

Inoculation of maize with phosphate solubilizing bacteria: effect on plant growth and yield

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Abstract

Phosphate-Solubilizing Bacteria (PSB) improve plant growth, yield and phosphorus content of several crops, and may be used as bioinoculant to enhance sustainable production. We evaluated the response of maize (*Zea mays* L.) to PSB inoculation under controlled and field conditions in Tucumán, Argentina. A pot culture experiment was conducted to investigate the effects of seven previously isolated PSB on early development of plants. Seeds were treated with each bacterial strain, and seedlings were harvested 30 days after inoculation. All strains showed a positive effect on plant growth. A significant increment in plant height (45%), shoot dry weight (40%) was determined in plants treated with *Pseudomonas tolaasii* IEXb, while *Pseudomonas koreensis* SP28 has remarkably increased P content compared to the uninoculated control. IEXb strain was selected and evaluated under field conditions in combination with triple superphosphate (TSP) as P fertilizer. The presence of IEXb strain stimulated seedling emergence (8%), shoot length (19%), grain yield (44%), 1000-grain weight (18%), total dry biomass (32%) and P content (56%) of maize plants. In general, *P. tolaasii* IEXb inoculation was more efficient as bioinoculant without P fertilizer than with TSP. These results provide baseline information for future studies of *P. tolaasii* IEXb as bioinoculant to promote an eco-friendly and sustainable agriculture.

Keywords: Bioinoculant, phosphate solubilizing bacteria, *Pseudomonas tolaasii* IEXb, *Zea mays* L., Growth promotion, sustainable agriculture

Abbreviations:

PGPR	Plant	growth-promoting
rhizobacteria		
PSB	Phosphate	solubilizing
bacteria		
TSP	Triple superphosphate	
IAA	Indole-3-acetic acid	
DAS	Days after sowing	

1. Introduction

Phosphorus (P), one of the main plant nutrients, is essential for plant growth and development. It is involved in several key plant functions, such as energy metabolism, photosynthesis, respiration, nitrogen fixation, enzyme regulation, nutrient movement within the plant and transfer of genetic characteristics (DNA) from one generation to the next. Therefore, P is important in cell division and development of new tissue (Hameeda *et al.*, 2008). Plants absorb P either as HPO_4^{2-} or $\text{H}_2\text{PO}_4^{1-}$ ions. However, in most soils, the concentration of soluble orthophosphates is low, normally 1 mg kg^{-1} or lower ($10 \text{ M H}_2\text{PO}_4^{1-}$; Rodríguez and Fraga, 1999) and must be replenished from other pools of soil P to meet plant requirements (Richardson *et al.*, 2009). To counterbalance this nutritional deficiency in crops, P is supplemented via different chemical fertilizer formulations. Only a low percentage of these agrochemicals can be used efficiently by plants, with the remaining fraction being rapidly converted into insoluble complexes in the rhizosphere, without the expected impact on agricultural yield (Fernández *et al.*, 2012). In this era of

sustainable agricultural production, the role of the rhizosphere has become important given the need to restrict fertilizer use and therefore improve the energetic balance of the system. Rhizosphere activity allows the transformation, mobilization and solubilization of nutrients from a limited pool in the soil and subsequent uptake of essential nutrients by plants to achieve crop genetic potential (Hinsinger *et al.*, 2011). In the rhizosphere, microorganisms interact with plants in several ways. Bacteria that act positively on plant growth and development, through direct or indirect mechanisms, are collectively known as Plant Growth-Promoting Rhizobacteria (PGPR) (Kloepper and Schroth, 1981). This group includes Phosphate-Solubilizing Bacteria (PSB), which are rhizobacteria that convert insoluble phosphates into soluble forms through acidification, chelation, exchange reactions and production of organic acids (Rodríguez and Fraga 1999). They are found in soil but usually they are not enough in the rhizosphere of plants. Therefore, inoculation of plants by a target microorganism at higher concentration than that normally found in soil is necessary to take advantage of the property of phosphate solubilization for plant yield enhancement (Vessey, 2003). Accordingly, several works reported that inoculation with PSB belonging to the genera *Achromobacter*, *Agrobacterium*, *Aerobacter*, *Bacillus*, *Burkholderia*, *Escherichia*, *Erwinia*, *Enterobacter*, *Flavobacterium*, *Micrococcus*, *Pseudomonas*, *Paenibacillus*, *Pantoea*, *Serratia* and *Rhizobium* resulted in improved growth, yield and P uptake in several crops (Ahemad and Kha 2010; Castagno *et al.*, 2011; Hameeda *et*

