Soil Biology and Biochemistry 41 (2009) 1768–1774

Contents lists available at ScienceDirect

Soil Biology and Biochemistry

journal homepage: www.elsevier.com/locate/soilbio

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

Mauricio Shoebitz [a,1], Claudia M. Ribaudo [b,1], Martín A. Pardo [b], María L. Cantore [c,*], Luigi Ciampi [a], José A. Curá [b]

[a] Instituto de Producción y Sanidad Vegetal, Facultad de Ciencias Agrarias, Universidad Austral de Chile, Valdivia, Chile
[b] Cátedra de Bioquímica, Facultad de Agronomía, Universidad de Buenos Aires, Buenos Aires, Argentina
[c] Instituto de Investigaciones Bioquímicas y Fisiológicas (IByF), CONICET, Facultad de Agronomía, Universidad de Buenos Aires, Avenida San Martín 4453 (DSE 1417), Ciudad Autónoma de Buenos Aires, Argentina

ARTICLE INFO

Article history:
Received 5 September 2007
Received in revised form
14 December 2007
Accepted 30 December 2007
Available online 6 May 2008

Keywords:
Enterobacter ludwigii
Plant growth promotion
Lolium perenne
Biocontrol
Fusarium solani

ABSTRACT

The capability of native bacterial strains isolated from *Lolium perenne* rhizosphere to behave as plant growth promoting bacteria and /or biocontrol agents was investigated. One strain (BNM 0357) over 13 isolates from the root tips of *L. perenne* resulted proved to be nitrogenase positive (ARA test) and an IAA producer. Conventional tests and the API 20E diagnostic kit indicated that BNM 0357 behaves to the Enterobacteriaceae family and to the *Enterobacter* genus. Molecular identification by 16S rRNA sequence analysis indicated that BNM 0357 had the highest similarity to *Enterobacter ludwigii* (EN-119). Isolate BNM 0357 had the capability to solubilize calcium triphosphate and to antagonize *Fusarium solani* mycelial growth and spore germination. Strain BNM 0357 also showed the ability to improve the development of the root system of *L. perenne*. This study disclosed features of *E. ludwigii* BNM 0357 that deserve further studies aimed at confirming its putative importance as a PGPR.

1. Introduction

Lolium perenne constitutes one of the most important native pastures for the feeding of cattle in prairies of southern Chile. The soils in this region are derived from volcanic ash (Andisol) and their low productivity is usually improved by periodic fertilization (Teuber, 1997). As in other types of soils, the capability of biological nitrogen fixation (BNF) depicted by forage legumes as *Trifolium repens* contributes to improving growth of grasses in Andisol. However, these classes of soil show high acidic levels and cations and micronutrient composition that can inhibit BNF of nodulated legumes (Schenke et al., 1973, 1974; Osores and Rodríguez, 1986). It has been shown that liming and fertilizers and micronutrient addition improve forage quality of the *L. perenne* and *T. repens* combination (Campillo et al., 2005).

The interest in the use of biological approaches to replace chemical agents in fertilizing soils or improve plant resistance against phytopathogens is at present in continuous growth. In this regard the use of plant growth promoting rhizobacteria (PGPR) has a potential role in developing sustainable systems for crop production (Sturz et al., 2000). The mechanisms used by PGPR leading to plant growth promotion are of such diverse nature as nonsymbiotic nitrogen fixation (Boddey and Döbereiner, 1988), phosphorus solubilization (Reyes et al., 2002), production of phytohormones (Bent et al., 2001) or excretion of diverse compounds (e.g., antibiotics or lytic enzymes), with deleterious properties against pathogenic organisms (Compant et al., 2005, and references therein; Romero et al., 2007).

Some strains of soil micro organisms like *Azospirillum* sp., *Enterobacter* sp., *Pseudomonas sp., Klebsiella* sp., *Serratia* sp. and *Pantoea* sp. have been reported in association with a variety of grasses and other plants depicting growth promotion capabilities (Lemanceau, 1992; Okon and Labandera-González, 1994; Koeppler et al., 1999; Kämpfer et al., 2005).

Inoculation of grass and cereals with *Azospirillum* has received special attention, the increase in grain yield and dry matter content being some of the consequences of inoculation (O'Hara et al., 1981; Rennie and Rennie, 1983; Kapulnik et al., 1985). However, little is known about the response of grasses to native bacterial inoculation. The aim of this investigation was to isolate strains from the rhizosphere of *Lolium perenne* and characterize them in view of their putative capability of being a PGPR for *L. perenne* and other grasses. We have isolated a diazotrophic, AIA synthesizing strain that, besides promoting the growth of *L. perenne* in hydroponic

* Corresponding author. Tel.: +54 11 4524 8061; fax: +54 11 4514 8741.
E-mail address: cantore@agro.uba.ar (M.L. Cantore).
[1] These authors contributed equally to this work.

0038-0717/$ – see front matter © 2008 Published by Elsevier Ltd.
doi:10.1016/j.soilbio.2007.12.031

experiments, has the capability to control growth of the pathogen *Fusarium solani in vitro*.

2. Materials and methods

2.1. Bacterial strains and culture conditions

The grass (*Lolium perenne*) used for the isolation of the strain studied in this work was collected in the province of Valdivia (Chile) in a field containing natural grasses (70% *L. perenne* and 30% *Trifolium repens*) which had not been fertilized for years. The type of soil is of volcanic origin and corresponds to the Andisol. The pH of soil is 5.9 with 9.9% of organic matter and nitrogen content of 24.6 mg kg^{-1} ($N\text{-}NO_3+NH_4$). Areas of 10 cm^2 of soil were selected at random and plants therein were removed together with 10 cm depth soil.

