

Plant growth promoting properties of a strain of *Enterobacter ludwigii* isolated from *Lolium perenne* L. rhizosphere.



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INTRODUCTION

The interest in the use of biological approaches to replace chemical agents in fertilizing soils is at present in continuous growth. In this regards the use of plant growth promoting rhizobacteria (PGPR) have a role in developing sustainable systems for crop production (Sturz *et al.*, 2000).

The aim of this investigation was to isolate strains from the rhizosphere of *Lolium perenne* L. and characterize them from the point of view of their putative capability of being a PGPR for *L. perenne* and other grasses.

MATERIALS AND METHODS

Isolation strain. The grass (*L. perenne*) used for the isolation of the strain studied in this work was collected in the province of Valdivia (Chile). For the isolation, plant roots were washed and 1cm fragments from the main roots were cut and incubated in NFb (Nitrogen Free Broth) (Döbereiner *et al.*, 1976) and red conge medium (RC) (Rodríguez-Cáceres, 1982). Wild-type *Azospirillum brasilense* FT326 was originally supplied by EMBRAPA, Brazil.

Identification strains. Biochemical tests, morphology and motility were determined for each isolate. Conventional API 20 E (Biomérieux) diagnostic kit and molecular identification by 16S rRNA sequence analysis were performed. The resulting was compared in a BLAST search with those in the National Library of Medicine (Bethesda, MD, USA) database (Altschul *et al.*, 1997).

Biological nitrogen fixation (BNF). Nitrogenase activity was measured according acetylene reduction assay (ARA) to Hardy *et al.*, 1968.

Phosphate solubilization. Mineral phosphate solubilization was assayed on agar plates containing insoluble tricalcium phosphate (5 and 1 g L⁻¹). Development of a clear zone around the colony was evaluated at 48 h (Goldstein and Liu, 1987).

Production indol acetic acid (IAA). Isolates were grown in trypticase soy broth supplemented with tryptophan. Productions of indole compounds were measured using Salkowski reagent according to Torres *et al.* (2000).

Plant inoculation. The seeds of *L. perenne* were sterilized and transferred to Hoagland flasks. Effect of bacterial inoculation in growth root and shoot parameter were evaluated in 20 days after inoculation. The statistical differences were measured with the test of Punnet with 5% of confidence.

RESULTS

a) Thirteen isolates differing in colony morphology in RC medium were evaluated by its ability to reduce acetylene. Just one of them, referred to as strain BNM 0357, resulted positive for this character (Table 1).

b) The capability to synthesize indol acetic acid is an important feature for a strain to be considered a PGPR; it is well known that the hormone participates in promotion of plant growth by increasing the radical surface of the inoculated plants. Fig. 1 shows that the strain BNM 0357 synthesizes IAA at a level comparable to that of the control *A. brasilense*.

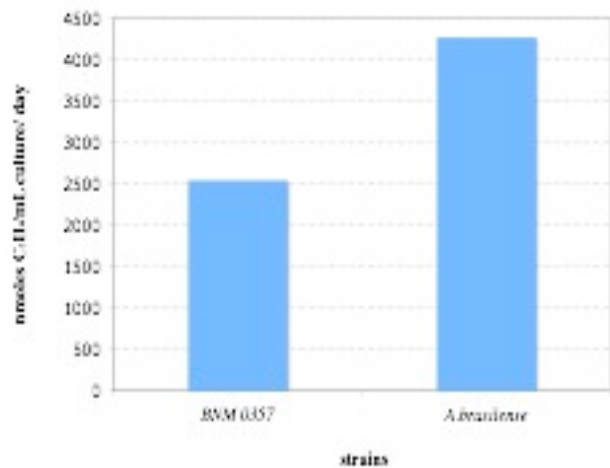
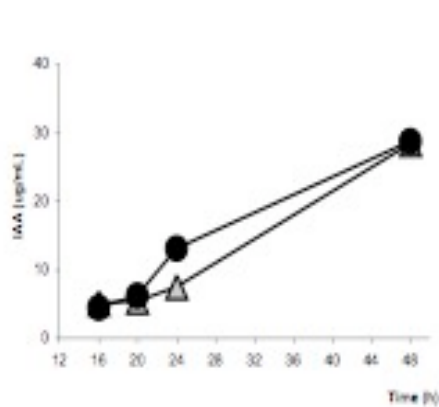


Figure 1. Indol acetic acid production by BNM 0357 and *A. brasilense*. **Triangles**, data for strain BNM 0357 and **circles**, data for *A. brasilense*.

Table 1. Biological nitrogen fixation of strain BNM 0357 and the control *A. brasilense*.

c) Identification of strain BNM 0357.

