

Motility and biofilm production involved in the interaction of phosphate solubilizing endophytic strains with peanut, maize and soybean plants

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ABSTRACT

Endophytic plant growth promoting bacteria are of great interest since the ability to colonize the internal tissues of plants gives it an adaptive advantage. To confer any beneficial effect on the plant, bacteria must present a successful colonization and thus be competent in the rhizosphere and finally infect internal tissues. In this sense, bacterial motility is an essential property involved in plant-microorganism interaction where bacteria can colonize the root and at the same time form biofilms. Within plant beneficial bacteria those that present phosphate solubilizing activity are of great interest due to the contribution of phosphorus to plants. The objective of this work was to evaluate the motility and biofilm formation properties of the strains *Serratia* sp. S119 and *Enterobacter* sp. J49 growing in the presence of peanut, maize and soybean root exudates. Results obtained indicated that both strains have flagella, possess swimming, swarming and twitching motilities and showed differential chemotactic attraction against root exudates. In addition, the strains under study showed the ability to form biofilms, being this ability greater in minimal media, in which a greater decrease was also seen by the addition of root exudates in the first hours of growth. The changes produced by the addition of root exudates in the chemotaxis and biofilm formation of the strains suggests that compounds released by the plants are detected by these bacteria and could be part of the molecular dialogue involved in their interaction with the roots of plant.

1. Introduction

Plants and their associated microorganisms, which can be rhizospheric, epiphytic and endophytic, are characterized by varied and complex interactions and have been the subject of extensive research and various applications (Glick, 2012). These microorganisms can be classified according to their effect on plants and the way they interact with them. Thus, some are pathogenic, while others trigger beneficial effects (Saharan and Nehra, 2011). Bacteria that promote plant growth are known as “Plant Growth Promoting Bacteria” (PGPB) (Bashan and Holguin, 1998). They are defined as those that, in the soil, the rhizosphere, the rhizoplane, the phyllosphere and/or within the plant tissue, under certain conditions, are beneficial for the plants. Within PGPBs are insoluble phosphate solubilizing and/or mineralizing bacteria (PSB). They constitute an important group in the natural cycle of phosphorus

(P), mainly due to the contribution of this nutrient to plants. The mechanism of solubilization of inorganic phosphate compounds by PSB is generally associated with the release of low molecular weight organic acids, which through their hydroxyl and carboxyl group, chelate the cations (Al^{3+} , $\text{Fe}^{2+/3+}$ and Ca^{2+}) linked to phosphates making them soluble (Behera et al., 2014). On the other hand, generally the mineralization of organic compounds of P is carried out by enzymatic mechanisms through the production of enzymes such as phosphatases (phosphohydrolases) or phytases (Behera et al., 2014; Rodríguez et al., 2006).

Within PGPB, endophytic microorganisms are of great interest since it is considered that the ability to colonize the internal tissues of plants gives to the bacteria an adaptive advantage (Hardoim et al., 2008). Before being able to confer any beneficial effect on the plant, endophytic PGPBs must accomplish a successful colonization in the rhizosphere. For

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this, they need to be competent in the rhizosphere and/or rhizoplane (Whipps, 2001) and finally infect internal tissues. Both rhizospheric and rhizoplane colonization are intimately linked to exudates released by the roots. Compounds released by the roots are carbohydrates, amino acids, organic acids, which constitute a source of nutrients for soil bacteria (Zhalnina et al., 2018). Root exudates produce variable patterns of molecules along the root, making bacterial colonization not uniform (Hayat et al., 2017).

It has been described that the infection of internal tissues by endophytic bacteria would involve specific characteristics such as the ability to produce enzymes degrading plant cell walls, detoxification mechanisms, twitching or flagellate-mediated motility, lipopolysaccharide production and, in the specific case of rhizobia, nod genes (Compant et al., 2010). Nevertheless, in some cases endophytic colonization occurs passively through fissures that occur in emerging roots, created by pathogenic microorganisms or at the tip of the root (apical meristem) (Reinhold-Hurek and Hurek, 2011). In the fight for survival, the ability to colonize different environments can provide new resources and opportunities to bacteria. Bacterial motility towards root exudates plays an important role in the colonization of the rhizosphere and the rhizoplane (Lugtenberg and Kamilova, 2009). Swimming, swarming and twitching they are within the most studied motilities (Harshey, 2003). Swarming and swimming motility are a mode of bacterial movement that is propelled by rotating flagella. Twitching motility is produced on surface driven by the extension, adhesion to the surface and retraction of the type IV pili by pulling the cell towards the junction site (Jarrell and McBride, 2008).

In addition to motility, chemotaxis, which is the response to chemical agents, allows bacteria, among other things, to search for nutrients and transport themselves effectively to grow and survive (Mazumder et al., 1999). It has been studied in detail in flagellated bacteria, although some without flagella are also chemotactic (Lux and Shi, 2004). Swimming motility is known as the fundamental basis of the process denominated as chemotaxis (Lux and Shi, 2004). Another aspect linked to bacterial colonization in its interaction with plants, is the ability to produce biofilm. This bacterial structure gives competitive advantages as it allows to survive desiccation and other types of environmental stress (Molina et al., 2003), leading to efficient colonization of the interior of the plant. Thus, biofilm constitute a protected mode of growth and development, being their behaviour and physiology significantly different from those microorganisms that grow free in the environment (Costerton et al., 1999). Bacteria that reside in the biofilm matrix are protected from various environmental stress factors, such as extreme pH, UV radiation, antimicrobial substances, dehydration and osmotic shock, and presence of predators (Romanova et al., 2006; Costerton et al., 1987).