For the isolation of bacteria, plant roots were washed with distilled water and 1 cm fragments from the main roots were cut and incubated in NFb semisolid medium with malate as carbon source (Döbereiner et al., 1976); cultures were maintained at 33 °C until the development of a white and dense film on the surface of the medium. In order to enrich the diazotrophs, bacteria from the pellicle were subcultured five times in the semisolid NFb medium. After the fifth subculture, appropriate dilutions of the pellicle were spread on RC medium (Rodríguez-Cáceres, 1982) and incubated at 33 °C for 96 h. Red scarlet colonies depicting dry consistency, rough surface and irregular edges were subcultured to obtain pure cultures. Isolates that resulted positive for nitrogenase activity and for the production of IAA (indole acetic acid) were finally grown for 24 h in NFb medium supplemented with NH_4Cl (1 g L^{-1}).

Azospirillum brasilense FT326 (EMBRAPA, Seropédica, Brazil) was used as a control strain in some experiments.

2.2. Fungus strain and culture conditions

The *Fusarium solani* strain used in this study was a kind gift from Ing. M. Mitidieri, Laboratorio de Fitopatología, INTA, San Pedro, Argentina. The fungus was routinely cultured in potato dextrose agar (PDA) at 25 °C.

2.3. Morphological characterization and biochemical test

Morphology and motility were determined for each isolate. Biochemical tests were also performed. Nitrate reductase was determined according to García de Salamone et al., (1996), and urea hydrolysis according to Christensen (1946). The test for decarboxylation of amino acid was performed in Basal Medium, with 0.05% amino acid (Asis and Adachi, 2003). Tests for citrate utilization, indole production and gelatin liquefaction were conducted according to DIFCO Manual of Bacteriology. Oxidase activity was measured using the reactive discs from Britania, Argentina. Utilization of different sugars in medium O/F were performed according to the Microbiology Manual Merck, 1996. API 20E test (Biomèrieux) was used for the identification of those negative Gram rods that were recognized as pertaining to the Enterobacteriaceae family.

2.4. Molecular characterization

Isolates were grown at 25 °C for 24 h in BHA and after lysing cells, the DNA was extracted using a microbial DNA isolation kit (Ultra Clean; Mo Bio Laboratories Inc.). The DNA was checked for purity using standard methods (Sambrook et al., 1989).

DNA templates were amplified in a Genius thermocycler (Techne), using universal primers amplifying a 1000 bp region of the 16S rDNA, 616F: 5′-AGA GTT TGA TYM TGG CTC AG-3′, 699R: 5′-RGG GTT GCG CTC GTT-3′ (Invitrogen). These primers are located at positions 8–25 and 1099–1113 (*Escherichia coli* numbering), respectively. The amplification mixture (100 μl) contained 2 μl (50 pmol μl^{-1}) each of 616F and 699R primers, 0.5 μl (2 U μl^{-1}) of Taq DNA Polymerase (Finnzymes), 10 μl of 10× reaction buffer (Finnzymes), 10 μl of dNTP mixture containing 1 mmol L^{-1} each of dATP, dGTP, dCTP and dTTP (Roche), 70 μl of sterile filtered water (Milli-Q purification system, Millipore) and 100 ng of DNA template. Reactions were run for 10 min at 94 °C followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 10 min. Controls devoid of DNA were included in the amplification process. The integrity of PCR products was controlled through the development of a single band after electrophoresis in 2% (w/v) agarose gels in TBE buffer at 5 V cm^{-1} for 1 h. Amplicons were purified using an UltraClean PCR clean-up kit (Mo Bio Laboratories), and subsequently sequenced using an Abi Prism 3730 automated sequencer using the Big Dye Terminator v3.1 cycle sequencing kit, premixed format. Sequencing primers were the same used in the amplification reaction but diluted ten times (5 pmol L^{-1}). The resulting 16S rDNA sequences were compared in a BLAST search with those in the National Library of Medicine (Bethesda, MD, USA) database (Altschul et al., 1997).

2.5. Nucleotide sequence accession number

The 16S rDNA sequence determined for strain BNM 0357 was submitted to the GenBank database under the accession number EU006530.

2.6. Nitrogenase activity

Isolates and *A. brasilense FT 326* were grown in NFb and serially diluted to obtain suspensions with the same concentration of bacteria/ml. Samples of 100 μl each were inoculated by puncturing in 5 ml semisolid NFb medium contained in 10 ml vials and incubated overnight at 33 °C. The medium contained malate as source of carbon. After the incubation, the head space air was replaced with 10% acetylene, the vials were hermetically sealed and incubated again for 24 h; 1 ml of the head space gas was withdrawn and ethylene formation was measured according to Hardy et al. (1968) using a gas chromatograph Series II 5890 (Hewlett Packard) fitted with a flame ionization detector (FID) and a stainless-steel Porapak N column (3.2 mm × 2 m; 80/100 mesh). The injector, oven and detector temperatures were 110 °C, 90 °C and 250 °C respectively. N_2 was used as carrier gas; linear gas velocity was 4.5 cm s^{-1}. The nitrogenase activity was expressed as mol ethylene produced per ml of culture in 24 h.

2.7. Mineral phosphate solubilization

Mineral phosphate solubilization was assayed on agar plates containing insoluble tricalcium phosphate (Goldstein and Liu, 1987). Bacteria were inoculated inside a well in the agar medium that contained glucose 10 g L^{-1}; NH_4Cl 5 g L^{-1}; NaCl 1 g L^{-1}, $MgSO_4 \cdot 7H_2O$ 1 g L^{-1} and $Ca_3(PO_4)_2$ 5 mg ml^{-1}, pH 7.2. The plates were incubated at 33 °C. Development of a clear zone around the colony was evaluated at 48 h.

