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Non-rhizobial peanut nodule bacteria promote maize (*Zea mays* L.) and peanut (*Arachis hypogaea* L.) growth in a simulated crop rotation system

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ABSTRACT

The term "Plant Growth Promoting Bacteria" or PGPB designates a diverse group of prokaryotic microorganisms that can increase plant growth by diverse mechanisms. Some PGPB are capable of colonizing root inner tissues and constitute endophytic populations. Incorporation of these microorganisms into agricultural practices may constitute a valid alternative to increase crop productivity in a sustainable and environmentally friendly production scheme, reducing the application of agrochemicals. In a previous work, we described the characterization of bacteria belonging to *Pseudomonas*,*Enterobacter* and *Klebsiella* obtained from surface sterilized peanut nodules. In addition, we showed that some of these isolates were able to promote several peanut growth and symbiotic parameters. Bounded to the results from this particular study, and considering their potential ability to interact with different plant species, in this work we assessed the effects of their inoculation in maize (*Zea mays* L.) under controlled conditions. Furthermore, we analyzed growth promotion in a simulated peanut–maize crop rotation system. Finally, we determined the plant growth promoting (PGP) properties present in the isolates. Results indicated that all bacteria are able to significantly promote maize and peanut growth, and that they also displayed plant growth promotion activity in maize growing in a peanut–maize crop rotation sequence.

1. Introduction

Soil is a critical resource for agricultural production, and it harbors a great diversity of living organisms. Among the soil prokaryotic microorganisms, Plant Growth Promoting Bacteria or PGPB are able to exert positive effects on plants by various mechanisms, which can be classified as direct or indirect (Bashan and Holguin, 1998). Direct mechanisms include either facilitating nutrient acquisition (nitrogen, phosphorus and essential minerals) or modulating plant hormone levels. Indirect mechanisms involve decreasing the deleterious effects of pathogens on plant growth and development (Glick, 2012).

The rhizosphere represents the portion of soil surrounding the roots that is physical, chemical and biologically influenced by plants (Sørensen, 1997), and is well known for hosting a variety of PGPB. In addition, some bacterial populations from the rhizosphere can invade the root inner tissues and become endophytes (Hallmann, 2001). Endophytes are, in general, more likely to induce plant growth promoting (PGP) effects than bacteria exclusively colonizing the rhizosphere (Chanway et al., 2000; Conn et al., 1997), and it has been suggested that plants can attract specific bacterial populations for their own ecological and evolutionary benefit (Bais et al., 2006; Schulz and Boyle, 2006). From an ecological perspective, endophytic bacteria could become better protected from biotic and abiotic stresses than their rhizospheric counterpart (Hallmann et al., 1997).

Bacterial non-rhizobial isolates evaluated in this work were previously obtained from the interior of surface sterilized peanut nodules. Genotypical and phenotypical characterization of this population indicated that it is highly diverse and includes microorganisms belonging to *Pseudomonas*, *Enterobacter* and

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Klebsiella (within γ-Proteobacteria Class). In addition, we showed that some of these isolates are able to promote growth and symbiotic performance of peanut.

Benefits of agricultural systems cultivating legumes together with cereals have been well documented (Jensen, 1996; Karpenstein-Machan and Stuelpnagel, 2000; Martin et al., 1991; Sing et al., 1996). Moreover, peanut–maize rotation is recommended to be used in the growing region of Argentina (Bongiovanni, 2008). In this context, and considering the potential ability of endophytic bacteria to interact with different plant species, we assessed the effects of their inoculation in maize. Moreover, we also analyzed growth promotion in a simulated peanut–maize crop rotation system and determined the presence of PGP properties in the nodule endophytic isolates. Results indicated an interesting potential for maize and peanut growth promotion, both individually and in crop rotation experiments.