al., 2008; Hu *et al.*, 2010; Minaxi *et al.*, 2013; Selvakumar *et al.*, 2008; 2011; Yu *et al.*, 2012). In Argentina, agricultural production generates a significant income to the country. Indeed, more than 50% of all Argentine exports come from the agricultural sector (Tomei and Upham, 2011). As a consequence of the intensive agricultural activity, fertilizer use in the 2010/11 cropping season was approximately 3.37 million t, being 5% higher than in 2009/2010 (Velasco *et al.*, 2012). Indiscriminate use of chemical fertilizers is producing negative environmental effects, such as nitrogen (N) leaching and run-off, and P-fixation in the soil (Adesemoye and Kloepper, 2009). The use of bioinoculants may be a better alternative and a complement to chemical fertilizers, since they promote rhizospheric activity, are economical and environmentally friendly, and may be easily obtained from the rhizosphere.

In our laboratory, we isolated seven PSB strains identified as *Serratia marcescens* EV1, *Pantoea eucalypti* EV2, *Pantoea agglomerans* EV3, *Pantoea eucalypti* EV4, *Pseudomonas tolaasii* IEXb, *Enterobacter aerogenes* IEY and *Pseudomonas koreensis* SP28, all of them from Puna grassland, north-western Argentina (Viruel *et al.*, 2011). These bacterial strains present several plant growth promotion traits, such as P solubilization (by acidification and phosphatase production) and production of Indole-3-acetic acid (IAA) and siderophore. The objective of this study was to screen a bacterial strain able to

enhance growth and yield in maize crop, both in growth chamber and under field conditions, as a first stage in the development of commercial formulations of bioinoculants.

2. Materials and Methods

2.1. Plant experiments

2.1.1. Growth chamber trial

To evaluate the use of bacteria as bioinoculants for promoting growth in maize, seven bacterial strains previously selected for their *in vitro* characteristics (Viruel *et al.*, 2011) were tested in growth chamber. All strains were maintained at -20°C in Luria Bertani broth containing 20% (v v⁻¹) glycerol. They were transferred to synthetic medium under aseptic conditions (Viruel *et al.*, 2011), and incubated in rotatory shaker for biomass propagation at 150 rpm and 30°C during 24 h.

Seeds of hybrid maize (*Zea mays* L. DK 390 MG RR2 Dekalb®) were used as host plant to evaluate the performance of PSB. Bacterial cultures (10⁹ CFU ml⁻¹) of each strain were used as bioinoculants at a final concentration of 50 ml Kg⁻¹ seed.

Glass pots (500 cm³) previously sterilized with 20% sodium hypochlorite solution were filled with sieved (< 2 mm) and sterile soil. Seeds were first sterilized in 80% ethanol for 2 min followed by 0.1% HgCl₂ for 10 min; finally, they were rinsed

10 times in sterile distilled water before bacterial treatment. For inoculation assays, seeds were soaked for 30 min in the bacterial suspension and placed at the same depth (approximately 2.5 cm below the soil surface) in all pots. The pots were arranged in a completely randomized factorial design in the growth chamber. Ten replicates (pots) per bacterial inoculum were made. The control treatment consisted of water-treated seeds (without bacteria inoculation). Plants were grown for 30 days under controlled conditions: $25 \pm 3^\circ\text{C}$, day-night cycle of 12–12 h with artificial light ($140 \mu\text{Em}^{-2} \text{s}^{-1}$) and 70% relative humidity; they were daily watered with sterile water and a modified Hoagland solution (Hoagland and Arnon, 1950), where the P source was changed by tricalcium phosphate (50 mg l^{-1}). At harvest, the following parameters were recorded in maize plants: height, shoot and root dry weight, and P content (Murphy and Riley, 1962) after digestion in nitric and perchloric acid (Johnson and Ulrich, 1959).

2.1.2. Field trial

The experiment was conducted at Instituto de Investigación Animal del Chaco Semiárido (IIACS), INTA Leales, Tucumán, Argentina ($27^\circ 11' 34''\text{S}$ $65^\circ 14' 47''\text{W}$, 435 masl) from January to June 2012. The soil was classified as silt loam; at the beginning of the experiment the soil contained 2.39% of oxidable organic matter (Walkley-Black Method), 0.1% of total N (Kjeldhal technique), 16.5 ppm of available P (Bray-Kurtz I Method), $1.54 \text{ me } 100 \text{ g}^{-1}$ of available K (Morgan Method), 0.5% of carbonate content (Gasometric Method), 1.5 dS m^{-1} of electrical conductivity of the saturated soil-paste extract and pH (H_2O 1:2.5) of 8.0. Monthly accumulated rain (mm) and monthly mean daily temperature ($^\circ\text{C}$) (Table 1) were recorded by the Weather Station Davies Vantage Pro 2 of the IIACS and processed using the software Weatherlink.