2.8. Indole acetic acid production

Isolates were grown at 33 °C in the dark with gently shaking in trypticase soya broth supplemented with tryptophan (0.01 g L^{-1}). At different times (16, 20, 24 and 48 h) 1 ml samples were withdrawn and centrifuged at 5000 rpm for 15 min. The supernatant was used to evaluate the production of indolic compounds according to Torres et al. (2000) using indole 3-acetic acid as standard.

2.9. Antifungal activity of the strain BNM 0357

2.9.1. Antagonism

Bacterial cultures (isolate and *A. brasilense FT 326*) grown in liquid NFb were sprayed on a PDA plate. A 0.9 cm diameter agar plug containing *F. solani* mycelium was placed in the middle of the plate and incubated at 25 °C. The diameter of the inhibition zones was registered for 9 days and the percentage of inhibition relative to the control (without bacteria) was evaluated (Landa et al., 1997).

2.9.2. Antibiosis

A 0.9 cm diameter agar plug containing *F. solani* mycelium was placed near the border of a plate containing PDA medium. Plates were incubated at 25 °C for 48 h and then the isolate or *A. brasilense FT 326* were streaked in a straight line on the opposite side of the plate. The inhibitory effect on fungal growth was evaluated after additional 7 days incubation at 25 °C.

2.9.3. Inhibition of F. solani spore germination

A fungal conidial suspension was obtained by adding 5 ml of sterile NaCl (0.85%) to a culture of *F. solani* grown in PDA and scraping the surface with a glass rake. The suspension thus obtained was centrifuged at 8000 rpm, the spores were resuspended in sterile distilled water, counted and adjusted to 1.7×10^7 conidia per ml. To assess the capability of bacteria to inhibit spore germination, 45 μl of the spore suspension were added to 200 ml of YPD medium (1% yeast extract, 2% peptone, and 2% dextrose) containing bacteria at 10^4 CFU ml^{-1}. Cultures were incubated at 25 °C and germinated conidia were scored at different times (4, 24 and 48 h). The experiment was repeated twice.

2.10. Plant inoculation

The seeds of perennial *L. perenne* (Nui variety) were superficially sterilized with a solution of NaClO 30% and 0.1% Tween 80 for 15 min, followed by three 10 min washes with sterile water. Afterwards, 5 seeds were transferred to a 500 ml culture flask containing 100 ml of 0.5% p/v agar Hoagland and maintained at 25 °C. After 48 h the seeds were inoculated with 30 μl of the bacterial suspension (10^7 CFU ml^{-1} in sterile 0.85% p/v NaCl). Controls received 30 μl of the NaCl solution. Five replicates were run per treatment.

The plants were grown for 20 days at 25 °C with 16 h of light. The roots were then washed smoothly with distilled water and separated from the aerial part.

2.11. Plant growth parameters

To evaluate the response to bacterial inoculation the following growth parameters were evaluated: fresh weight of shoots and roots, shoot height, main root length and root surface. The latter was assessed according to Ansari et al. (1995). Each experiment was repeated three times. The statistical differences between the treatments were measured with the test of Punnet with $P < 0.05$ of confidence.

3. Results

Thirteen isolates differing in colony morphology in RC medium were evaluated in terms of their ability to reduce acetylene. Just one of them, referred to as strain BNM 0357, resulted as positive for this character. In fact, strain BNM 0357 depicted 40% of the BNF capability of *A. brasilense* used as control (2538.8 and 4264.6 nmol C_2H_4 ml^{-1} culture per day respectively as an average of two determinations).

The capability to synthesize IAA 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*.

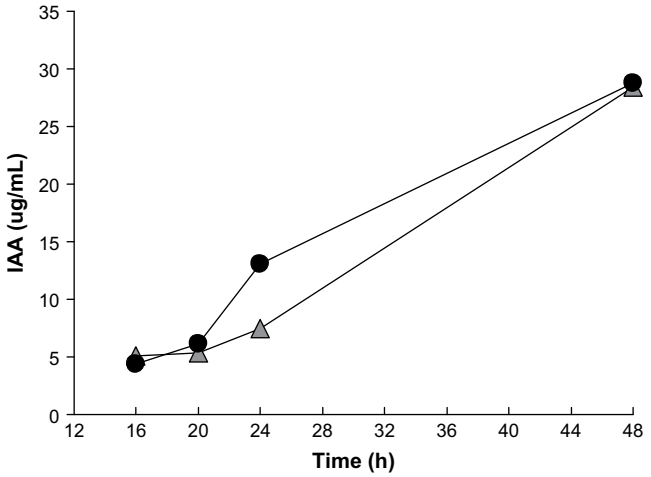

Fig. 1. IAA production by BNM 0357 and *A. brasilense*. Bacteria were grown as indicated in Section 2 and IAA accumulation was measured at the indicated times. Triangles, data for strain BNM 0357; circles, data for *A. brasilense*.

The results on the BNF capacity and IAA biosynthesis obtained for strain BNM 0357 supported the notion that we were dealing with a PGPR. Therefore, strain BNM 0357 was further characterized.

3.1. Identification of strain BNM 0357

Morphologically the isolate corresponded to mobile, round rod shaped cells that stained Gram negative, grows a 37 °C in RC medium and rendered white colonies in potato agar.