PSB constitute a biotechnological tool for the improvement of agricultural crops yield. Thus the study of mechanisms involved in their interaction with plant is an important aspect to explore. Considering, that endophytic colonization is a desirable trait when looking for a potential inoculant, it is important to inquire about all the steps involved in this process.

Strains used in this study are two efficient phosphate solubilizers (Taurina et al., 2010) that promote the growth of peanut, maize (Anzuay et al., 2013, 2017; Ludueña et al., 2017), and soybean (data not shown). Given the promising effects produced by the inoculation of these strains, further studies were attempted to gain more knowledge in the mechanisms of the interaction between beneficial endophytic bacteria and agronomical important plants, especially those related to bacterial infectivity. This work was based on the hypothesis that in beneficial non-symbiotic bacteria, the mechanisms involved to enter the host plant involve more than one type of motility and the ability to produce biofilm and that root exudates influence on them. In this regard, the objective of this study was to evaluate the motility and biofilm formation properties of the *Serratia* sp. S119 and *Enterobacter* sp. J49, growing in the presence of peanut, maize and soybean root exudates.

2. Materials and methods

2.1. Bacterial strains, media and growth conditions

Strains used were *Serratia* sp. S119 and *Enterobacter* sp. J49 (Taurina et al., 2010). Selection of these strain was based on their ability to efficiently solubilize phosphate *in vitro* and on its ability to promote the growth of peanut (Anzuay et al., 2013, 2017), maize (Ludueña et al., 2017) and soybean plants (unpublished data). Bacteria were grown on LB (Tryptone 10 g l⁻¹, yeast extract 5 g l⁻¹, NaCl 5 g l⁻¹, Miller, 1972); TY (Tryptone 5 g l⁻¹; yeast extract 3 g l⁻¹; CaCl₂·6H₂O 1.3 g l⁻¹; Beringer, 1974); NBRIP (glucose 10 g l⁻¹; Ca₃(PO₄)₂ 5 g l⁻¹; MgCl₂·6H₂O 5 g l⁻¹; MgSO₄·7H₂O 0.25 g l⁻¹; KCl 0.2 g l⁻¹ (NH₄)₂SO₄ 0.1 g l⁻¹; Mehta and Nautiyal, 2001); and NBRIP-Psol (glucose 10 g l⁻¹; KH₂PO₄ 3 g l⁻¹; MgCl₂·6H₂O 5 g l⁻¹; MgSO₄·7H₂O 0.25 g l⁻¹; KCl 0.2 g l⁻¹; (NH₄)₂SO₄ 0.1 g l⁻¹) media and maintained in 20% glycerol (v.v⁻¹) at -80 °C. For the determination of the grow generation time 3 mL of an inoculum in LB medium of each bacteria (approximately 10⁹ CFU ml⁻¹) were transferred to 60 ml of LB medium and were grown at 28 °C, with 200 rpm of agitation. Samples were taken every 2 h and at each incubation time, CFU. ml⁻¹ by microdrop plate method (Somasegaran and Hoben, 1994) in LB medium was determined. This assay was performed three times independently, with a six replicates each one.

2.2. Phosphate solubilizing capacity

One ml aliquot (OD_{660nm} = 0.3) of an overnight culture of each bacterium grown in LB medium was transferred to an Erlenmeyer containing 60 ml of NBRIP medium. Cultures were incubated at 28 °C with agitation (200 rpm). At 24, 48, 72, 96, 120, 144 and 196 h a 1 ml aliquot was aseptically taken to quantify the P solubilized by the bacteria. The aliquot was centrifuged at 10,000 rpm for 12 min to settle the bacteria and the insoluble P. Soluble P was determined in the supernatant by the colorimetric technique of Fiske and Subbarow (1925) adapted to small volumes. This assay was performed two times independently, with eight replicates each one.

2.3. Motility assays

Swimming, swarming and twitching motilities were determined. For swimming and swarming motilities the methodology described by Nievas et al. (2012) of the strains under study were followed. Petri dishes with TY medium diluted 1:10 in distilled water, with 0.3% and 0.5% Britania agar, for swimming and swarming respectively, were used. For the swimming test, the plates were inoculated with a sterile pointed wire from a bacterial culture colony grown on plates with LB (1.5% agar). For the swarming assay, plates were inoculated with 3 µl of a liquid culture in LB medium in stationary phase. Both were incubated at 30 °C for 72 h. The images were taken with a Syngene G: BOX image analyser.

To determine twitching motility, the methodology described by Haley et al. (2014) was followed. Bacteria colonies grown in Petri dishes with LB medium (1.5% agar) for 24 h at 28 °C, were transferred by means of a puncture with a sterile pointed tip on plates containing LB or TY medium (1% agar). The plates were incubated at 37 °C for 48 h, then at room temperature for an additional 48 h. The agar was removed from the plate and the twitching area was visualized by staining with 1% crystal violet.

2.4. Electron microscopy

Microscopic observations was performed through a confocal microscopy service with a Leica TCS SP5 Confocal Laser Microscope (CLSM) equipped with 7 laser lines, AOTF (Acousto-Optic Tunable Filter), AOB (Acousto-Optical Beam Splitter) and scanning system (Tandem-scanning system SP5) for High resolution work suitable for *in vivo* material (School of Exact Sciences, Universidad Nacional de La Plata).