2. Material and methods

2.1. Bacterial strains and growth conditions

Seven isolates belonging to a collection of non-rhizobial peanut nodule endophytic bacteria were used (Ibañez et al., 2009). Bacteria belong to the genera *Pseudomonas* (isolates NVAM24, NCHA33 and NCHA35), *Enterobacter* (NMAN11 and NONC13) and *Klebsiella* (NTI31 and TT001). Bacteria were grown on TY medium supplemented with 40 $\mu g\,ml^{-1}$ chloramphenicol, since the previous characterization revealed that the bacterial isolates are resistant to this antibiotic. *Serratia* sp. 119, *Azospirillum brasilense* Cd and *Bradyrhizobium* sp. (*Arachis hypogaea* L.) SEMIA6144 were used as reference strains.

2.2. Surface sterilization of maize and peanut seeds and plant growth conditions

Uniform sized Hybrid NK900 TD MAX maize seeds and peanut var. Runner Cv. Tegua seeds were surface-sterilized according to Pereira et al. (2011) and Vincent (1970), respectively. Afterwards, seeds were germinated and seedlings were grown under controlled environment as previously described (Ibañez et al., 2009).

2.3. Maize growth promotion analysis

Bacteria were inoculated individually or coinoculated (in groups formed according to their taxonomic affiliation) on maize plants. Three inoculation groups were tested: *Pseudomonas* (coinoculation of *Pseudomonas* sp. NVAM24, *Pseudomonas* sp. NCHA33 and *Pseudomonas* sp. NCHA35), *Enterobacter* (*Enterobacter* sp. NMAN11 and *Enterobacter* sp. NONC13) and *Klebsiella* (*Klebsiella* sp. TT001 and *Klebsiella* sp. NTI31). For inoculation, 3 ml of broth cultures in stationary growth fase (10^9 CFU ml^{-1}) of each of the isolates was deposited on roots of 7 days old plants growing in pots containing approximately 300 g of sterile volcanic sand as substrate. A total of 8–9 plants per treatment were used in the individual inoculation, while 4–5 replicates per treatment were tested in the coinoculations. At 30 days post-inoculation (PI), plants were harvested and several growth parameters were determined.

2.4. Growth promotion analysis in a peanut–maize crop rotation trial

Peanut plants were cultivated in pots containing approximately 1 kg of a sterile low phosphate (7 ppm) Entic Haplustoll soil obtained from an agricultural field located in the South of Córdoba Province, Argentina. A total of 8–9 plants per treatment were coinoculated with mixtures of the symbiont *Bradyrhizobium* sp. SEMIA6144 and groups of the endophytic isolates cultures. For inoculation, 3 ml of each bacterial culture were deposited at the root crown of peanut plants. At 80 days PI, plants were harvested and growth and nodulation parameters were determined. Subsequently, maize seeds were sown in the same pots and three plants per treatment were reinoculated with 3 ml of the correspondent non-rhizobial isolate, in order to increase the number of viable bacteria in the substrate. At 30 days PI, maize plants were harvested and several growth parameters were evaluated.

2.5. Determination of the number of viable microorganisms in soil after peanut harvest

In order to quantify the number of inoculated microorganisms that remained viable in soil after peanut harvest, CFU g^{-1} soil were determined according to Somasegaran and Hoben (1994) in TY media supplemented with chloramphenicol (40 $\mu g\,ml^{-1}$). ERIC-PCR profiles (de Bruijn, 1992) of the isolates obtained were compared to those of the inoculated bacteria in order to confirm their identity.

2.6. Isolation of endophytic microorganisms

Isolation of endophytic microorganisms from maize roots was performed according to Pereira et al. (2011). To confirm the identity of the bacterial isolates, ERIC-PCR profiles (de Bruijn, 1992) of the isolates obtained in the experiment were compared to those of the inoculated bacteria.

2.7. Evaluation of bacterial PGP activities in vitro

The method described by Schwyn and Neilands (1987) was used to assess siderophore production.

Ability of the isolates to solubilise inorganic phosphate was analyzed in NBRIP-BPB medium (National Botanical Research Institute's phosphate growth medium) (Metha and Nautiyal, 2001).

Production of indoleacetic acid (IAA)-like molecules was assessed by the method described by Bric et al. (1991).

2.8. Statistical analysis

Experiments were laid out in complete randomized design. Data were subjected to analysis of variance (ANOVA) and Dunnet's multicomparison test using SigmaStat v3.5 software. A $p \leq 0.05$ significance level was used throughout.