Morphologically the isolate corresponded to mobile, round rod shaped cells that stained Gram negative, grows at 33°C in RC medium and rendered white colonies in PDA. The biochemical characteristics summarized in Table 2, strongly suggest that strain BNM 0357 can be a member of the Enterobacteriaceae family. Based on their reaction to API 20 E diagnostic tests, the isolate was identified as *Serratia liquefaciens* with 82.5 % identity.

Table 2. Biochemical characteristics of strain BNM 0357.

Test	+/-	Test	+/-
Voges-Proskauer	+	Lisine descarboxilasa	+
Oxidase C	-	Growth on	
Catalase	-	Growth on Brilliant Green Bile Broth	-
NO ₃ ⁻ reductase	+	Growth on MacCONKEY broth	+
Desnitrication	-	Glucosa	+
Gelatin hydrolysis	+	Maltose	+
Citrate utilization	+	Lactose	+
Urease activity	-	Sucrose	+
Use of sucrose in N free medium	+	Myoinositol	+
H ₂ S production (TSI)	-		

d) Molecular identification based on 16S rRNA sequence.

Comparing the 16S rRNA sequence of BNM 0357 (1017 bp) with the NCBI database, the highest similarity was obtained to Enterobacteriaceae family: *Pantoea agglomerans* (98.5%) and the recently described taxon *Enterobacter ludwigii* strain type EN-119 (99.8 %).

e) Mineral phosphate solubilizing ability of the bacterium.

Strains BNM 0357 and *A. brasilense* used as control, were tested for their phosphate solubilizing ability. To that end, solubilization of precipitated tricalcium phosphate. As it can be seen in Fig. 2, BNM 0357 showed a good phosphate solubilizing ability whereas *A. brasilense* did not.

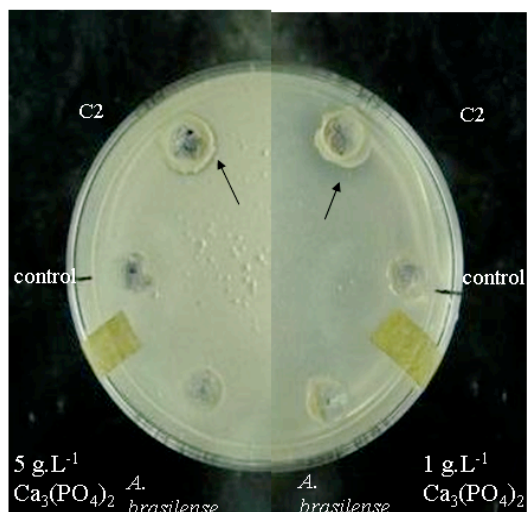


FIGURE 2. Extracellular solubilization of tricalcium phosphate (5 and 1g.L⁻¹). The arrows point to the clear zones surrounding the BNM 0357 colony.