2.5. Root exudates collection

Peanut (*Arachis hypogaea* L. cultivar Granoleico), maize (*Zea mays* SYN860 TD/TG from Syngenta) and soybean (*Glycine max* NS4611 STS from Nidera) seeds were disinfected following the method of Vincent (1970). They were immersed in 96° ethanol for 30 s, the alcohol was discarded and hydrogen peroxide (H₂O₂) 15% was added for 15 min. Finally, six washes were performed with sterile distilled water. Disinfected seeds were deposited in sterile Petri dishes containing moist cotton and paper filter and incubated at 28 °C in darkness until radicle reached a length of 2–3 cm. The pregerminated seeds were transferred to Jensen tubes containing 15 ml of Hoagland liquid medium (diluted 1:4 with water) (Hoagland and Arnon, 1950) and after 7 days of incubation in a growth chamber (16 h day/8 h night cycle, at a constant temperature of 28 °C), the liquid medium containing the root exudates (RE) was collected. RE were concentrated by lyophilisation and suspended in sterile Milli-Q water in a volume necessary to achieve a 20X concentration of each plant. RE were sterilized by filtration with a cellulose acetate membrane of 0.22 µm in pore diameter and conserved at –20 °C.

2.6. Chemotaxis assays

The chemotaxis assay was performed through a capillary following the technique described by Mazumder et al. (1999). One hundred µl of a bacterial culture with an approximate concentration of 3×10^8 CFU ml⁻¹ was placed in a 200 µl disposable pipette tip used as chamber. A 1 ml BD Plastipak syringe was used, with 25G 5/8 needle as a chemotaxis capillary containing 100 µl of the different RE (10X). As controls, a syringe with 100 µl of Milli-Q water and another containing 10X Hoagland medium were used.

The “needle + syringe” capillary was inserted into the pipette tip containing the bacterial cell suspension. After 45 min of incubation at room temperature, the “needle + syringe” was removed from the bacterial suspension and the content was diluted in physiological solution. The dilutions were seeded through the micro drop technique on Petri dishes with solid LB medium. The accumulation of bacteria in the capillaries was calculated as CFU. ml⁻¹ obtained according to the micro-drop technique described above.

2.7. Biofilm bacterial production in different culture media and in the presence of root exudates

The biofilm formation assay was performed following the methodology proposed by O’toole and Kolter (1998). To evaluate the formation of biofilm in different culture media, LB and TY were used as rich media, NBRIP-Psol and NBRIP as minimal media.

Two hundred µl of a culture (OD_{620nm} = 0.01) obtained from a dilution of an overnight culture, with or without root exudates (final concentration 1X), was added to each well of 96-well polystyrene microplates. The plates were incubated at 36 °C for 48 h and then at 25 °C during additional 48 h.

Planktonic cells were gently homogenized and bacterial growth was determined by measuring O.D. at 600 nm. Wells were washed with 200 µl of PBS (phosphate buffered saline) and stained for 15 min with 200 µl of 0.1% crystal violet solution (CV) in 5% ethanol. Each well was then rinsed with water. The retained dye in the adhered cells was resuspended with 200 µl of ethanol: acetone (80:20) solution added to the wells and incubated at room temperature for 30 min. The O.D. at 570 nm of resuspended CV was determined using an Epoch™ Microplate spectrophotometer. The amount of biofilm produced, based on the number of bacteria contained in each well, was estimated by determining the Biofilm formation Index (BI) from the following formula: $BI = OD_{570}/OD_{600}$

2.8. Statistical analysis

Data were analysed using the software INFOSTAT (Di Rienzo et al., 2018). Previous to analysis, data were controlled to comply with the assumptions of normality and homocedasticity. Variables were analysed with multifactorial ANOVA considering as factors the (1) independent assays, (2) treatments with addition of RE. Comparison of means was conducted using the protected test of Fisher (i.e., LSD), with a significance level of 0.05.

3. Results

3.1. Motility of native endophytic phosphate solubilizing strains *Serratia* sp. S119 and *Enterobacter* sp. J49

It was observed that both strains have swarming (Fig. 1A and B), swimming (Fig. 1C and D), and twitching (Fig. 2) motility. For the former, and based on the description of Kearns (2010), both bacteria presented a colony of swarming motility in the form of dendrites. Since swarming and swimming motility are associated with the action of flagella, a microscopic observation of both strains was performed. Electronic microscopy confirmed the presence of flagella in both bacterial strains (Fig. 1E and F). In relation to twitching that was analysed in two different media, this motility was greater in TY medium than in the LB medium for both strains (Fig. 2).