Table 1. Monthly accumulated rain and monthly mean daily temperature at the experimental site of field inoculation trial during the crop growing season (Dec–Jul) (Data by IIACS, INTA Leales).

Date	Monthly accumulated rain (mm)	Monthly mean daily temperature ($^\circ\text{C}$)
Dec-11	89.6	24.2
Jan-12	220.4	24.9
Feb-12	273.7	23.5
Mar-12	103.4	22.1
Apr-12	63.2	20.0
May-12	9.0	16.2
Jun-12	24.0	12.4
Jul-12	10.0	11.4

Based on the growth chamber test results and information in the literature (Brady *et al.*, 2009; Krey *et al.*, 2013), *Pseudomonas tolaasii* IEXb was selected for field experiment. Seed inoculation was carried out as described above (2.1.1). Four treatments with three replicates were established in completely randomized blocks. Each plot was 8 m long \times 3 m wide, with a distance between rows of 0.52 m. Nitrogen in the form of urea (100 kg N ha⁻¹) was applied in two-split doses (sowing and flowering) to all treatments. Phosphorus (50 kg P ha⁻¹) was applied as triple superphosphate (TSP) in a single dose at sowing. The experiment consisted of the following treatments: (i) uninoculated control (C), (ii) uninoculated control with P fertilizer (C + TSP), (iii) *P. tolaasii* IEXb (IEXb), (iv) *P. tolaasii* with P fertilizer (IEXb + TSP).

The emergence of seedlings was recorded 7 days after sowing (DAS). At harvest (170 DAS), one quadrat (0.25 m² of plant material) per plot was randomly cut to ground level and plant growth parameters were measured.

Shoot dry weight was determined by drying the plant material at 60°C for 5 days. Shoot and cob were separated and grain yield was calculated. For chemical analysis, plant samples were re-dried at 80°C for 48 h and ground to a powder (2 mm). These powders (0.5 g) were digested and analyzed for P content, as described in 2.1.1.

2.2. Statistical analysis

All the recorded data were subjected to an analysis of variance (ANOVA) and means were separated by the Fisher's protected LSD test using the Infostat Analytical Software for Windows (Di Rienzo *et al.*, 2011). A 0.05

significance threshold was used for all statistical tests.

3. Results

3.1. Growth chamber trial

Results of shoot length, shoot and root dry weight and P content of plants grown in the growth chamber for all treatments are shown in Figure 1. Under controlled conditions, plants inoculated with *P. tolaasii* IEXb had the highest aerial height, with significant differences compared with the other treatments (Figure 1A). Plants inoculated with this bacterial strain were 45% taller than uninoculated plants. Treatment with EV4 strain also stimulated growth, with an increase of 38% with respect to control. Although control plants had the smallest shoot length, there were no significant differences between this treatment and EV1, EV2, EV3 or SP28 inoculation treatments. Shoot dry weight was 40% higher in plants inoculated with IEXb than in the control treatment, whereas in the IEY inoculation treatment this parameter was 15% lower than in the control (Figure 1B). Root dry weight of plants inoculated with SP28 was significantly higher than that of the other treatments, being more than double with respect to control plants (Figure 1C).

All bacterial inoculation treatments showed a remarkable increase in plant P content (Figure 1D). The highest P concentration was found in plants inoculated with SP28 strain (8.1 mg P g⁻¹ plant), a value that was 145% higher than that of the control treatment. However, two different types of responses were observed among the remaining inoculation treatments, with an

intermediate P content for IEXb, EV2 and EV1, and a lower one for EV3, EV4 and IEY.

