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Effect of the Phosphorus-Solubilizing Bacterium Enterobacter Ludwigii on Barley Growth Promotion

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Abstract

Phosphorus (P) is essential for plant growth and development but is often a limiting nutrient in soils. Thus, Pi acquisition from the soil by plant roots is a subject of considerable interest in agriculture. One ecological alternative is the use of P-solubilizing bacteria, which make P available to plants through different mechanisms. Thus, the aim of the present study was to investigate the role of the P-solubilizing bacterium *Enterobacter ludwigii* in the growth promotion and P content of *Hordeum vulgare* (barley) under field conditions. Plants were inoculated with *E. ludwigii* and then its growth promotion effects were compared with those of the reference strain *Azospirillum brasilense.* The effect of bacterial inoculation showed a beneficial effect on the dry weight, P assimilation and barley yield, especially in *E. ludwigii*-inoculated plants. The plant P content at 60 DAS was 38% to 56% higher in *E. ludwigii* -inoculated plants with respect to non-inoculated plants. The application of bacteria without fertilizer led to the same biological yield (3,795 kg/ha) and increase in one thousand seed weight as the maximum dose of chemical fertilizer applied, while the application of bacteria along with the intermediate fertilizer dose led to a significant increase in grain size (83% of plump grains larger than 2.75 mm wide, whereas 76% of the grains of the control plants reached that size). Endophyte populations of the inoculated bacteria were observed in plants growing under field conditions. The results demonstrated that the inoculation of with *E. ludwigii* is a promising option to increase P levels in plants and could be a technique for application in agricultural industry.

Keywords: Rhizobacteria; Phosphate-solubilizing bacteria; Phosphorous chemical fertilizer; Barley; Yield.

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1. Introduction

In Argentine grasslands *Hordeum vulgare* (barley) is one of the cereals most used in the beer industry or as nutritional supplement in animal feed. In this grass, the practice of fertilization to achieve acceptable levels of production entails considerable costs, not only economically, but also environmentally. In this regard, nitrogen and phosphorus fertilization leads to the pollution of the phreatic layer and eutrophication of water bodies [1] Phosphorus (P) is a key nutrient required for plant growth. However, in the soil, P is present in the form of inorganic phosphate (Pi) at low available concentrations (around 1–10 mM in the soil solution), which represents less than 1% of the total P pool in soils [2]. Thus, P is usually applied as chemical fertilizer. The disadvantage is that a large portion of the soluble form of P applied is initially available for plant uptake but rapidly reacts with the soil and becomes progressively less available due to immobilization into insoluble forms, particularly $CaHPO_4$, $Ca_3(PO_4)_2$, and $FePO_4$ [3]. Thus, the interest in the use of biological approaches to replace chemical agents to fertilize soils as well as to 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 [4; 5]. The mechanisms used by PGPR to promote plant growth include nonsymbiotic nitrogen fixation, P solubilization, production of phytohormones and antibiotics, and excretion of lytic enzymes [6]. On the other hand, plants have evolved complex adaptive responses to cope with Pi limitations, including a positive interaction with P-solubilizing microorganisms. A wide range of microorganisms are able to solubilize Pi [7; 8] via exudation of organic acids that dissociate Ca^{2+}-bound P [9; 10]. In this regard, phosphate-solubilizing bacteria (PSB) have been seen as the best ecological alternative for P nutrition of crops. Considerable populations of PSB are present in the soil and in plant rhizospheres, and strains of the genera Pseudomonas, Bacillus and Rhizobium are among the most powerful PSB. Although several bacteria have been identified as PSB, their performance under in situ conditions is not reliable and it is therefore necessary to search for and characterize new PSB strains that can improve the growth of crops. *Enterobacter ludwigii* is an endophytic nitrogen-fixing bacterium with growth-promoting abilities isolated from the *Lolium perenne* rhizosphere [11]. Such abilities include not only the promotion of plant growth [12; 13; 14;], but also the biocontrol of plant diseases [11]. In addition, *E. ludwigii* plays a significant role in the remediation of metal-contaminated soils, promoting the phytoextraction of metals [15; 16] and even as an industrial microorganism catalyzing the conversion of sugarcane molasses into 2,3-Butanediol production via fermentation [17]. In previous studies, we isolated *E. ludwigii* BNM0357 from ryegrass rhizospheric soil, and found it to have various plant growth-promoting traits [11]. We also studied the effect of *E. ludwigii* inoculation on the growth and quality of *Festuca arundinacea* (tall fescue), but never tested its plant growth promotion capability or its direct contribution of P to other grass. Thus, the aim of the present study was to investigate the effect of *E. ludwigii* on the growth promotion of *Hordeum vulgare* (barley) under field conditions in soils deficient in available P, as well as the contribution of this bacterium to the P content of plants. This was assessed in terms of biomass, yield, Pi uptake and PSB colonization for their potential use to develop bio-fertilizers for sustainable agriculture.