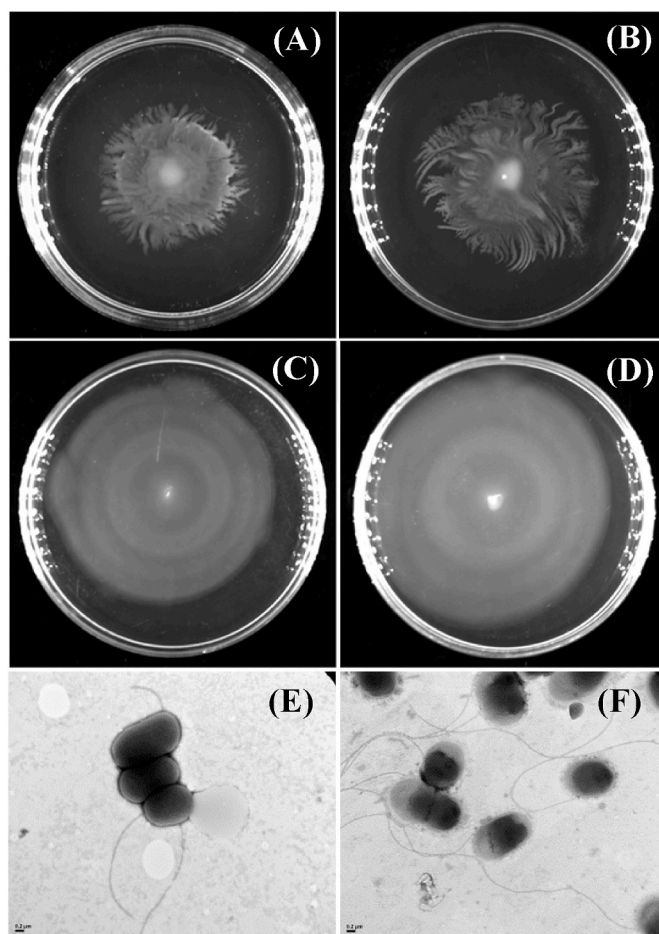


Fig. 1. Swarming (A and B) and swimming (C and D) motility on TY plates containing 0.5% and 0.3% agar respectively, after 72 h of incubation at 30 °C of *Enterobacter* sp. J49 (A and C), *Serratia* sp. S119 (B and D). Electron microscopy of flagellar structures of *Enterobacter* sp. J49 (E) and *Serratia* sp. S119 (F). Magnification $\times 30000$.

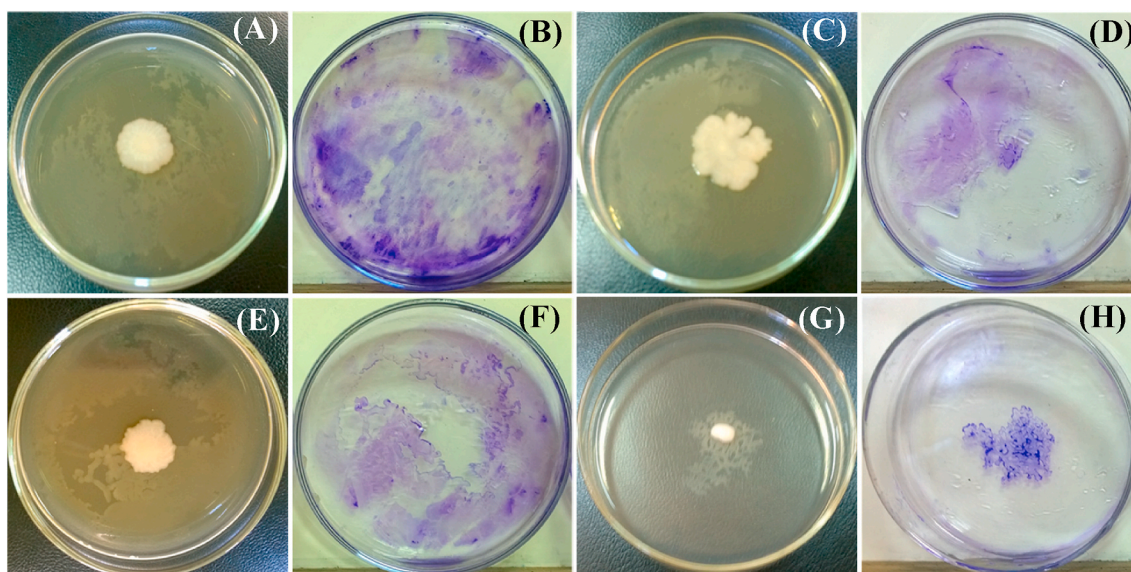


Fig. 2. Twitching motility on TY (A, B, E and F) and LB (C, D, G and H) plates containing 1% agar of *Enterobacter* sp. J49 (A and C: Petri dish with agar, B and D: Petri dish without agar and stained with 1% crystal violet) and *Serratia* sp. S119 (E and F: Petri dish with agar, G and H: Petri dish without agar and stained with 1% crystal violet). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

3.2. Chemoattractant effect of root exudates

In order to select an incubation time in the experiments to study chemotactic motility, growth curves of both strains were performed and the maximum generation time (GT_{max}) was determined. It was possible to determine that the GT_{max} of *Serratia* sp. S119 and *Enterobacter* sp. J49 were 60 and 66 min, respectively. Based on the GT values obtained from each bacterium, a capillary incubation time of 45 min was established for the chemotaxis test. It was possible to observe a chemoattractant effect of soybean and maize RE towards *Serratia* sp. S119 (Fig. 3A). In the case of *Enterobacter* sp. J49, peanut and soybean root exudates had an attractive effect on this bacterium (Fig. 3B).

3.3. Production of biofilm by *Serratia* sp. S119 and *Enterobacter* sp. J49 in the presence of root exudates

In parallel to biofilm formation analysis, the phosphate solubilization of both strains was quantified in order to establish possible relationship between this capacity and the formation of biofilm in the different minimal culture media. The maximum amount of phosphate solubilized by the native isolate *Serratia* sp S119 was $0.936 \pm 0.114 \text{ g l}^{-1}$

P obtained at 48 h of growth (Table 1). This value was higher than that reached by strain *Enterobacter* sp. J49, which obtained a maximum solubilization value of P of $0.326 \pm 0.015 \text{ g l}^{-1}$ P at 144 h of growth.

Initially, it was observed that in rich media *Serratia* sp. S119 presented a significantly higher Biofilm formation Index (BI) in TY than in LB at all tested times (Fig. 4A). *Enterobacter* sp. J49 showed no difference

Table 1

Average phosphorus concentrations solubilized by *Enterobacter* sp. J49 and *Serratia* sp S119 in NBRIIP medium.

Time (hours)	Solubilized phosphorus (g/l)	
	<i>Serratia</i> sp. S119	<i>Enterobacter</i> sp. J49
24	0.383 ± 0.056	0.112 ± 0.008
48	0.936 ± 0.114	0.157 ± 0.005
72	0.876 ± 0.073	0.207 ± 0.013
96	0.873 ± 0.050	0.269 ± 0.031
120	0.922 ± 0.087	0.316 ± 0.047
144	0.816 ± 0.121	0.326 ± 0.015
168	0.883 ± 0.136	0.324 ± 0.015

The data represent the mean \pm SE (n = 8).

