq* promoter activity with MN-RE-TCP at 4 h of growth with respect to that observed with MN-RE-SOLP.

Table 1. PQQ operon promoter activity in *Serratia* sp. S119 strain growing in the presence of peanut or maize root exudates

RE concentration (µg.ml^{-})		m-Cherry fluorescence/OD_{620nm}			
		Time of growth			
Treatment		4 h	6 h	8 h	24 h
Without root exudates		7.62 ± 0.02 a	7.86 ± 0.02 a	7.92 ± 0.01 b	7.59 ± 0.003 b
Peanut root exudates					
MN-RE-TCP	50	8.49 ± 0.21 a	8.02 ± 0.05 a	7.85 ± 0.04 a	7.81 ± 0.004 a
MN-RE-SOLP		8.39 ± 0.24 a	8.07 ± 0.01 a	7.82 ± 0.11 a	7.77 ± 0.03 a
MN-RE-TCP	100	7.73 + 0.08 ab	8.08 + 0.03 b	7.78 + 0.01 a	7.56 + 0.01 a
MN-RE-SOLP		7.81 + 0.03 b	8.01 + 0.06 b	7.86 + 0.01 c	7.54 + 0.01 a
Maize root exudates					
MZ-RE-TCP	50	7.90 + 0.06 b	8.07 + 0.06 b	7.85 + 0.01 a	7.50 + 0.02 a
MZ-RE-SOLP		7.83 + 0.06 b	8.15 + 0.02 b	7.84 + 0.02 a	7.45 + 0.03 a
MZ-RE-TCP	100	8.03 + 0.02 c	8.19 + 0.01 b	7.87 + 0.01 a	7.56 + 0.004 a
MZ-RE-SOLP		7.92 + 0.04 b	8.14 + 0.04 b	7.85 + 0.01 a	7.55 + 0.01 a

RE: root exudates. Different letters indicate significant differences between treatments. Data are means ± SE of 3 replicates (n=3), $p < 0.05$ according to LSD test ($p < 0.05$)

ACC deaminase activity of Serratia sp. S119 is stimulated by root exudates from P-deficient grown plants

Initially, the ACC deaminase activity of *Serratia* sp. S119 strain was analyzed at 24 h of growth in NBRIP medium supplemented with ACC as a sole source of N without RE. Results showed that S119 exhibits ACC deaminase activity with a mean value of 132.76 nM of α-ketobutyrate.mg^{-1} of protein. The impact of RE on this enzymatic activity was assayed using 100 µg ml^{-1} and 50 µg ml^{-1} of peanut and maize RE, respectively. Results indicated that the highest enzyme activity of S119 strain was detected when it was grown with RE from plants grown under P deficient conditions (MN-RE-TCP and MZ-RE-TCP) (Figure 3E and 3F). In presence of both MN-RE-TCP and MN-RE-SOLP it was possible to observe a significant increase in ACC activity of S119 with respect to control conditions (without RE) being significantly higher when it was exposed to RE-TCP with respect to that observed with RE-SOLP (Figure 3E). The ACC desaminase activity detected when S119 strain was grown in the presence of MZ-RE-TCP was significantly higher with respect to ACC activity quantified in the presence of MZ-RE-SOLP and control conditions (Figure 3F).

Discussion

Phosphorus (P) is an essential plant nutrient required for many cellular functions like carbon metabolism, energy generation and transfer, enzyme activation, membrane formation and nitrogen fixation (Schachtman et al., 1998). Although P is abundant in soils, only a small fraction is available to organisms (Alori et a., 2017; Sharma et al., 2013). This P deficiency is the result of its natural fixation to soil insoluble components (Richardson et al., 2001). Currently, in order to increase crop yield, there is a high demand for chemical phosphate fertilizers. Nevertheless, it is important to highlight that only 10-25% of applied agrochemicals are utilized by plants (Robert and Johnson, 2015) which leads to an excessive use of chemical phosphate fertilizers in agriculture. In addition, this practice generates other negative effects such as soil nutrition imbalance, heavy metal accumulation and modification of the structure and genetic diversity soil microbiota (Huang et al., 2017; Park et al., 2021; Mei et al., 2021). As mentioned earlier, PSB can solubilize or mineralize insoluble inorganic and organic phosphate, respectively into available forms of P that can be then absorbed by plants (Paratesh et al., 2019; Bi et al., 2020). In this manner, PSB play an important role in the biogeochemical cycle of P. On the other hand, plant roots influence PSB population as well as other microbial communities modifying the composition of root exudates according to plant requirements, such as the response to nutrient limitation (Walker et al., 2003; Haichar et al., 2014). Thereby, root exudates show a distinct role in the physiological ecology of plant-bacteria interaction. The present study is the first

one that analyzes the interaction of a PSB with agronomic important plants in a P deficient environment. Bacterial plant colonization was evaluated under this stress condition as well as the effect of REs obtained under this nutritional deficiency on bacterial chemotaxis, biofilm production, phosphate solubilization and ACC deaminase activity. In addition, genetic studies related to bacterial phosphate solubilization were analyzed in order to correlate results observed in the phosphate solubilization ability of the PSB strain used.