3.2. Field trial

The present results are similar to those reported for inoculation with strains of the genera *Pseudomonas* and *Pantoea* on several crops under controlled conditions (Hu *et al.*, 2010; Minaxi *et al.*, 2013; Selvakumar *et al.*, 2008; 2009). However, strains of the genus *Pantoea* are closely related to plant diseases, like Stewart's vascular wilt caused by *Pantoea stewartii* subsp. *stewartii*, crown and root gall disease of *Gypsophila* spp. and beet caused by *P. agglomerans* and *P. ananatis*, which causes a number of diseases on a wide range of hosts, including stem necrosis of rice and brown stalk rot of maize (Brady *et al.*, 2009). Notwithstanding, in our experiment, inoculation with *Pantoea eucalypti* EV4 did not cause any sign of pathogenesis in maize plants. However, considering this background and the obtained results, the bacterial strain selected to be tested as bioinoculant in the field trial was *Pseudomonas tolaasii* IEXb.

Significant differences in seedling emergence at 7 DAS were observed among treatments (Figure 2A). The maximum percentage of

plant emergence was found in plots inoculated with IEXb strain (89.1%); this value was 4% higher than the same bacterial treatment with P fertilizer (IEXb + TSP). Uninoculated plots (C) had the lowest seedling emergence percentage (80.8%) and were only 2.2% lower than the uninoculated plots with P fertilizer (C + TSP).

The activity of IEXb strain without TSP had a considerable influence on maize growth; indeed, a 19% increase in shoot length was recorded with respect to untreated control (C) (Figure 2B). IEXb + TSP treatment also significantly promoted shoot biomass growth, with an increment of 15% in shoot length at the end of the experiment. Only inoculated plants, both with and without P fertilizer, reached shoot lengths of over 250 cm at harvest. As mentioned above, the lowest shoot length value (225.3 cm) was recorded in uninoculated plants (C).

Interestingly, the maximum P content was observed in plants treated with IEXb without TSP (62.8 g P mg⁻¹ plant), with significant differences with respect to the counterpart fertilized bacterial treatment (IEXb + TSP: 56.1 g P mg⁻¹ plant). The minimum P content value was determined in uninoculated plants without P fertilizer (C: 40.3 g P mg⁻¹ plant). No significant difference was found between control treatments (Figure 2C).

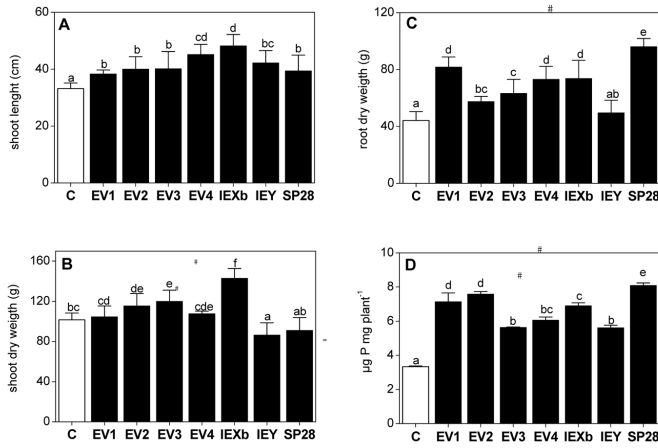


Figure 1. Effects of inoculation with phosphate solubilizing bacteria on shoot length (A), shoot (B) and root (C) dry weight and P content (D) of maize plants grown under controlled conditions, 30 days after sowing. White bar: uninoculated seeds, black bars: inoculated seeds. Data represent mean \pm standard error of mean (SE).

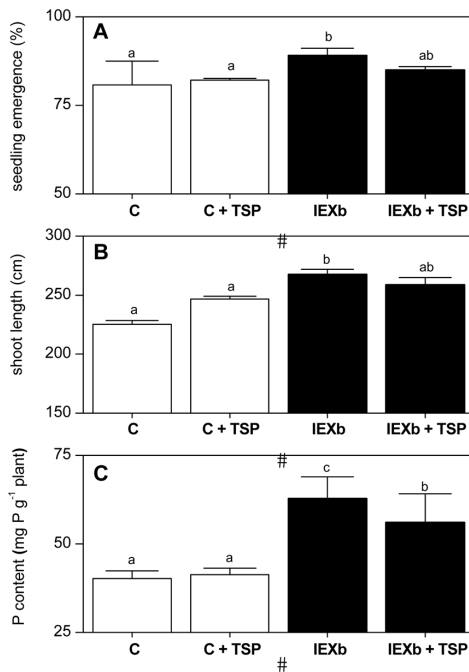


Figure 2 Effects of inoculation of maize seeds with *Pseudomonas tolaasii* IEXb on growth parameters: seedling emergence (A), shoot length (B) and P content (C) of maize plants grown under field conditions. White bar: uninoculated seeds without (C) or with triple superphosphate (C + TSP); black bars: seeds inoculated with IEXb or IEXb + TSP. Data represent mean \pm standard error of mean (SE).