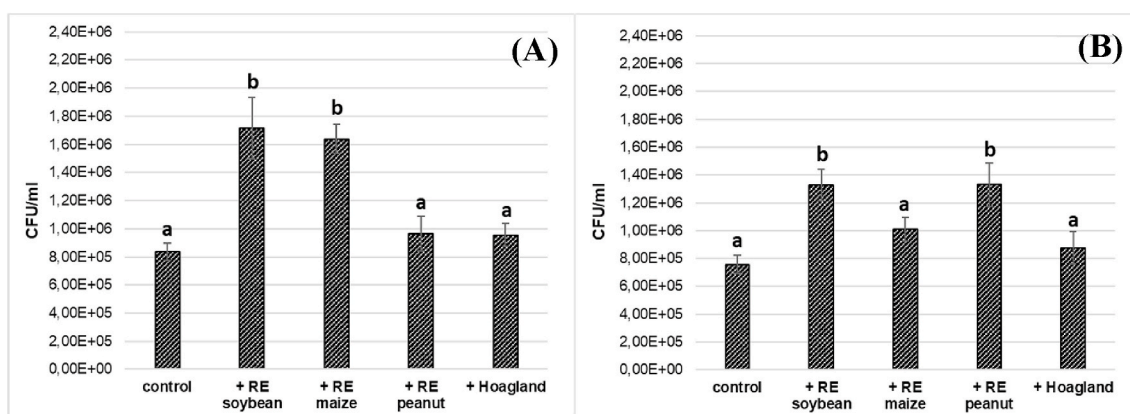


Fig. 3. Number of cells of strain (A) *Serratia* sp. S119, (B) *Enterobacter* sp. J49 expressed in CFU. ml^{-1} indicating chemotactic response to the different root exudate (RE) treatments of soybean, maize and peanuts. Control treatments: H_2O Milli-Q and solution Hoagland. Each data is the average \pm 1 standard error (SE; n = 18). Different letters indicate significant differences ($p > 0.05$) between treatments.

in BI between rich media in most of the times analysed (Fig. 4B). In minimal media, it was possible to observe that *Serratia* sp S119 produced higher BI in NBRIP-Psol medium in contrast to NBRIP. On the contrary, *Enterobacter* sp. J49 strain showed a greater BI in this last medium.

Analysis of the effect of root exudates on biofilm formation indicated that *Serratia* sp. S119 showed variability over the time evaluated (Fig. 5A) on LB medium and against the different RE used. The addition of peanut RE in the four times evaluated produced the lowest BI values. In the case of the treatment without addition of RE, the BI increased during the days reaching a significantly higher value at 96 h. It was also possible to observe that after 72 h the BI values in the presence of the three RE decreased in relation to control treatment. In TY medium, *Serratia* sp. S119 showed higher BI than in LB medium and the effect of RE was not significant (Fig. 5B). In most treatments, the maximum BI value was reached after 24 h. As with LB medium, the addition of RE produced a decrease on the BI after 72 h. In NBRIP minimal medium, *Serratia* sp. S119 showed a decrease of more than 50% in BI when it grew in the presence of each of the three RE in the four times evaluated (Fig. 5C). In the NBRIP-Psol medium, the highest BIs were detected for treatment without RE after 48 h (Figs. 4A and 5D). The decrease in BI due to the addition of REs could be observed after 48 h (Fig. 5D).

Regarding *Enterobacter* sp. J49, the addition of RE in LB rich medium produced a significant decrease in BI at 48 h, which was reversed at 72 h, time in which the four treatments reached highest BI value (Fig. 5E). At 96 h a new decrease in BI was observed for treatments with maize and peanut RE. In the case of TY rich medium, the addition of peanut RE at all times evaluated showed no significant differences with respect to the control treatment (Fig. 5F). The RE of soybean and maize, significantly decreased the BI of the strain at 48 h. On both minimal media, this parameter was significantly decreased with the addition of the three RE at all times tested (Fig. 5G and H).

4. Discussion

Rhizosphere colonization is an important trait when looking for microorganisms to be applied for beneficial purposes for plants. The ineffectiveness of PGPB in the field has often been attributed to their inability to colonize plant roots (Benizri et al., 2001; Bloemberg and Lugtenberg, 2001). In addition, PGPB generally improve plant growth by colonizing the root system and preventing the establishment or suppression of harmful microorganisms of the rhizosphere (Schroth and Hancock, 1982). For this, these bacteria must be able to compete with soil microorganisms and efficiently colonize the rhizosphere of plants. Bacterial motility is one of the features necessary for a successful colonization. In this study, it was determined that both strains analysed showed swimming, swarming and twitching motility. The motility capacity mediated by flagella coincides with previous studies for these bacterial genera (Mishra et al., 2016; Choi et al., 2015). Thus, structures related to swarming and swimming motility types have been reported in

other studies to be present in bacteria of the *Serratia* and *Enterobacter* genera (Péramé et al., 2015; Alberti and Harshey, 1990; Eberl et al., 1999). In addition and considering that both strain are endophytes it is important to consider that flagellar motility (swimming and swarming) and contraction motility (twitching) has been described to play an important role in the colonization of the root (Harjai and Sabharwal, 2017).