3. Results and discussion

3.1. Analysis of maize growth promotion

Two sets of experiments were conducted. In the first, isolates were inoculated individually while, in the second, they were coinoculated in groups conformed considering their taxonomic affiliation.

Results from individual inoculation indicated that all isolates significantly increased at least one of the growth parameters analyzed when compared to uninoculated control (Supplementary Table S1). In particular, *Klebsiella* sp. TT001 increased most of the maize growth parameters evaluated. In the second trial (coinoculation) all treatments significantly increased the fresh weight of the root, and the shoot length and fresh and dry weight, while only one treatment (*Pseudomonas*) significantly increased the radical dry weight compared to the uninoculated control (Table 1). Comparison of the results from both experiments indicates that coinoculation represents a more efficient growth promotion strategy, suggesting the existence of a synergistic effect among inoculated bacteria.

Table 1
Growth parameters of coinoculated maize plants.

Inoculation group	Root		Shoot		
	Fresh weight (g)	Dry weight (g)	Fresh weight (g)	Dry weight (g)	Length (cm)
Enterobacter	6.30 ± 0.60^{b}	0.95 ± 0.14^{a}	3.86 ± 0.35^{b}	0.76 ± 0.01^{b}	34.64 ± 1.12^{b}
Klebsiella	6.44 ± 0.50^{b}	0.81 ± 0.01^{a}	4.18 ± 0.30^{b}	0.85 ± 0.01^{b}	38.00 ± 1.78^{b}
Pseudomonas	6.08 ± 0.44^{b}	1.43 ± 0.28^{b}	4.20 ± 0.46^{b}	0.73 ± 0.06^{b}	42.5 ± 1.69^{b}
Uninoculated control	3.05 ± 0.22^{a}	0.55 ± 0.06^{a}	1.77 ± 0.12^{a}	0.31 ± 0.01^{a}	26.20 ± 0.86^{a}

Data represent mean ± SE (n = 4–5). Letters in columns indicate statistically significant differences with respect to the uninoculated control according to Dunnet's test ($p \leq 0.05$).

In addition, the ability of the isolates to colonize the interior of maize roots was also analyzed. ERIC-PCR profiles of endophytes recovered from maize plants were compared to those of the inoculated bacteria. Results indicated that isolates Enterobacter sp. NMAN11, Enterobacter sp. NONC13, Pseudomonas sp. NCHA33, Klebsiella TT001 and Klebsiella NTI31 are capable of colonizing the root inner tissues of maize seedlings, in orders that range from 10^2 to 10^3 CFU g^{-1} fresh weight. Since these isolates were originally obtained from peanut nodules, their ability to colonize the interior of maize roots suggests that they possess general traits allowing the invasion of taxonomically distant plant species. It has not been resolved whether plants benefit more from an endophyte than from a rhizospheric bacterium (Rosenblueth and Martínez-Romero, 2006). Results from this work indicate that there is not a direct relationship between the efficiency of the plant growth promotion and the endophytic ability of the isolates. However, from an ecological perspective, endophytic capacity offers microorganisms a niche more stable and inaccessible to other microbiota components than soil, favoring their persistence in the agroecosystems.

3.2. Plant growth promotion in a peanut–maize crop rotation sequence

In a first stage, isolates were coinoculated on peanut plants in groups formed by taxonomic affiliation together with *Bradyrhizobium* sp. SEMIA6144. Results obtained confirmed the previously described ability of most of the isolates to promote peanut growth (Ibañez et al., 2009), and indicated that the endophytes inoculation did not affect nodulation process induced by the bradyrhizobial strain. Coinoculation of *Bradyrhizobium* sp. SEMIA6144 plus *Klebsiella* strains or plus *Pseudomonas* strains increased several growth parameters not only compared to the uninoculated control, but also with plants inoculated with *Bradyrhizobium* sp. SEMIA6144 alone (Table 2).