The biochemical characteristics summarized in Table 1 strongly suggest that strain BNM 0357 can be a member of the Enterobacteriaceae family and based on their reaction to API 20E diagnostic

Table 1
Biochemical characteristics of strain BNM 0357

Test	+/−
Voges–Proskauer	+
Oxidase C	−
Catalase	+
NO_3 reductase	+
Denitrification	−
Gelatin hydrolysis	+
Citrate Simmon's	+
Urease activity	−
Use of sucrose in N-free medium	+
H_2S production (TSI)	−
Lysine decarboxylase	+
Growth on Brilliant Green Bile Broth	−
Growth on MacConkey broth	+
Carbohydrate metabolism (Hugh and Leifson)	
Glucose	+
Maltose	+
Lactose	+
Sucrose	+
Myo-inositol	+
L-Fucose	−
α-D-melibiose	+
α-L-rhamnose	−
Dulcitol	−
Adonitol	−
α-methyl-D-glucoside	+
Esculin hydrolysis	+

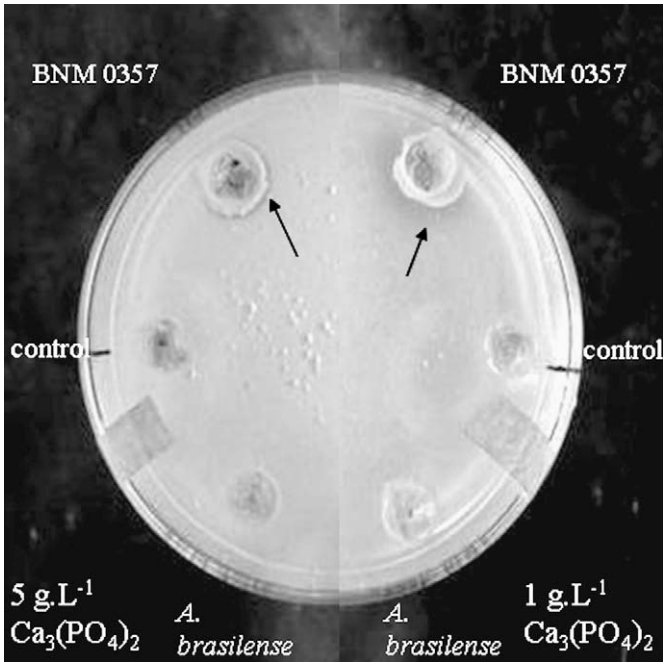

Fig. 2. Extra cellular solubilization of tricalcium phosphate present in the medium at two concentrations (5 and 1g.L^{-1}). Strain BNM 0357 or A. brasilense were inoculated inside wells performed on the agar surface. The arrows point to the clear zones surrounding the BNM 0357 colony. Control means without bacteria.

tests, the isolate could be identified as *Serratia liquefaciens* with 82.5% identity. Nevertheless, it is noteworthy that BNM 0357 differed from *S. liquefaciens* in that it is Voges–Proskauer positive and growth in lactose, myo-inositol, α-L-rhamnose and α-methyl-D-glucoside while the later did not (Table 1 and Bergey et al., 1984).

3.2. Molecular identification based on 16S rDNA sequence

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

3.3. 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 present at 1 or 5 mg ml^{-1} in a medium that contained glucose as a sole carbon source was evaluated. As can be seen in Fig. 2, BNM 0357 showed a good phosphate solubilizing ability whereas *A. brasilense* did not.

3.4. Biocontrol activity of the BNM 0357 strain

Mycelial growth of *F. solani* was inhibited by strain BNM 0357. Inhibition became evident from day 4 onwards reaching a value of 35% inhibition at day 9 (Fig. 3A). As Fig. 3B shows, no effect was observed in the presence of *A. brasilense*.

Strain BNM 0357 inhibits *F. solani* growth even when bacteria and fungi are not in contact, suggesting that the inhibitory effect is mediated by some diffusible metabolite segregated by the bacterium. As can be seen in Fig. 4, in the *F. solani* pure culture a dense layer of mycelia covered the surface of the agar reaching the limits of the plate (Fig. 4B). In the presence of BNM 0357, growth of the fungus was scarce and a clear zone was evident in the vicinity of the bacterium streak (Fig. 4A). No inhibition of *F. solani* mycelial growth was observed in dual cultures of the fungus and *A. brasilense* (Fig. 4C).

Strain BNM 0357 also inhibited fungal spore germination. Fig. 5 shows that at 4 h incubation in rich medium spore germination were 67% inhibited in the presence of BNM 0357; at longer incubation times inhibition decreased to 20%. When the fungus was challenged with *A. brasilense* a 20% inhibition was observed at 24 and 48 h.

3.5. 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 after inoculation. Fig. 6A and B show that, though relatively small, a statistically significant increase in shoot fresh weight and in shoot height (14% and 20% respectively) is evident in plants inoculated with strain BNM 0357 while no effect was detected in *A. brasilense* inoculated plants.

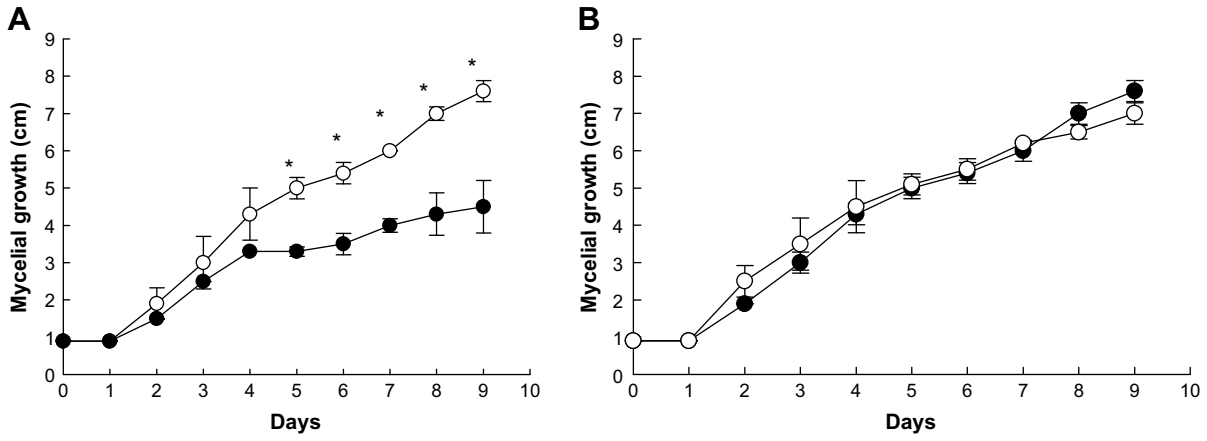

Fig. 3. Inhibition of *F. solani* mycelial growth in the presence of strains BNM 0357 or *A. brasilense*. Fungal growth was assessed by measuring the diameter of the growth zone reached by mycelia from the plug placed on the bacterial lawn. * Significant differences ($P \leq 0.05$ Tuckey test). The bars indicate the standard error. Panel A, black circles, strain BNM 0357; panel B, black circles, *A. brasilense*. In both panels, white circles, control without bacteria.