Considering twitching motility, several studies determine that this bacterial capacity is possible to be shown through staining with Crystal Violet of bacterial growth that occurs at the interface between the agar medium and the Petri dish (Rotcheewaphan et al., 2016; Tomada et al., 2016). In addition, studies with mutants of *Azoarcus* spp., defective in the production of type IV pili, demonstrated the importance of this cell structure in twitching motility, not only to join the surface of the plant but also to colonize the inside of the root (Böhm et al., 2007). The mutants showed a strongly reduced ability to invade the roots compared to the wild type of bacteria. Considering that the strains of the present study were isolated from the inside of peanut root nodules, it is possible to suggest that their endophytic colonization capacity could be mediated by twitching motility. In the present work twitching motility was studied by using this strategy and it was analysed on different bacterial media. Through these assays, it was possible to observe that both bacteria showed greater twitching motility in TY medium with respect to the LB medium being more evident for *Serratia* sp. S119 strain than for the *Enterobacter* sp. J49 strain. The most noticeable difference between the media used is in the salt that is added. LB contains NaCl and TY CaCl₂ so the observed differences in twitching motility could be attributed to the difference between cation composition of the media. In particular, calcium (Ca²⁺) has been described to modulate a variety of bacterial phenotypes, among which are motility (Parker et al., 2015; Guragain et al., 2013), support adherence (Mongiardini et al., 2008; Ausmees et al., 2001) and the synthesis of EPS (Patrauchan et al., 2007). In particular, in a study by Cruz et al. (2014), a specific regulatory function of Ca²⁺ in twitching motility was identified. In this study authors found that Ca²⁺ binds to a motif of the pili type IV protein and produced a greater contraction.

Chemotaxis of beneficial bacteria generated by root exudates is a necessary requirement for efficient root colonization (Allard-Massicotte et al., 2016; Scharf et al., 2016). Studies on attraction and migration of beneficial rhizosphere bacteria provide important information on ecological traits for root colonization. Chemical signalling between plant roots and other soil organisms, including the roots of neighboring plants, is often based on chemical substances derived from the roots. A variety of compounds present in root exudates, attract a wide range of microbial populations to survive on and within their tissues, including the aerial parts, the vascular network and the radical tissues located beneath the soil (Khan et al., 2017). Plant exudates are released at specific sites, and many bacteria prefer to mobilize and colonize different positions on the plant surface with optimal nutrient availability

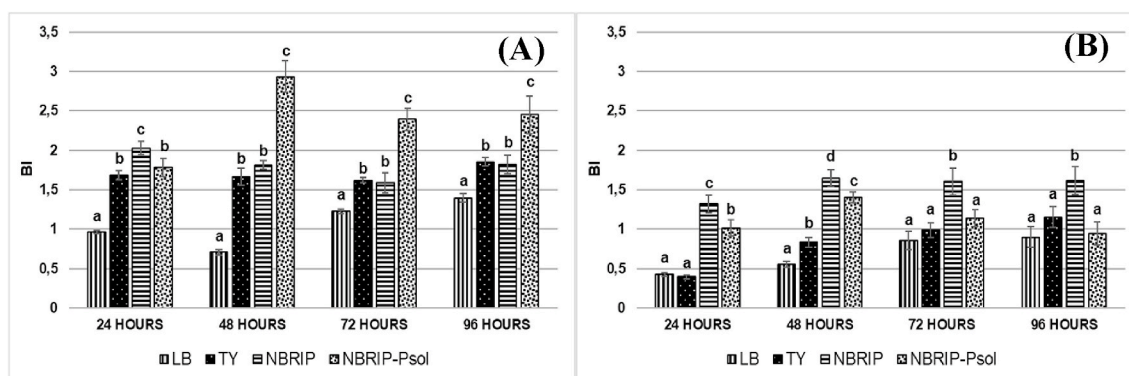


Fig. 4. Effect of the culture medium on biofilm formation index (BI) of strain (A) *Serratia* sp. S119 and (B) *Enterobacter* sp. J49.