2. Materials and methods

2.1 Microorganisms

The following strains were used in all the experiments: *Enterobacter ludwigii* BNM0357 and *Azospirillum brasile*nse FT326. The strains were cultured in NFb medium amended with 0.1 g/l of NH_4Cl for 48 h at 30ºC [18].

2.2 Plant inoculation

Seeds of *Hordeum vulgare* (cv. Shakira) were surface-disinfected with 30% w/v sodium hypochlorite solution with 0.1% w/v Triton X-100 for 30 min, followed by three washes of 10 min each with sterile distilled water. Bacterial inoculation was carried out before sowing, inoculating each seed with 10^8 colony-forming units (CFU)/ml bacterial suspension. Sterile distilled water instead of the bacterial inoculum was used as a negative control. Field experiments were conducted in the town of Hughes, Santa Fe, Argentina. The soil type at the experimental site was a luvic Phaeozem. The nutrient composition of the soil at sowing was: 2.55% organic matter [19] 24.3 ppm of available P (0–20 cm) [20]; 19.9 ppm of available N , 16.9 ppm of S, and 0.15 salinity (dSml), pH 6.2. The experiment was arranged in a 3 x 3 factorial model (bacteria x dose of fertilizer). For the factor bacteria, the three following treatments were evaluated: a) two biofertilization treatments with *E. ludwigii* and *A. brasilense,* and one control without bacteria. The experiments were carried out according to a completely randomized block design for each bacterium and with the main plots divided according to a factorial arrangement (bacteria x dose of P). For the bacterial factor, three levels were used: a bacterial suspension containing *Enterobacter ludwigii* BNM0357, a bacterial suspension containing *Azospirillum brasilense* FT326, and a control treatment without bacteria, whereas three levels of P dose were used: 0 kg/ha, 35 kg/ha and 85 kg/ha of diammonium phosphate (DAP). The plants were sown in plots of 231 m^2/treatment (4.62 m x 50 m), at a seeding density of 115 kg/ha. The distance between rows was 21 cm. The design was a block design with three repetitions. The plants were fertilized with urea (80 kg/ha) at 40 days after sowing (DAS). The plant material was evaluated at 60 DAS, during the tillering of the crop, and at 135 DAS.

2.3 Determination of plant P content

The stem and roots of *H. vulgare* were used for P determination using 100 mg of dry weight of each tissue, according to the authors in [21].

2.4 Determination of bacterial endophytism

The bacterial endophytism was measured at the end of the experiments (135 DAS). Ten plants of each species were taken at random and the roots were macerated in saline solution. The number of CFU was determined by serial dilution plating on Congo red nutrient agar [22]. Counting of microorganisms was performed in triplicate. Controls were performed with non-inoculated plants.

2.5 Statistical analysis

The experiments were carried out according to a completely randomized block design for each bacterium and with the main plots divided according to a factorial arrangement date x dose of P. The results were analyzed using the Infostat statistical software [23]. Analysis of variance (ANOVA) and the Fisher LSD tests were

performed at a significance level of $p<0.05$.

3. Results

3.1 Plant growth promotion

We first evaluated the effect of bacterial inoculation on barley growth at 60 DAS. A statistically significant ($p<0.05$) increase in shoot dry weight of *E. ludwigii*-treated plant was higher than in control plants and similar to that of *A. brasilense*-treated plants (57% and 43% respectively over non-inoculated plants) (Figure 1).