The native strain used in this study; *Serratia* sp. S119, was isolated from peanut root nodules and exhibits a strong *in vitro* ability to solubilize inorganic and mineralize organic phosphates (Taurian et al., 2010; Anzuay et al., 2013, 2017). In addition, S119 strain promotes the growth of peanut (*Arachis hypogaea* L.) (Taurian et al., 2010, Anzuay et al., 2015; Ludueña et al., 2016) and maize (*Zea mays* L.) plants (Ludueña et al., 2016) under controlled phosphate deficient growth conditions. In the present study, we aimed to analyze specific bacterial properties associated to the interaction that would explain the beneficial effect of this PSB on peanut and maize plants. We started from the hypothesis that when bacteria interact with plant roots, initially they should sense signal molecules present in the root exudates. Several studies have demonstrated the chemotaxis effect of root exudates. Thus, Vora et al. (2021) reported a prominent chemotaxis of *Rhizobium* sp. IC3109 to maize-pigeon pea intercropping root exudates. In the particular case of peanut, Ankati and Podile (2019) informed the presence of molecules in root exudates of this legume that may be responsible for inducing positive chemotaxis of *Bacillus sonorensis* RS4 and *Pseudomonas aeruginosa* RP2 to its root tissues. In another study by Feng et al (2018) it was detected that some amino acids like serine and threonine present in peanut root exudates were associated with the attraction of *Bacillus amyloliquefaciens* SQR9. It is important to stand out that none of these authors have studied the plant-bacteria interaction under nutrient stress conditions. This is the first study that evaluates the effect of peanut and maize root exudates obtained under phosphate deficient conditions on the chemotaxis, biofilm and colonization of a native PSB. The molecular dialog established between both partners will allow PSB movement toward root tissues thanks to a process called chemotaxis (Chagas et al., 2018). Our results demonstrated that both peanut and maize root exudates have specific molecules that may act as signals attracting S119 strain to the root surface. Nevertheless, only in the presence of peanut root exudates obtained from plants grown under phosphate deficiency was it possible to detect a significant increase in chemotaxis towards these plant compounds. This could be explained by the fact that S119 strain is a very efficient phosphate solubilizer strain that can be beneficial for plant nutrition and for this reason, it is recruited to the peanut and maize rhizosphere.

Once bacteria reach the root surface, the interaction between the bacterium and plant continues with the colonization process (Vanbleu and Vanderleyden, 2010). The areas of major colonization are the sites where most of the exudates are released, like the zone of emergence of secondary roots and root hairs (Haichar et al., 2014). Bacterial colonization and establishment can be verified by counting bacterial cells and by evaluating biofilm development (Taulé et al., 2021). *Serratia* sp. S119 strain colonized epiphytically and endophytically all plant tissues assayed, being more abundant on the surface than inside of those tissues. However, the most interesting result was the fact that, regardless of plant location, colonization of S119 strain was higher in plants grown under P deficient conditions than in plants grown with an available source of P. This bacterial behavior may be due to specific signal molecules present in peanut and maize RE grown under P deficiency that recruit S119 strain and stimulate its colonization. Similarly, Cesari et al. (2019) reported positive chemotaxis, stimulated adhesion and colonization of bacteria in the presence of peanut root exudates obtained from plants grown under water restriction. Our results showed that the biofilm formation of *Serratia* sp. S119 strain was stimulated in the presence of peanut and maize RE obtained mainly from plants grown under phosphate deficient conditions. In a similar study, Sampedro et al. (2020) observed that different concentrations of REs from the halophyte *Salicornia hispanica* significantly increased the biofilm production of *Halomonas anticariensis* FP35. Other studies demonstrated that tomato and *Arabidopsis* REs increase biofilm formation in different species of the genus *Bacillus* (Rudrappa et al., 2008; Chen et al., 2012).

The major colonization of S119 strain on maize plants with respect to peanut plants, from which the strain was isolated, can be explained by the fact that it probably establishes a better mutualistic relationship with this plant. This explanation is supported by previous results in which S119 strain promoted more efficiently the growth of maize than peanut plants (Anzuay et al., 2017; Ludueña et al., 2017a). *Serratia* sp. S119 belongs to a phosphate solubilizing bacterial collection obtained from peanut plants grown in Cordoba soils were maize is used as rotative crop (Anzuay et al.2017; 2021). It is highly probable that this strain was isolated from peanut because this oilseed was used as a plant tramp without being its preference host plant. In addition, S119 strain is very well adapted to Cordoba's soil conditions as well as the pesticides used in this agricultural area (Anzuay et al., 2017) and for this reason is able to efficiently solubilize soil phosphates and stablish mutualistic relations with the two mainly cultivated crops in this region; peanut and maize.