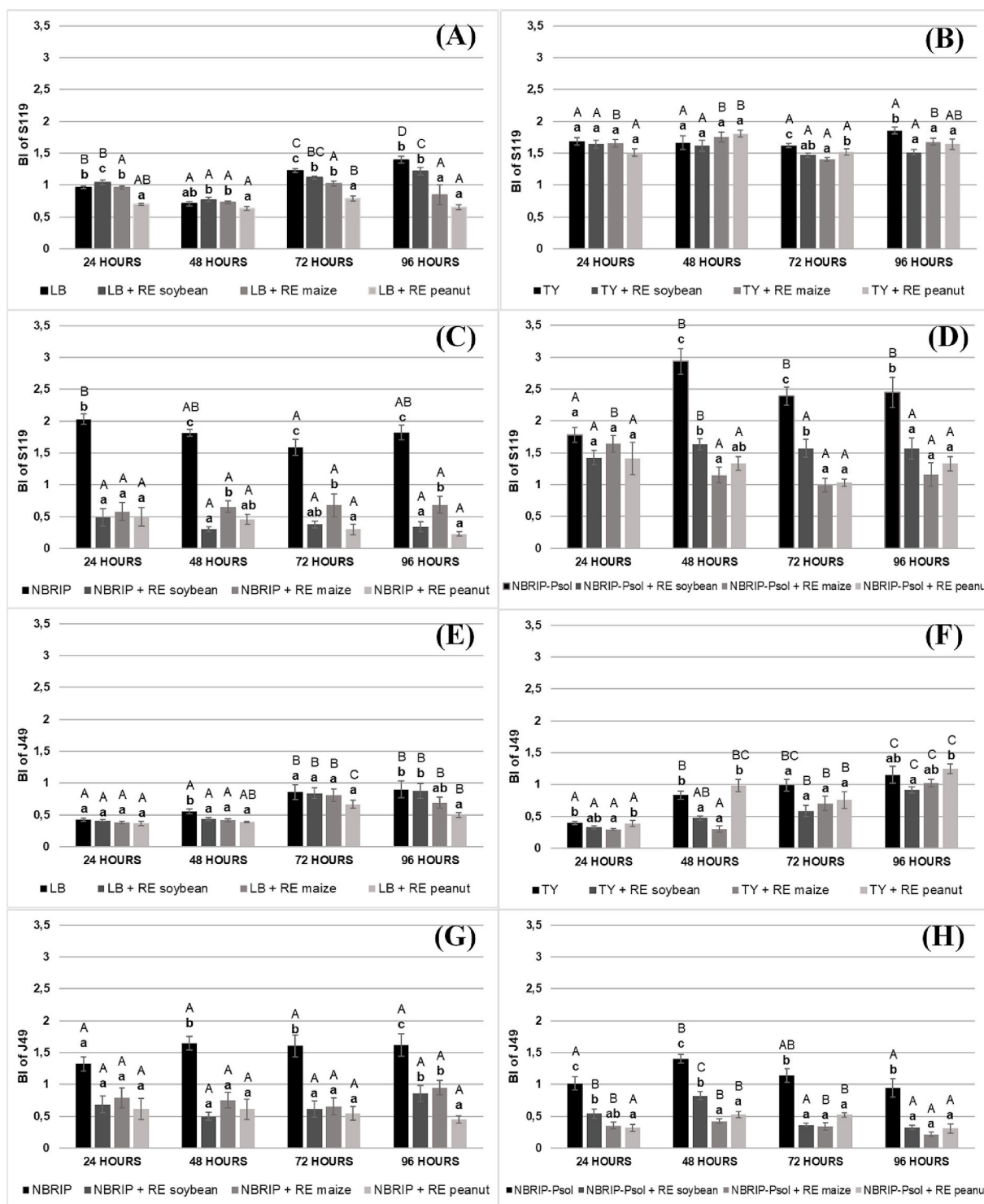


Fig. 5. Biofilm index of *Serratia* sp. S119 (A–D) and *Enterobacter* sp. J49 (E–H) growing in the presence of root exudates (RE) of soybean, maize and peanut plants on the biofilm formation index (BI) in (A and E) LB, (B and F) TY, (C and G) NBRIP and (D and H) NBRIP-Psol media. The data represent the mean ± SE (n = 16). Different letters above in uppercase indicate significant differences (p < 0.05) between times for the same treatment. Different lowercase letters below indicate significant differences (p < 0.05) between treatments for the same time.

(Danhorn and Fuqua, 2007). Han and Cooney (1993) found that an isolation of *Serratia* sp. Gil-1 showed positive chemotaxis against different concentrations of aspartate. On the other hand, *Enterobacter aerogenes* presented a good chemotaxis for maltose and aspartate while a strain of *Serratia marcescens* showed no significant responses to maltose or aspartate, while it was attracted to casaminoacids (Dahl and Manson, 1985). As mentioned earlier, the results obtained from this study

indicate that endophytic peanut bacteria were differentially attracted to the RE of the plants to which they were exposed. *Enterobacter* sp. J49 strain showed a positive attraction towards RE of peanut, its host plant, to the same extent as with those of soybean, which also belongs to the legume group, while it was not chemoattracted towards RE of maize. There are reports by other authors that bacterial isolates are more attracted to the RE of their host plant, than those secreted by a non-host

plants (Yao and Allen, 2006; Mandimba et al., 1986). On the other hand, the strain *Serratia* sp. S119 was not attracted to peanut's RE, its host plant, while it did so against the RE of soybean and maize plants. Yaryura et al. (2008) found that *B. amyloliquefaciens* and *B. japonicum* did not show chemotaxis induced by the exudates of their macrosymbiont soybean. Nevertheless, Bacilio-Jiménez et al. (2003), suggested that the chemoattractant characteristics of the RE would favour the endophytic microorganisms and could induce the exclusion of other colonizing microorganisms from the ecological niche. These results demonstrate the differential attraction of soil bacteria to molecules secreted by plants and would explain the different chemotaxis observed by the strains.

The formation of bacterial biofilms is of importance for agriculture. Bacterial biofilms established in plant roots could protect colonization sites and reduce the availability of root exudates and nutritional elements to decrease pathogen development and subsequent root colonization (Weller and Thomashow, 1994). The analysis of biofilm formation produced by the strains was performed on four different culture media in order to determine the effect that different growth conditions have on the biofilm formation index (BI). For this rich and minimal media were selected for comparing. In the case of rich media, LB and TY were chosen given that there were differential results in twitching motility of the both strains studied when growing on them. In addition to this, it is documented that the formation of biofilm is influenced by the presence of type IV pili that characterizes this motility (Koczan et al., 2009). It is possible to correlate the results obtained for the formation of biofilms in rich medium, with those obtained for the analysis of twitching, given that in medium TY presented greater motility and greater BI was seen. Relationship between the formation of biofilms and twitching motility is consistent with studies of other authors who found that the retractable movement of type IV pili, acts as cross-linking structures, allowing the bacteria to spread over the surface and form biofilms (Fong and Yildiz, 2015). In addition, it has been observed that mutants with poor synthesis of these type IV pili proteins cannot form biofilms (Khan et al., 2017; Elhenawy et al., 2015). Furthermore, Ca^{2+} supplementation, as occurs in the TY medium, has been shown to promote the formation of biofilms in different bacterial genera (Das et al., 2014; Cruz et al., 2012; Rinaudi et al., 2006), among them *Enterobacter cloacae* (Zhou et al., 2014). Cruz et al. (2012), observed an increase in the formation of biofilms when the medium was supplemented with at least 1 mM Ca^{2+} . Considering this, the observed difference regarding the formation of biofilms in the two rich culture media used, could be attributed to the Ca^{+2} contribution of the TY medium, in which a concentration of 5.94 mM Ca^{2+} is incorporated through of one of its components, CaCl_2 , a value that is above the reported concentrations.