The number of the inoculated microorganisms that remained viable in the soil after peanut harvest was determined, and ERIC-PCR profiles of the recovered bacterial isolates were compared to those of the correspondent inoculated bacteria to confirm their identity. Results indicated that isolates are able to survive in the soil, ranging between 10^4 and 10^5 CFU g^{-1} (Supplementary Table S2). As peanut plants were harvested at 80 days PI (time at which nodules are senescent), and considering that the isolates were originally described as peanut nodule endophytes, we speculate that their presence in the substrate could be maintained as a consequence of the microorganisms that are released from senescent nodules.

Subsequently, maize seeds were sown in the same pots in which the peanut plants have previously been grown. Results indicated that all treatments significantly increased at least one of the maize growth parameters in rotation with peanut (Table 3). In particular, *Enterobacter* treatment increased three growth parameters. However, most promising results were obtained when the isolates were reinoculated. All treatments involving reinoculations showed a significant increase in all the growth parameters evaluated with respect to the uninoculated control. In addition, data from almost all growth parameters from reinoculation assays showed statistically significant differences with their corresponding treatment without reinoculation, with the exception of shoot length. Therefore, although bacteria are able to survive in the substrate after peanut harvest and promote maize growth, their reinoculation produces a substantial effect. This indicates that increasing the number of viable bacteria between crops is a better strategy in order to improve the growth promotion of the second crop. However, it would be interesting to analyze the effects of reinoculation over a longer period of time. There is a chance that, after several years of bacterial inoculation on the same field, reinoculation between crops would not be crucial to observe an efficient maize growth promotion.

3.3. In vitro identification of plant growth promoting properties in the isolates

We analyzed the presence of three well-known PGP traits in the isolates of the collection. Results show that all isolates displayed some degree of phosphate solubilizing ability, being *Pseudomonas* sp. NCHA35 the strain that displayed the greatest activity (Supplementary Table S3). Solubilization levels are in agreement with those reported by Taurian et al. (2010) for isolates obtained from peanut plants. Three isolates (*Pseudomonas* sp. NCHA35,

Table 2
Growth and nodulation parameters of peanut plants from the peanut–maize rotation experiment.

Treatment	Root		Shoot			Nodule number/plant
	Fresh weight (g)	Dry weight (g)	Dry weight (g)	Fresh weight (g)	Length (cm)	
Pseudomonas	85.75 ± 1.81^{b2}	1.54 ± 0.07^{b2}	4.13 ± 0.17^{b}	182.87 ± 7.05^{b}	32.50 ± 0.50^{b}	355.00 ± 17.00^{1}
Klebsiella	73.10 ± 3.47^{a1}	1.70 ± 0.09^{b2}	4.12 ± 0.21^{b}	177.89 ± 9.54^{b}	28.20 ± 0.35^{a}	401.11 ± 16.00^{1}
Enterobacter	71.00 ± 5.44^{a1}	1.24 ± 0.07^{a1}	4.28 ± 0.21^{b}	171.75 ± 14.3^{a}	29.44 ± 0.9^{b}	267.87 ± 31.40^{1}
Bradyrhizobium sp. SEMIA6144	66.60 ± 3.37^{a1}	1.23 ± 0.09^{a1}	4.09 ± 0.15^{b}	178.00 ± 7.55^{b}	30.12 ± 0.96^{b}	325.60 ± 8.23^{1}
Uninoculated control	65.67 ± 1.94^{a1}	1.08 ± 0.06^{a1}	3.28 ± 0.15^{a}	137.80 ± 6.16^{a}	24.78 ± 0.29^{a}	0.00 ± 0.00

Data represent mean ± SE (n = 8–9). Letters in columns indicate statistical significant differences compared to the uninoculated control according to Dunnet's test ($p \leq 0.05$). Numbers in columns indicate statistically significant increase compared to plants inoculated with *Bradyrhizobium* sp. SEMIA6144 according to Dunnet's test ($p \leq 0.05$).

Table 3
Growth parameters of maize plants from the peanut-maize rotation experiment.