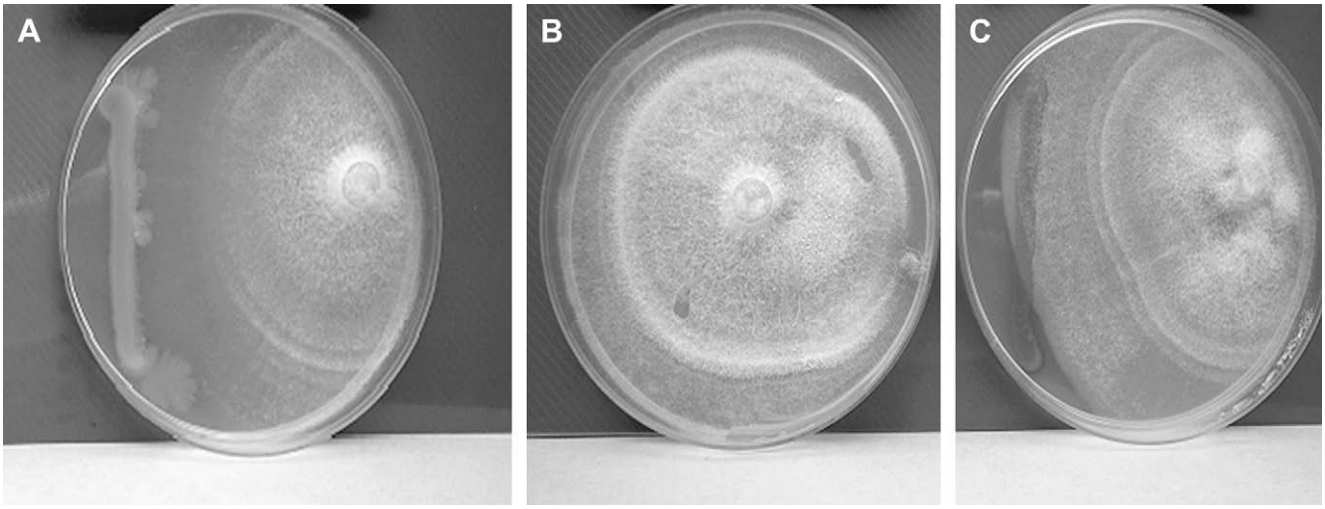

Fig. 4. Antibiotic activity of strains BNM 0357 and *A.brasilense* on *F. solani* growth in PDA plates. Left plate (A), a culture of *F. solani* in the presence of a strike of strain BNM 0357. Middle plate (B), a pure culture of *F. solani*. Right plate (C), a culture of *F. solani* in the presence of a strike of *A. brasilense*. Plates were incubated for 7 days at 28° C. These results are representative of tree independent experiments.

The effect of inoculation on root development was also evaluated. The inoculation with BNM 0357 promoted an increase in root fresh weight of around 50% (Fig. 6C) but no effect was observed on roots length (Fig. 6D). As can be seen, both treatments slightly improved the density of roots (Fig. 6E).

4. Discussion

Although *Azospirillum* predominates among PGPRs colonizing rhizosphere of grasses, none of the 13 isolates obtained from *L. perenne* roots correspond to this genus, even though the RC medium suited for the isolation of *Azospirillum* was used. Just one of the 13 isolates obtained from the 1 cm root tip segments matched the characteristic properties of a diazotrophic bacterium, e.g. it synthesizes IAA and possesses nitrogenase activity. Concerning IAA synthesis, isolate BNM 0357 reached the same level of hormone production as the control strain *A. brasilense*. Regarding nitrogenase activity, values in BNM 0357 were lower than those measured in *A. brasilense*. However, this activity is on the same order of magnitude

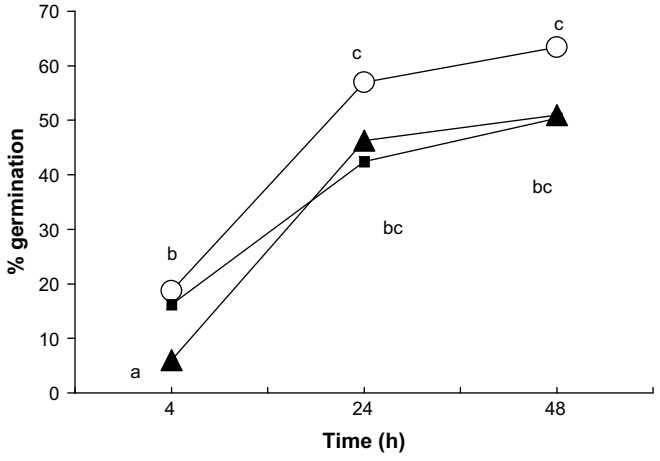

Fig. 5. Effect of BNM 0357 on *F. solani* conidia germination. The percentage of spores germinated in the presence of bacteria was measured at different times of incubation in YMD medium. The results are the average of two independent repetitions. Circles, control (without bacteria); triangles, germination in the presence of BNM 0357; squares, germination in the presence of *A. brasilense*. Same letters at each time mean no significant differences ($P \leq 0.05$, Tukey test).

reported by Seldin et al. (1984) for plant bacteria of genus *Bacillus* isolated from roots and soil of grasses and wheat that was considered an efficient nitrogen fixing bacteria. Therefore, it is possible that BNM 0357 provides at least part of the nitrogen available for the ryegrass in vivo. More studies are needed to quantify the extent of this contribution.