On the other hand, the NBRIP minimal medium (Mehta and Nautiyal, 2001) was selected because it is a selective medium for phosphate solubilizing bacteria, PGPB property of the strains under study, and compared with the same medium replacing the insoluble phosphate source ($\text{Ca}_3(\text{PO}_4)_2$), for KH_2PO_4 , which is a soluble source of P (NBRIP-Psol). Phosphate solubilizing capacity of the strains under study has been previously described (Luduena et al., 2017; Taurian et al., 2010). However, in this work it was quantified again in parallel to Biofilm formation Index (BI) estimation in order to establish possible relationship between this capacity and the formation of biofilms in the different culture media. It was possible to observe that both strains showed the same pattern of phosphate solubilization curves than those previously reported but reaching higher values of solubilized P because higher stirring conditions was used in this study. The comparison between the amount of P solubilized by the bacteria, with that incorporated as soluble P in the NBRIP-Psol medium, allows us to infer in which of the two conditions it generates greater stress due to deficiency of P. As described, the strain *Serratia* sp. S119 showed maximum solubilization at 48 h of growth reaching values of soluble P in the NBRIP medium that exceed the concentration of this nutrient added in the NBRIP-Psol medium (3 g.l^{-1} of KH_2PO_4 , which is equivalent to a P concentration of

0.68 g l^{-1}). Only at 24 h, a lower P content than the NBRIP-Psol medium was detected and would explain the greater BI at this grow time. In the case of *Enterobacter* sp. J49, which reached its maximum phosphate solubilization at 144 h of growth (0.326 g l^{-1} of P), its solubilization capacity would give it a lower concentration of P in the four tested times than that added in the NBRIP-Psol medium. The impact of low P availability on biofilms depends on the organism and its role in the ecosystem. P limitation has been reported to increase the density of the biofilm of *A. tumefaciens* (Danhorn et al., 2004) and *R. leguminosarum* bv. *trifolii* (Janczarek and Skorupska, 2011). Considering that a lower availability of P would generate a stress condition, and to induce the formation of biofilms as a form of survival, it is feasible to infer that the results obtained could be explained from the point of view of the availability of this nutrient in the culture media used.

Recent studies on the root-microorganism interaction have shown that bacteria can colonize the root and at the same time form biofilms. This phenomenon is considered a survival strategy by rhizobacteria which provide protection for themselves and the plant against stress conditions (Timmusk and Nevo, 2011). Motility is involved not only in the beginning and development of biofilms, but also in their dispersion being an essential mechanism for the propagation and colonization of new habitats (Sauer et al., 2002) such as the rhizosphere. For this purpose, another property analysed in the bacteria was its ability to produce biofilm and the effect that root exudates have on this property. In the epiphytic or endophytic colonization process, several steps occur such as the attraction of microbes to the root, adhesion, colonization and growth (Nihorimbere et al., 2010). While, at a distance from the root, exudates improve transcription levels of the chemoreceptor genes promoting chemotaxis, this process is reversed in the vicinity of the root, where the need for chemotaxis towards the root may be less important (López-Farfán et al., 2019). Accordingly, Corral-Lugo et al. (2016) established a link between the absence of chemotaxis and the formation of biofilms, since the mutation of specific chemoreceptor genes resulted in a greater formation of biofilm. Numerous studies have investigated the impact of root exudates on transcription levels of chemotaxis and motility genes, and showed that almost all chemoreceptor transcripts decreased at a high concentration of RE (Webb et al., 2016; Oku et al., 2012). In this regard, in the present study chemotaxis of the bacteria towards RE was analysed employing a concentration of 10X and a chemoattractant effect was observed. On the other hand, biofilm production was analysed using a 1X RE concentration and a decrease of BI was observed. Thus, this could suggest that at the 1X RE concentration used in this work, the biofilm formation genes of the bacteria were probably not induced, with those of chemotaxis prevailing. It is possible to speculate that probably genes related to chemotaxis are activated on both 1X and 10X RE concentrations and a greater concentration higher than 10X is required to observe an increase in BI. Another aspect to consider is that eukaryotic organisms, including plants, produce different molecules capable of interfering with bacterial quorum sensing systems (Gao et al., 2003). Therefore, it is possible to speculate that in the RE of the plants under study there were molecules that interfere with the quorum sensing communication of the bacteria, reducing their ability to form biofilms.

The results of the present study, although preliminary, allow to suggest that non-symbiotic endophytic bacteria present more than one type of motility and that chemotaxis toward RE is variable as hypothesized. In addition, biofilm production of bacteria are also influenced by RE concentration and the composition suggesting a nutrient dependent phenotype as mentioned. Further studies are necessary to confirm what it is speculated and to gain more knowledge on what happens in the soil environment. Other factors could be influencing motility and chemical signalling processes such as the other microorganisms inhabiting the same ecosystem both in a negative or positive manner.

5. Conclusion

The motility and biofilm formation properties of the native peanut phosphate solubilizing strains *Serratia* sp. S119 and *Enterobacter* sp. J49 could be involved in their capacity of endophytic colonization. The changes produced by the addition of root exudates in the production of chemotaxis and biofilms of the strains suggest that compounds released by the plants are detected by these bacteria and could be part of the molecular dialogue involved in their interaction with the roots of plant.

Declaration of competing interest

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

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