The amount of total shoot dry matter in maize was higher in seed treated with IEXb bacterial strain (2980 Kg ha⁻¹) than in the counterpart uninoculated control (2132.7 Kg ha⁻¹), with a significant (32%) increase in biomass production (Figure 3A). Regarding fertilized treatments, there were no significant differences between inoculated fertilized treatment (IEXb + TSP) and fertilized control (C + TSP).

Under unfertilized conditions, *P. tolaasii* IEXb caused a 45% increase in grain yield compared to the control (C) (Figure 3B). The treatment IEXb + TSP (9750.9 Kg ha⁻¹) was statistically

similar to C + TSP treatment (9904.7 Kg ha⁻¹), with 21% higher yield than that of its counterpart control (8164.1 Kg ha⁻¹). There were significant differences in 1000-grain weight between treatments, and similar to the grain yield, the best performance was observed with IEXb without TSP (414.8 g), whereas the lowest values corresponded to the untreated control (348.8 g) (Figure 3C). Overall, *P. tolaasii* IEXb inoculation treatment without the application of TSP was more efficient in stimulating maize growth and yield than the other treatments, including the same bacterial treatment with TSP.

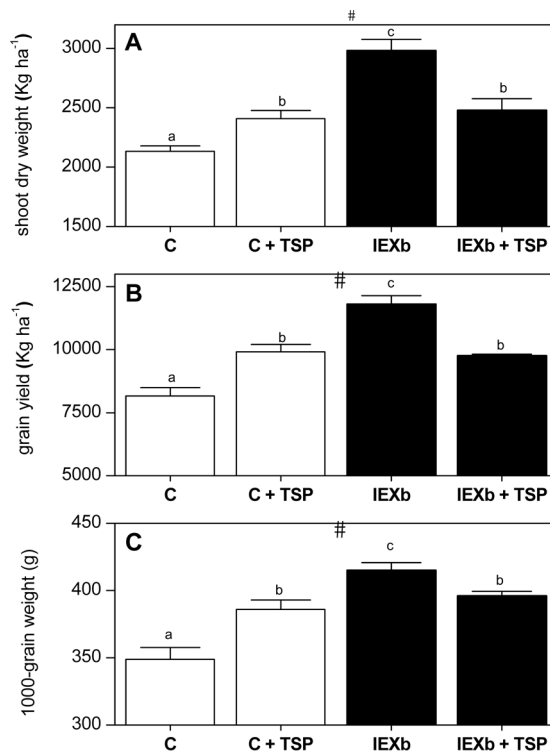


Figure 3 Effects of *Pseudomonas tolaasii* IEXb inoculation in maize seeds on yield parameters: shoot dry weight (A), grain yield (B) and 1000-grain weight (C) of maize plants grown under field conditions. White bar: uninoculated seeds without (C) or with triple superphosphate (C + TSP); black bars: seeds inoculated with IEXb or IEXb + TSP. Data represent mean \pm standard error of mean (SE).

4. Discussion

The first stage of the experiment consisted of evaluating the effect of inoculating maize seeds with PSB in growth chamber. Two strains of the genus *Pseudomonas* and one strain of *Pantoea* had significant growth promoter effect on plant development. In general, seed inoculation with *Pseudomonas tolaasii* IEXb and *Pantoea eucalypti* EV4 was found to have positive effects on aerial biomass, whereas inoculation with *Pseudomonas koreensis* SP28 increased the development of root biomass and P content in maize plants. This growth promoter effect could be attributed to the potential of these strains to increase the availability of nutrients, such as phosphorus, and siderophore and phytohormone production (Viruel *et al.*, 2011), as well as to their capacity to colonize the root system and interact positively with the plant. Similar results of inoculation with strains of the genera *Pseudomonas* and *Pantoea* on several crops under controlled conditions have been reported (Carlier *et al.*, 2008; Hu *et al.*, 2010; Minaxi *et al.*, 2013; Schoebitz *et al.*, 2013; Yu *et al.*, 2012). *Pseudomonas aurantiaca* JD37 was found to effectively colonize the rhizosphere and internal tissues of roots of maize and promote maize growth (Fang *et al.*, 2012). Strains of *Pseudomonas tolaasii* have been reported as bacteria with biocontrol activity and plant growth promotion of rice (Adhikari *et al.*, 2001) and showed ability to enhanced growth and yield of canola in the presence of cadmiun (Dell'Amico *et.al.*, 2008). *Pantoea dispersa* 1A promoted shoot and root length, shoot and root dry biomass and N, P and K uptake on wheat with respect to uninoculated control (Selvakumar *et*