The morphological and biochemical features tested placed the isolate as belonging to the family of Enterobacteriaceae, and the API system identified it as *S. liquefaciens* with the highest percentage of identity (82.5%). Nevertheless, Voges–Proskauer test and lactose utilization resulted as positive for BNM 0357 (Table 1). Since both tests are decisive in classifying bacteria at the genus level, we conclude that the isolate belongs to the *Enterobacter* and not to *Serratia* genus. Identification at the molecular level was assessed through the analysis of the 16S rDNA sequence. The BNM 0357 sequence achieved the highest percentage of similarity (99.8%) with *E. ludwigii* EN-119 (Hoffmann et al., 2005). Morphological and biochemical characteristics of the isolate summarized in Table 1 also matched, with a high score (13/17) with those of *E. ludwigii* EN-119 (Hoffmann et al., 2005). Taken as a whole these results allow us to propose that we are dealing with a strain of *E. ludwigii* 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, a series of tests were run in order to find out whether this strain directly and/or indirectly stimulates *L. perenne* growth.

It is widely known that a variety of bacterial species, including PGPRs, can act as biocontrol agents protecting plants from bacterial and fungal diseases (Romero et al., 2003, 2007; Ren et al., 2006). *P. agglomerans*, a strain closely related to *E. ludwigii* as shown by its 16S rDNA sequence, is able to antagonize *Verticilium dahliae*, *Rizoctonia solani*, and *Sclerotinia sclerotiorum* (Berg et al., 2002). To assess whether the strain isolated in this work has the capability to act as a biocontrol agent, antagonism towards *F. solani* was investigated. BNM 0357 strain depicted fungicidal activity on *F. solani* vegetative growth in mixed cultures as well as in cultures in which BNM 0357 and *F. solani* are not in contact. This behavior suggests that bacteria compete with fungal development (e.g., for nutrients in the culture) and also that a diffusible compound could be responsible for the fungal growth inhibition. As expected, *A. brasilense* showed no fungal inhibiting activity. *F. solani* spore germination was also inhibited by BNM 0357 strain. These results indicate that *E. ludwigii* BNM 0357 is an efficient fungal antagonist

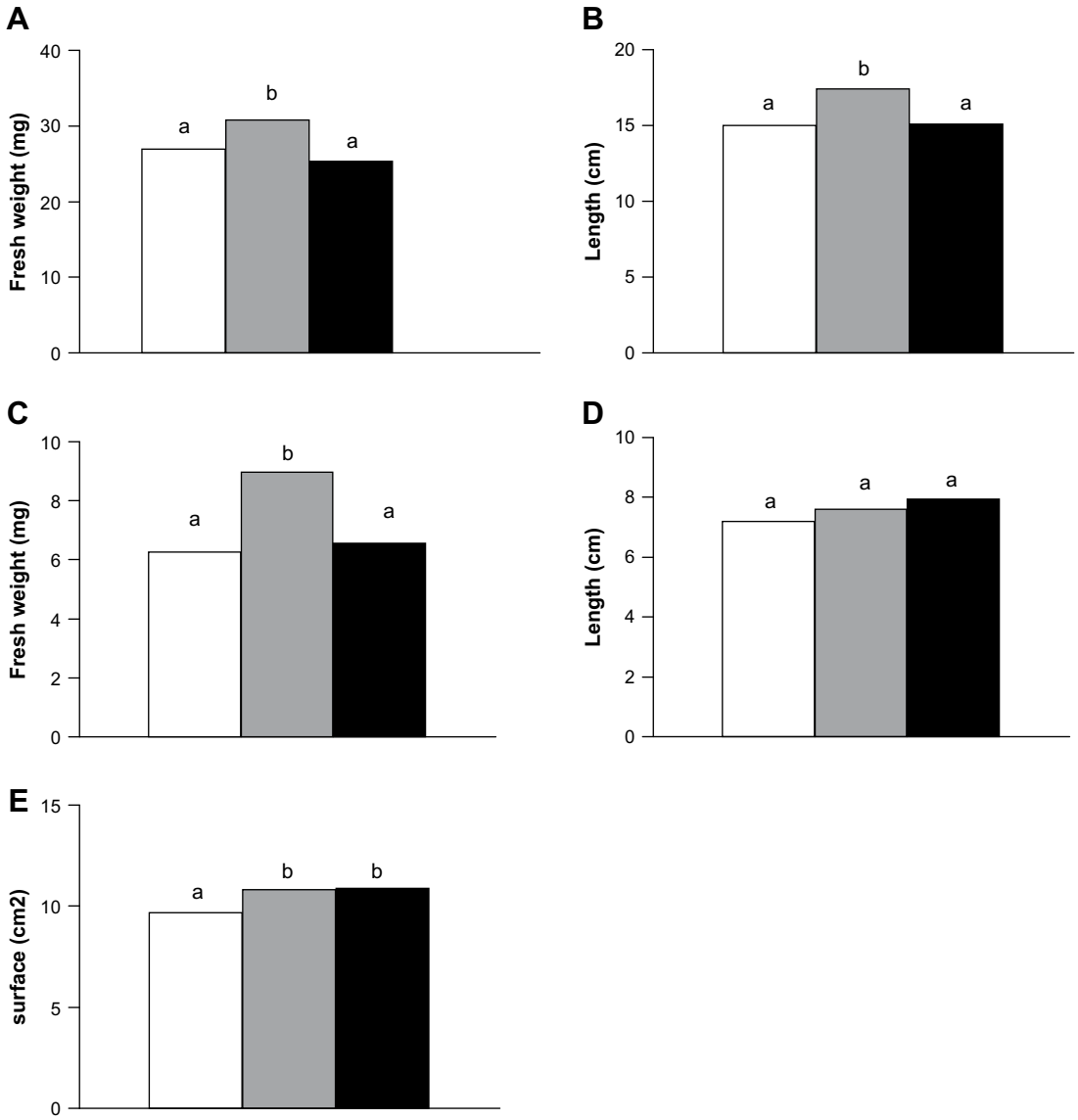

Fig. 6. Effect of inoculation with strain BNM 0357 and with *A. brasilense* on the growth of *L. perenne*. (A, B) Fresh weight and length of *L. perenne* shoots. (C, D) Fresh weight and length of *L. perenne* main root. (E) Root surface. White bars: control plants; gray bars: BNM 0357 inoculated plants; black bars: *A. brasilense* inoculated plants.