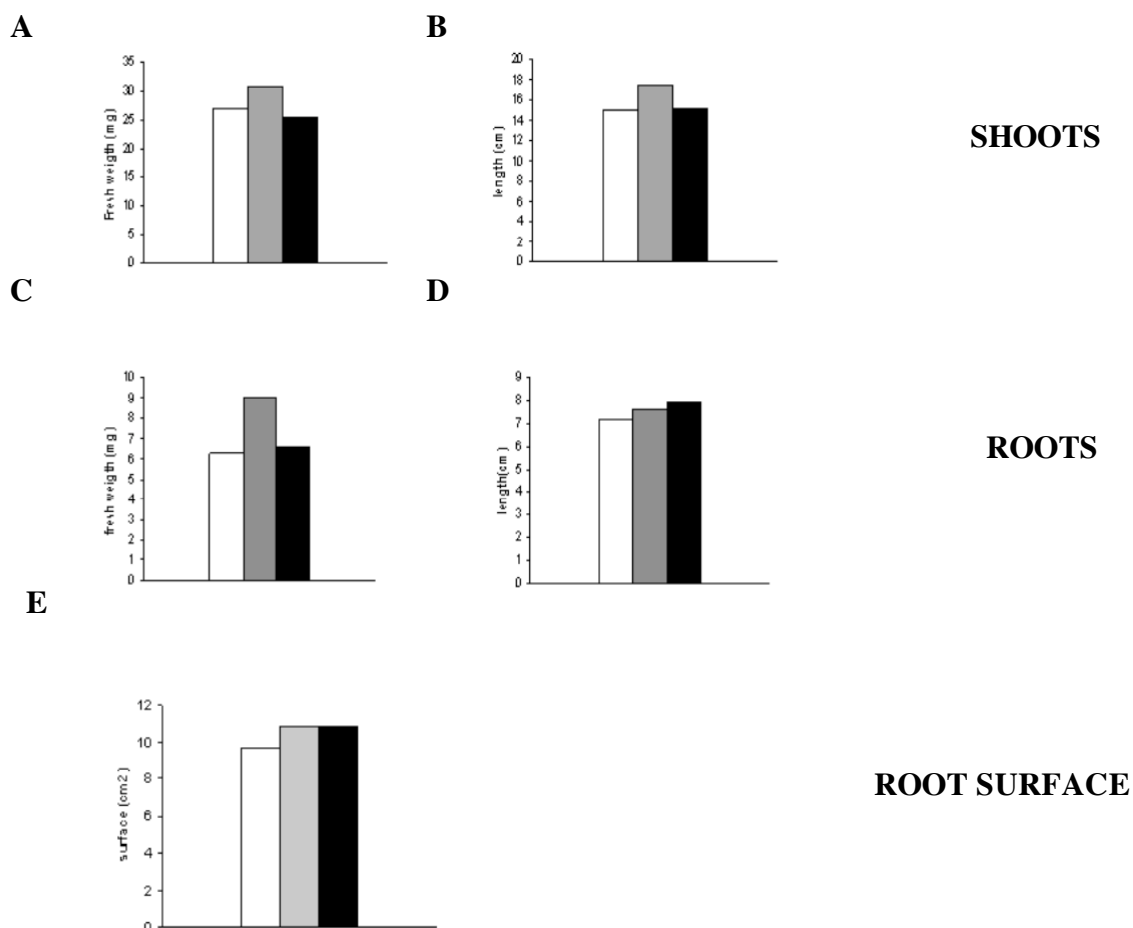


FIGURE 3. Effect of inoculation strain BNM 0357 and *A. brasilense* on the growth of *L. perenne*. **A** and **B**, weight and length of shoots. **C** and **D**, weight and length of root. **E**: root surface. White bars: control; grey bars, BNM 0357 inoculated plants; black bars, *A. brasilense* inoculated plants.

f) Effects of the inoculation with BNM 0357 on growth of *L. perenne*

Growth parameters were measured to assess the growth promotion capability of strain BNM 0357. Fresh weights of roots and shoots, shoot height, main root length and root surface in plants inoculated with strain BNM 0357 were assessed at 20 days. Fig. 3, A and B show that, even though relatively small, a statistically significant increase in shoot fresh weight and in shoot height (14 and 20% respectively) are evident in plants inoculated with strain BNM 0357 while no effect was detected in *A. brasilense* inoculated plants. The inoculation with BNM 0357 promoted an increase in root fresh weight of around 50 % (Fig. 3C) but no effect was observed on roots length (Fig. 3D). As it can be seen, both treatments improved slightly the density of roots (Fig. 3E).

DISCUSSION

Although *Azospirillum* predominates among PGPRs colonizing rhizosphere of grasses, none of the 13 isolates obtained from *Lolium* roots corresponds to this genus, even though the RC medium suited for the isolation of *Azospirillum* was used. Concerning IAA synthesis, isolate BNM 0357 reached the same level of hormone production than the control strain *A. brasilense*. The biochemical features tested placed the isolate as belonging to the family of Enterobacteriaceae. Identification at the molecular level was assessed through the analysis of the 16S rRNA sequence. The BNM 0357 sequence achieved the highest percentage of similarity (99.8%) with *E. ludwigii* (Hofman *et al.*, 2005). However, since in spite of this high degree of similarity, among the biochemical features tested in both strains there exist a few differences (e.g. strain BNM 0357 is catalase negative and gelatinase positive) we consider that we are dealing with a strain of *E. ludwigii* (*E. ludwigii* BNM 0357) different from the reference strain *E. ludwigii* EN-119 DSM 16688^T.

In order to determine the possibility that isolate BNM 0357 matches the properties of a PGPR, it underwent a series of tests conducted to know whether this strain directly and/or indirectly stimulates *L. perenne* growth. On the one hand, BNM 0357 inoculation promoted a light, statistically significant rise in shoots fresh weight and height, effect that was not observed at all in plants inoculated with *A. brasilense*. A relatively more important effect, also restricted to BNM 0357 inoculation, was observed on root fresh weight that increases 50%. Strengthening of the root system in addition to the capability of the isolate to facilitate solubilization of mineral phosphate as shown in Fig. 2, could be an important trait for BNM 0357 to improve plant growth.

This research disclosed the first features of *E. ludwigii* like a PGPR and the future work will concern in bioencapsulation process.

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