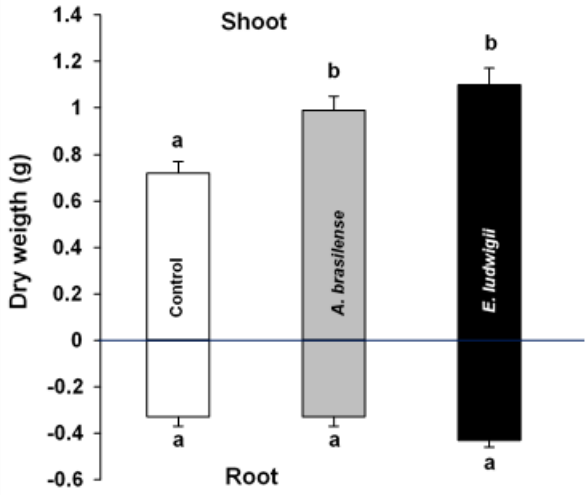

Figure 1: Growth promotion in Hordeum vulgare inoculated with PGPRs.

The dry weight of shoots and roots was measured at 60 DAS. White bar: Control plant without inoculation, grain bar: *Azospirillum brasilense* inoculated plants, black bar: *Enterobacter ludwigii* inoculated plants. The bars represent the media of 8 determinations. Different letters at top bar indicate significant differences between treatments with $P < 0.05$ (LSD, Fischer test). No root growth promotion was evident with any of the inoculation treatments and no significant differences were found between *E. ludwigii*- and *A. brasilense* -inoculated plants.

3.2 Effect of bacterial inoculation on the growth of barley plants fertilized with Pi

Diammonium phosphate (DAP) is the world's most widely used P fertilizer in farming. Thus, in this study, we used it to test the ability of bacteria to provide crops with available P from the barely soluble form in the soil, at the following doses: 0, 35 and 85 kg/ha. We evaluated the dry weight of barley at 60 DAS upon the inoculation treatments. *E. ludwigii* inoculation resulted in significant differences in barley growth at all Pi doses studied when compared to control plants. The non-fertilized inoculated plants showed 77% and 38% higher weight than control plants for *E. ludwigii*- and *A. brasilense*-treated plants, respectively. The increase in aerial biomass due to *A. brasilense* inoculation was similar to that achieved by *E. ludwigii* inoculation at 35 and 85 kg/ha chemical fertilizer applied. Both bio-fertilized treatments had a positive effect on barley dry weight when combined with DAP at 35 and 85 kg/ha (around 40% higher weight, Table 1). The bacterial inoculation stimulated the root

growth and was more evident in *E. ludwigii*-inoculated plants at 0 kg/ha fertilizer dose (more than two fold).

Table 1: Effect of Pi doses on shoot and root growth promotion of inoculated *Hordeum vulgare*.

	Biomass	
Treatment	**Aerial**	**Root**
	mg.plant^{-1}	
C^0	570.1 ± 45 ^{a}	190 ± 40 ^{a}
C^{35}	730.3 ± 70.3 ^{ab}	364 ± 41 ^{abc}
C^{85}	850.1± 60.2 ^{ab}	441 ± 11 ^{bc}
Ab^0	790.2 ± 91 ^{ab}	310 ± 54 ^{abc}
Ab^{35}	1100 ± 50 ^{cd}	381 ± 120 ^{bc}
Ab^{85}	1091 ± 70 ^{cd}	305 ± 30 ^{ab}
El^0	1011 ± 130 ^{bcd}	484 ± 11 ^{c}
El^{35}	1071 ± 70 ^{cd}	411 ± 70 ^{bc}
El^{85}	1220 ± 170^{d}	403 ± 32 ^{bc}

The dry weight of *Hordeum vulgare* was evaluated at 60 DAS. C: Control plants (without inoculation). El: Plants inoculated with *Enterobacter ludwigii*. Ab: Plants inoculated with *Azospirillum brasilense* and with three levels of fertilization with $(NH_4)_2(HPO_4)$: 0; 35 and 85 kg.ha^{-1}. Representation of means ± standard deviation (n = 3). Different letters in each column indicate significant differences between treatments with $P < 0.05$ (LSD Fischer test).