at least towards *F. solani*. Since the *Fusarium* spp. are the causative agent of serious diseases in a variety of plants of economic interest, it would be interesting to further investigate the capability of *E. ludwigii* BNM 0357 to antagonize the fungus and extend the study to other species of *Fusarium*.

BNM 0357 inoculation promoted a slight, statistically significant rise in shoot fresh weight and height, an effect which 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 depending on the nutritional resources of the soil. In this concern, it is important to emphasize that the Andisol from where BNM 0357 was isolated usually shows high phosphorus sorption capacity (Campillo et al., 2005) making the presence of phosphorus solubilizing microorganisms highly profitable for crops in this soil.

Taken together, the results presented in this paper show that, in laboratory conditions, *E. ludwigii* BNM 0357 isolated from *L. perenne* behaves as a PGPR, especially concerning its ability to solubilize insoluble phosphorus and to control *F. solani* growth. The fact that BNM 0357 has been isolated from the soil of a low temperature region indicate that it persists and proliferates in an environment in which fungal phytopathogens could seriously affect plant health and where mesophilic biocontrol strains could find difficulty in growing.

To our knowledge, this is the first time that the PGPR features of *E. ludwigii* sp have been reported. It is well known that the capability of any bacterium to act as a biocontrol agent could be different in laboratory or field conditions (Stephens et al., 1993). Therefore, it is necessary to perform experiments to assess the fungicidal ability of *E. ludwigii* against *F. solani,* and also on other fungi in the field.

Acknowledgments

We are grateful to Ing. M. Mitidieri for the kind gift of the *F. solani* strain and to Dra. J. Döbereiner for providing us with the *A. brasilense* FT326 strain used in this study. We thank Dr. David Ruiz Arahal for expert assistance in sequence analysis. This work

was principally funded by grant from the Universidad de Buenos Aires to J.A.C. and M.L.C. (UBACyT G028).

References

Altschul, S., Modden, T., Schafer, A., Zhang, J., Miller, W., Lipman, D., 1997. Gapped BLAST and PSIBLAST: a new generation of protein database search programs. Nucleic Acids Research 25, 3389–3404.

Ansari, S.A., Kumar, P., Gupta, B.N., 1995. Root surface area measurement based on adsorption and desorption of nitrite. Plant and Soil 175, 133–137.

Asis, C.A., Adachi, K., 2003. Isolation of endophytic diazotroph *Pantoea agglomerans* and nondiazotroph *Enterobacter asburiae* from sweetpotato stem in Japan. Letters in Applied Microbiology 38, 19–23.

Bent, E., Tuzun, S., Chanway, C.P., Eneback, S., 2001. Alterations in plant growth and in root hormone levels of lodgepole pines inoculated with rhizobacteria. Canadian Journal of Microbiology 47, 793–800.

Berg, G., Roskot, N., Steidle, A., Eberl, L., Zock, A., Smalla, K., 2002. Plant-dependent genotypic and phenotypic diversity of antagonistic rhizobacteria isolated from different *Verticillium* host plants. Applied Environmental Microbiology 68, 3328–3338.

Bergey, D.H., Holt, J.G., Krieg, N.R., 1984. Bergey's Manual of Systematic Bacteriology. Williams & Wilkins, Baltimore.

Boddey, R.M., Döbereiner, J., 1988. Nitrogen fixation associated with grasses and cereals: Recent results and perspectives for future research. Plant and Soil 108, 53–65.

Campillo, R., Urquiaga, S., Undurraga, P., Pino, I., Boddey, R.M., 2005. Strategies to optimise biological nitrogen fixation in legume/grass pastures in the southern region of Chile. Plant and Soil 273, 57–67.

Christensen, W.B., 1946. Urea decomposition as means of differentiating *Proteus* and *Paracolon* cultures from each other and from *Salmonella* and *Shigella* types. Journal of Bacteriology 52, 461–466.

Compant, S., Duffy, B., Nowak, J., Clément, C., Ait Barka, E., 2005. Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. Applied Environmental Microbiology 71, 4951–4959.

Döbereiner, J., Marriel, I.E., Nery, M., 1976. Ecological distribution of *Spirillum lipoferum* Beijerinck. In: Manual of Determinative Bacteriology. Williams & Wilkins, Baltimore, pp. 40–42.

García de Salamone, I.E., Döbereiner, J., Urquiaga, S., Boddey, R.M., 1996. Biological nitrogen fixation in *Azospirillum* strain in maize genotype associations as evaluated by ^{15}N isotope dilution technique. Biology and Fertility of Soils 23, 249–256.

Goldstein, A.H., Liu, S.T., 1987. Molecular cloning and regulation of a mineral phosphate solubilizing gene from *Erwinia herbicola*. Bio/Technology 5, 72–74.

Hardy, R.W.F., Holsten, R.D., Jackson, E.K., Burns, R.C., 1968. The acetylene ethylene assay for N_2 fixation: laboratory and field evaluation. Plant Physiology 43, 1185–1207.