Inoculation treatment	Root		Shoot		
	Fresh weight (g)	Dry weight (g)	Fresh weight (g)	Dry weight (g)	Length (cm)
Pseudomonas	5.24 ± 0.30^{a1}	1.06 ± 0.11^{a1}	4.18 ± 0.11^{a1}	0.78 ± 0.04^{a1}	58.30 ± 0.84^{b1}
Pseudomonas reinoculated	9.03 ± 0.62^{b2}	1.84 ± 0.14^{b2}	8.67 ± 0.52^{b2}	1.77 ± 0.12^{b2}	61.17 ± 1.59^{b1}
Klebsiella	5.18 ± 0.46^{a1}	1.21 ± 0.12^{a1}	5.18 ± 0.35^{b1}	0.85 ± 0.06^{a1}	62.33 ± 1.7^{b1}
Klebsiella reinoculated	9.17 ± 0.69^{b2}	2.09 ± 0.23^{b2}	9.67 ± 0.87^{b2}	1.92 ± 0.16^{b2}	65.17 ± 3.4^{b1}
Enterobacter	5.86 ± 0.30^{a1}	1.20 ± 0.14^{a1}	5.66 ± 0.22^{b1}	1.20 ± 0.07^{b1}	64.40 ± 1.50^{b1}
Enterobacter reinoculated	9.77 ± 0.26^{b2}	1.80 ± 0.03^{b2}	9.77 ± 0.32^{b2}	1.82 ± 0.06^{b2}	64.17 ± 1.70^{b1}
Uninoculated control	4.50 ± 0.22^{a}	0.93 ± 0.06^{a}	3.46 ± 0.14^{a}	0.65 ± 0.03^{a}	51.06 ± 1.46^{a}

Data represent mean ± SE (n = 8–9). Letters indicate statistically significant differences with the uninoculated control (Dunnet, $p \leq 0.05$). Numbers indicate statistically significant differences between reinoculated plants and the correspondent non-reinoculated treatment (Dunnet, $p \leq 0.05$).

Pseudomonas sp. NVAM24 and *Klebsiella* sp. TT001) were capable to produce siderophores. In addition, all isolates from our collection were capable of producing IAA-like molecules (Supplementary Table S3).

Taken together, results revealed that all isolates possess, at least, one of the PGP properties analyzed. These properties are similar to those described by Montañez et al. (2012) for diazotrophic maize endophytes. Moreover, isolates *Pseudomonas* sp. NCHA35, *Pseudomonas* sp. NVAM24 and *Klebsiella* sp. TT001 exhibit the three direct PGP properties examined. It is interesting to determinate the contribution of such properties determined in vitro to the growth promotion effect observed. *Klebsiella* sp. TT001 was the best maize growth promoter, being able to significantly increase most of the analyzed parameters. Remarkably, this isolate displayed the three PGP properties determined. However, *Pseudomonas* NCHA35 also displays these three PGP properties but only increases one growth parameter on maize plants. Therefore, no strong relationship between the presence of PGP properties in vitro and growth promoting effect could be found in the single inoculation experiment. In the coinoculated plants, the most significant promoting effect was achieved by those treated with *Pseudomonas* and *Klebsiella*, strains that, considered as a group, display the three promoting properties evaluated. Therefore, it is possible to speculate that a synergistic effect would contribute to the better performance of these endophytic isolates. However, further work is necessary to confirm this hypothesis.

The presence of *nifH* gene in *Klebsiella* TT001 and *Klebsiella* NTI31 genomes has been confirmed (Ibañez et al., 2009). Since this gene encodes for one of the enzymes of the nitrogenase complex, is possible to speculate that nitrogen fixation could be another of the PGP properties present in these isolates.

Understanding how plants shape the composition and functioning of their endophytic microbiome will contribute to develop biotechnological applications that allow to increase the growth and productivity of crops. Taken together, results from this work indicate that the peanut nodule endophytic microorganisms possess interesting PGP properties and some of them are also able to invade the interior of maize roots. Some isolates, particularly those from *Pseudomonas* and *Klebsiella* genera, are able to increase several peanut and maize growth parameters when inoculated. Moreover, these isolates were efficient to increase both peanut and maize growth in a simulated crop rotation sequence. However, reinoculation of the isolates after peanut harvest produces a substantial effect. Nevertheless, further field trials should be performed in order to confirm these promising results obtained under controlled conditions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.apsoil.2014.08.002.

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