al., 2008). Castagno *et al.*, (2011) reported the ability of *Pantoea eucalypti* strains to solubilize phosphate and to promote plant growth *in vitro* onto *Lotus tenuis*. Notwithstanding, *P. tolaasii* is related to pathogenesis in cultivated mushrooms (Cuthi *et al.*, 1984; Munsch *et al.*, 2000) and *P. eucalypti* is related to plant diseases of maize and eucalyptus (Brady *et al.*, 2009). Taking into account these backgrounds and the results of preliminary tests and growth chamber experiment, we selected the strain *Pseudomonas tolaasii* IEXb for the field trial.

The results of the field trial confirmed that *P. tolaasii* IEXb has a plant growth promoter effect on maize, as previously observed under controlled condition. The treatment inoculated with this bacterial strain improved the nutritional plant status, with the consequent significant increase in development of plant biomass and grain yield. Although *P. tolaasii* IEXb was found to have multiple plant growth promotion traits (Viruel *et al.*, 2011), we may attribute the increased nutrient uptake primarily to auxin production by the bacterium (as IAA), which has a cascade effect on root development and nutrient uptake, and secondly to phosphate solubilization capacity, which increases soil P availability. Early P uptake leads to an increase in crop yield by stimulating plant growth and development (Richardson *et al.*, 2009).

The results of the field trial revealed that the efficiency of this bacterial strain for improving growth and grain yield in maize was reduced with the application of P fertilizer to the soil. Only the control treatment showed a positive response to the chemical fertilizer. The inhibitory effect of the chemical fertilizer on the bioinoculant performance was already observed (Krey *et al.*,

2013; Shaharooma *et al.*, 2008). In both cases, significant negative linear correlations were recorded between percentage of increase in plant growth and grain yield parameters caused by inoculating wheat and maize, respectively, and increasing levels of fertilizer application.

Increased plant growth and nutrient uptake as a consequence of inoculation of PSB under field conditions were also reported in wheat (Selvakumar *et al.*, 2009; 2011), maize (Hameeda *et al.*, 2008; Kumar *et al.*, 2007), rice (Ashrafuzzaman *et al.*, 2009; Sharma *et al.*, 2014) and chickpea (Rudresh *et al.*, 2005). There is currently a considerable interest in species of the genus *Pseudomonas*, which are being exploited for better crop production due to their rapid colonization, production of growth-promoting substances, and prevention of pathogen establishment in the rhizosphere through antibiosis, siderophore production or secretion of hydrolytic enzymes (Kumar *et al.*, 2007; Selvakumar *et al.*, 2009; Shaharooma *et al.*, 2008). However, inoculation of maize with the *Pseudomonas* species studied in this work has still not been reported. In fact, to the best of our knowledge, this is the first report of the use of a *Pseudomonas tolaasii* strain as bioinoculant on maize in a field experiment. This work provides baseline information for future studies of PGPR candidates for development of bioinoculants to improve nutrient availability in soil, reduce chemical fertilizer application, minimize environmental pollution and promote an eco-friendly and low-input sustainable agriculture.

5. Conclusion

At present, the use of chemical fertilizers and manures cannot be eliminated without avoiding a consequent drastic decrease in food production. Hence, there is an urgent need for integrated management of nutrients that are incorporated into the soil as agricultural inputs to reduce the adverse environmental impacts of chemical fertilizers. The screening method used in growth chamber is an interesting technique to select the adequate bacterial strain for promoting growth in a particular crop and environment. These results also indicate that bacterial inoculation in maize seeds leads to a higher grain yield potential. In particular, P-solubilizing *Pseudomonas tolaasii* IEXb showed great potential for use as bioinoculant. The application of this bacterial strain had beneficial effects on growth, yield and P nutrition on maize plants. Regarding environmental pollution due to excessive use of chemical fertilizers and high costs of P fertilizer production, the bacterial strain tested may well be used as bioinoculant to enhance sustainable agricultural production. Our study also demonstrates the importance of evaluating potential growth-promoting bacteria under a variety of experimental conditions. These experiments, conducted both in growth chamber and in the field, are part of an ongoing project aimed at enhancing the use of this interesting technology to contribute to a sustainable agricultural system.

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