Hoffmann, H., Stindl, S., Stumf, A., Mehlen, A., Monget, D., Heesemann, J., Schleifer, K., Roggenkamp, A., 2005. Description of *Enterobacter ludwigii* sp. nov., a novel *Enterobacter* species of clinical relevance. Systematic Applied Microbiology 20, 206–212.

Kämpfer, P., Ruppel, S., Reiner, R., 2005. *Enterobacter radicincitans* sp. Nov., a plant growth promoting species of the family Enterobacteriaceae. Systematic Applied Microbiology 28, 213–221.

Kapulnik, Y., Gafny, R., Okon, Y., 1985. Effect of *Azospirillum* spp. inoculation on root development and NO_3 uptake in wheat (*Tritricum aestivum* L. cv. Miriam) in hydroponic systems. Canadian Journal of Botany 63, 627–631.

Koeppler, J.W., Rodríguez-Urbana, R., Zehnder, G.W., Murphy, J.F., Sikora, E., Fernández, C., 1999. Plant root bacterial interactions in biological control of soil born diseases and potential extension to systematic and foliar diseases. Australasian Plant Pathology 28, 21–26.

Landa, B.B., Hervás, A., Bettiol, W., Jiménez-Diaz, R.M., 1997. Antagonistic activity of bacteria from the Chickpea rhizosphere against *Fusarium oxysporum* f.sp. *ciceris*. Phytoparasitica 25, 413–434.

Lemanceau, P., 1992. Effects bènèfiques de rhizobactéries sur plantes: exemple des *Pseudomonas spp fluorescens*. Agronomie 12, 413–434.

O'Hara, G.W., Davey, M.R., Lucas, J.A., 1981. Effect of inoculation of *Zea mays* with *Azospirillum brasilense* strains under temperate conditions. Canadian Journal of Microbiology 27, 871–877.

Okon, Y., Labandera-González, C.A., 1994. Agronomic application of *Azospirillum*: an evaluation of 20 years worldwide field inoculation. Soil Biology and Biochemistry 26, 1591–1601.

Osores, G.M.A., Rodríguez, S.J., 1986. Fertilidad de los suelos de la Isla Grande de Chiloé. Ciencia e Investigación Agraria (Chile) 13, 213–227.

Ren, H., Gu, G., Long, J., Yin, Q., Song, T., Zhang, S., Dong, H., 2006. Combinative effect of bacterial type-III effectors and a biocontrol bacterium on rice growth and disease resistance. Journal of Biosciences 31, 617–627.

Rennie, R.J., Rennie, D.A., 1983. Techniques for quantifying N_2 fixation in association with nonlegumes under field and greenhouse conditions. Canadian Journal of Microbiology 29, 1022–1035.

Reyes, I., Bernier, L., Antoun, H., 2002. Rock phosphate solubilization and colonization of maize rhizosphere by wild and genetically modified strains of *Penicillium rugulosum*. Microbial Ecology 44, 39–48.

Rodríguez-Cáceres, E., 1982. Improved medium for isolation of *Azospirillum* spp. Applied Environmental Microbiology 44, 990–991.

Romero, A.M., Correa, O.S., Moccia, S., Rivas, J.G., 2003. Effect of Azospirillum-mediated plant growth promotion on the development of bacterial diseases on fresh-market and cherry tomato. Journal of Applied Microbiology 95, 832–838.

Romero, D., de Vicente, A., Rakotoaly, R.H., Dufour, S.E., Veening, J.W., Arrebola, E., Cazorla, F.M., Kuipers, O.P., Paquot, M., Perez-García, A., 2007. The iturin and fengycin families of lipopeptides are key factors in antagonism of *Bacillus subtilis* toward *Podosphaera fusca*. Molecular Plant-Microbe Interactions 20, 430–440.

Sambrook, J.E., Fritsch, F., Maniatis, T., 1989. Molecular Cloning: A Laboratory Manual, second ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Seldin, L., Van Elsas, J.D., Penido, E., 1984. *Bacillus azotofixans* sp. Nov. a nitrogen-fixing specie from Brazilian soils and grass roots. International Journal of Systematic bacteriology 34, 451–456.

Schenke, G., Baherle, P., Floody, H., Gajardo, M., 1973. Exploración de deficiencias nutritivas con suelos en macetas.XIV. Macronutrientes. Provincia de Chiloé. Agricultura Técnica (Chile) 33, 214–223.

Schenke, G., Baherle, P., Floody, H., Gajardo, M., 1974. Exploración de deficiencias nutritivas con suelos en macetas. XV. Comportamiento de algunas fórmulas de fertilización. Provincia de Chiloé. Agricultura Técnica (Chile) 34, 19–28.

Stephens, P.M., Crowley, J.J., O'Connell, C., 1993. Selection of pseudomonad strain inhibiting *Pythium ultimim* on sugar beet seeds in soil. Soil Biology and Biochemistry 25, 1283–1288.

Sturz, A.V., Christie, B.R., Novak, J., 2000. Bacterial endophytes: potential role in developing sustainable system of crop production. Critical Reviews in Plant Science 19, 1–30.

Teuber, N., 1997. Bases de un manejo eficiente de praderas de Chiloé. In Seminario: Diagnóstico nutricional de los suelos de la Isla de Chiloé. In: Campillo, R., Bortolameolli, G. (Eds.), Serie Remehue N, 65. Instituto de Investigaciones Agropecuarias, Centro Regional de Investigación Remehue, Osorno (Chile), pp. 47–57.

Torres, M., Valencia, S., Bernal, J., Martinez, P., 2000. Isolation of *Enterobacteria, Azotobacter* sp. and *Pseudomonas* spp., producers of Indole-3-Acetic Acid and Siderophores, from Colombian Rice Rhizosphere. Revista Latinamericana de Microbiologia 42, 171–176.