

Biochemical and molecular characterization of phosphate solubilizing bacteria and evaluation of its efficiency promoting the growth of *Lotus tenuis*

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Halophyte communities of saline-alkaline lowlands represent a variable proportion of breeding establishments, arriving sometimes to surpass 30% of its surface. It is therefore a clear need to increase the production capacity of these environments through the incorporation (adaptation and distribution) of leguminous species such as gender *Lotus*, by its condition of fixing nitrogen and its high nutritious value for ruminant (Quinos *et al.*, 1998).

The improvement of soil fertility is one of the most common strategies to increase agricultural production. Maintaining high levels of available nitrogen (N) and phosphorus (P), the two most limiting nutrients in soil, remains being a challenge.

Major researches on biofertilizers have concentrated on understanding and improving N₂ fixation. However, it is known that every aspect of the process of nodule formation is limited by the availability of P. Legumes like alfalfa and clover show a high positive response to P supplementation (Gyaneshwar *et al.*, 2002), but most of the supplemented P become unavailable when its reacts with soil components.

Many soil microorganisms are able to solubilize this unavailable P through their metabolic activities exuding organic acids, which directly dissolve the rock phosphate, or chelating calcium ions that release P to the solution. Production of microbial metabolites results in a decrease in soil pH, which probably plays an important role in the solubilization (Abd-Alla, 1994).

The discovery of mutual relationship between plants and phosphate solubilizing bacteria (PSB), in which bacteria provide soluble phosphate and plants supply rootborne carbon compounds (mainly sugars), that can be metabolized for bacterial growth; encouraged the development of new technologies, such as the use of PSB for biofertilization to improve crop yield (Pérez *et al.*, 2007) (Goldstein, 1995).

Our working hypothesis suggests that the use of phosphate solubilizing bacteria in saline-alkaline soils would increase the level of available phosphorus, contributing substantially to improve the implantation and development of *Lotus tenuis* in the region.

To test this hypothesis, activities undertaken were as follows:

Isolation and characterization of phosphate solubilizing bacteria. Samples of saline-alkaline soils were collected from the rizosphere of *Lotus tenuis* plants growing at fields close to IIB-INTECh and Estación Experimental de Manantiales (Latitude 35° 30' S. Longitude 58° 30' W). Isolations were made in NBRIP medium that contains $\text{Ca}_3(\text{PO}_4)_2$ as the sole P source and which allows the identification of PSB by the formation of a halo of solubilization in the culture medium (Nautiyal C. S.; 1999).

Determination of solubilized phosphate concentration. The concentration of solubilized phosphate was determined at different times (0, 24, 48 and 72 h.), allowing to observe the kinetics of solubilization of each isolate (Fiske C.H. & Subbarow Y.; 1925). Similarly the medium pH value was determined to try to establish a relationship between this parameter and the soluble phosphorus.

Based on the results, the isolates were classified into 3 groups:

	Activity		
	Low	Intermediate	High
Isolates	I26, I29, I35, I38, M22, M52	I17, M51, M56, M87	M25, M75, M76, M77, M78, M89, M91

Genetic diversity and molecular taxonomic identification of PSB. BOX-PCR fingerprinting using a BOX A1R primer (5'-CTA CGG CAA GGC GAC GAC GCT G-3'), was performed to assess the genetic diversity of the isolates, identify strains with different BOX profiles and dismiss those resulting redundant (Versalovic *et al.*, 1991). Then the taxonomic identification of strains with different BOX profiles was carried out through the amplification and subsequent sequencing of the gene coding for the ARNr 16s (Herrera-Cervera *et al.*, 1999).

In vitro evaluation of Plant Growth Promoting Rhizobacteria (PGPR) activity PSB M91, one of the isolates that showed high and reproducible phosphate solubilization activity was selected and inoculated onto *L. tenuis* seedlings grown in semisolid Evans medium (Evans *et al.*, 1970). The soluble phosphate source was replaced by $\text{Ca}_3(\text{PO}_4)_2$ and several P/N ratios were used, in order to simulate different growth-limiting conditions.

Dry weight and total phosphorus content in shoots were analyzed (Murphy J. and Riley JP.; 1962). The data was subjected to two-way analysis of variance (ANOVA) (P: 0.05).

Experiment 1: Inoculation with M91 varying P/N ratio in growth medium at pH 7. Inoculation with PSB isolate M91 significantly enhanced growth of *L. tenuis* plants, as compared with non-inoculated controls. Growth of plants inoculated with PSB isolate M91 was increased by high N levels (10 ppm) in the growth medium. Shoot P content (mg P g^{-1} dry weight) was similar for all treatments.

Experiment 2: Inoculation with M91 at three levels of pH (7, 8, 9) (10 ppm N). The PGPR activity of PSB isolate M91 was not affected by a pH shift from 7 to 8. On the contrary, a

further pH increase from 8 to 9 significantly reduced the PGPR activity of this isolate.

Experiment 3: Inoculation with M91 varying P/N ratio in growth medium at pH 8. As occurred at pH 7, growth of plants inoculated with PSB isolate M91 was increased by high N levels (100% N) when the pH of the growth medium was adjusted to 8.

Conclusions

Seventeen phosphate solubilizing bacteria were isolated, identified and characterized. Most of the bacteria were isolated from soil samples with pH values close to 8. 16s ARNr sequence analysis showed a high level of identity between the isolates and bacteria from genera *Pseudomonas*, *Erwinia*, *Pantoea* and *Rhizobium*, previously reported as phosphate solubilizing bacteria. There is a close relationship between the phosphate solubilizing activity and low pH levels in the growth medium. This suggests that phosphate solubilization could be the result of organic acids released from bacterial metabolism, as reported in literature. Results from assays at pH 7 and 8 clearly demonstrate that inoculation with PSB isolate M91 enhances the growth of *Lotus tenuis*. The plant growth promoting effect was also dependent on the N content in the nutrient solution.

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