Considering that S119 strain is a very efficient phosphate solubilizing bacteria, we evaluated the effect of peanut and maize root exudates on this plant beneficial trait. We found that the addition of peanut and maize

root exudates obtained mainly under phosphate deficient conditions significantly improved the phosphate solubilizing ability of S119 strain. Similar results were described by Ding et al. (2021) in which sweet potato REs enhanced phosphate solubilizing activity of several PSB. These authors suggested that sweet potato RE would increase this activity through the promotion of colonization by the PSB. We postulate that there are molecules in peanut and maize RE obtained under phosphate deficient conditions that trigger in *Serratia* sp. S119 strain a signaling pathway associated with phosphate solubilization mechanisms. To support this hypothesis, we evaluated the expression of *pqq*E gene and the activity of a *pqq* promoter region of *Serratia* sp. S119 by growing this strain in the presence of root exudates obtained from plants grown in P deficient conditions. We have previously demonstrated that PQQ cofactor is essential for the phosphate solubilizing phenotype in *Serratia* sp. S119 (Ludueña et al. 2016). The abolishment of PQQ production by this strain was obtained by interrupting the *pqq*E gene, which significantly reduced its phosphate solubilizing activity. *pqq*E gene expression with peanut 100 µg ml^{-1} root exudates indicated that even though both MN-RE-SOLP and MN-RE-TCP induced gene expression, the highest levels of *pqq*E expression was detected with RE obtained from plants grown with available P conditions. On the other hand, with maize 50 µg ml^{-1} of MZ-RE-TCP the highest *pqq*E gene expression was induced. Regarding the activity of *pqq* promoter, we used two different concentrations of peanut and maize RE obtained from plants grown with P deficiency or with an available source of P. Although we detected an increase in the activity of *pqq* promoter of S119, these results were dependent on the concentration of RE, bacterial time growth and the origin of RE used. Nevertheless, the greater activity of *pqq*E promoter in the presence of 100 µg/ml of MZ-RE-TCP also supports the results detected in *pqq*E gene expression analysis.

Finally, we also observed that root exudates obtained from plants grown under P deficiency stimulated ACC deaminase activity in S119 strain. Considering that P deficit is a stressful condition and that this enzymatic activity would be induced under an unfavorable growth environment it is expected to find an enhancement in its activity probably by the detection of changes in the composition of the REs of peanut and maize plants. Truyens et al. (2013) reported similar results by determining an increase in ACC deaminase activity in bacteria isolated from *Arabidopsis thaliana* seeds previously exposed to stress due to heavy metal contamination. These authors observed that the enzymatic activity in this group of bacteria was statistically higher than that quantified in bacteria from seeds that were not exposed to this stress. A similar observation was described by Timmusk et al. (2011) who determined a higher number of bacteria with ACC deaminase activity in the rhizosphere of rye plants grown under salt stress conditions compared

to the number of bacteria recovered from plants that grew without being exposed to these stressful conditions. To summarize, it is possible to infer that, under stress conditions, such as nutrient P deficiency, peanut and maize plants would recruit bacteria that produce the enzyme ACC deaminase through signal molecules present in their RE in order to reduce the harmful levels of ethylene generated during those stressful conditions. Future studies targeting the detailed profile of primary metabolites of root exudates under P deficient conditions would help to understand more deeply the molecular dialog between plant-bacteria interaction. These findings would permit to associate PSB behavior to a specific set of plant´s root molecules in the rhizosphere in order to improve P availability to plants.

Conclusions

It is possible to conclude that peanut and maize plants grown under P deficient condition exude compounds there are specific signal molecules that would be capable to recruit PSB to the rhizosphere of these plants. These signal molecules would favor the colonization and establishment of the PSB strain in the surface tissues as well as in inner tissues, stimulate phosphate solubilization ability and enhance ACC deaminase activity to decrease plant stress response.

Author contribution

TT and LL conceived and designed the experiments, and wrote the manuscript. LL and PFV performed the experiments. MSA, RD, JGA and GTT helped to analyzed results.

Aknowledgements

LL, M.S.A., J.G.A., G.T.T. and T.T. are members of research career of CONICET, Argentina. RD is a PhD student of CONICET and PFV is a PhD student of Agencia Nacional de Promoción Científica y Tecnológica (ANPCyT).

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