3.3 Barley yield

The number of grains is itself a function of the number of fertile shoots per unit area and the number of grains per head. Nutrients can have an impact on the number of grains/ear so a higher mineral fertilization with nitrogen, phosphorus and potassium causes an increase in the number of ears per m^2 [24]. In our study, the number of grains per ear was significantly affected by the bacterial inoculation (Table 2).

Table 2: Effect of Pi dose and PGPR inoculation on yield components of *Hordeum vulgare.*

Treatment	**Number of grains.m^{-2}**			**One thousand grains weight (gr)**
	Plants.m^{-2}	**Ears.plant^{-1}**	**Grains.ear^{-1}**	
C^0	253 ± 18.5 ^{a}	3.33 ± 0.53 ^{ab}	21.68 ± 0.48 ^{a}	41.80 ± 0.99 ^{ab}
C^{35}	263 ± 18.4 ^{a}	3.33 ± 0.48 ^{ab}	24.48 ± 0.54 ^{def}	43.76 ± 1.02 ^{bc}
C^{85}	255 ± 23.8 ^{a}	4.00 ± 0.80 ^{ab}	24.36 ± 0.49 ^{cdef}	40.34 ± 0.96 ^{a}
Ab^0	239 ± 5.8 ^{a}	3.73 ± 0.27 ^{ab}	21.94 ± 0.54 ^{ab}	48.00 ± 1.11 ^{de}
Ab^{35}	251 ± 19.0 ^{a}	3.60 ± 0.23 ^{ab}	23.34 ± 0.51 ^{cd}	47.34 ± 1.11 ^{de}
Ab^{85}	242 ± 9.7 ^{a}	3.33 ± 0.13 ^{ab}	24.94 ± 0.41 ^{efg}	46.10 ± 1.08 ^{cd}
El^0	243 ± 10.5 ^{a}	2.93 ± 0.27 ^{a}	23.16 ± 0.38 ^{bc}	48.82 ± 1.14 ^{def}
El^{35}	272 ± 16.7 ^{a}	3.87 ± 0.35 ^{ab}	24.76 ± 0.48 ^{ef}	51.36 ± 1.20 ^{f}
El^{85}	250 ± 22.0 ^{a}	5.33 ± 0.53 ^{c}	26.10 ± 0.37 ^{g}	48.88 ± 1.14 ^{def}

The number of grains.m^{-2} Plants.m^{-2} Ears.Plant^{-1} Grains.Ear^{-1} and One thousand Grains weight (gr) of *Hordeum*

vulgare was evaluated at 135 DAS. C: Control plants (without inoculation). El: Plants inoculated with *E. ludwigii.* Ab: Plants inoculated with *A. brasilense* and with three levels of fertilization with $(NH_4)_2(HPO_4)$:0, 35 and 85 $kg.h^{-1}$. Representation of means ± standard deviation (n = 3). Different letters in each column indicate significant differences between treatments with $P < 0.05$ (LSD, Fischer test). The maximum number of grains per ear was observed in *E. ludwigii*-inoculated barley, whereas the minimum number was observed in the control treatment, both without fertilizer. A synergistic interaction effect between DAP and the bacteria was observed with the application of 85 kg/ha of DAP and inoculation of seeds with *E. ludwigii.* The one thousand grain weight is an important quality criterion. The effect of DAP on this parameter was significantly affected by the inoculation treatment. The maximum one thousand grain weight was observed using bacteria at all Pi doses evaluated and was higher for barley plants inoculated with *E. ludwigii*. Our results are in agreement with that found by the authors in [25], who reported that PSB increase the nitrogen and phosphorus available in the soil and that this could enhance crop production. Grain yield, which is another productivity parameter, was significantly affected by the inoculation treatments. Figure 2 summarizes the barley yields obtained, expressed as grain production per hectare. A synergetic interaction between the bacteria and P fertilizer was detected. The maximum grain yield (4,650 kg/ha) was obtained using 85 kg/ha of DAP and *E. ludwigii* as well as with the treatment that included *A. brasilense* in combination with higher Pi fertilizer applied (4,026 kg/ha).

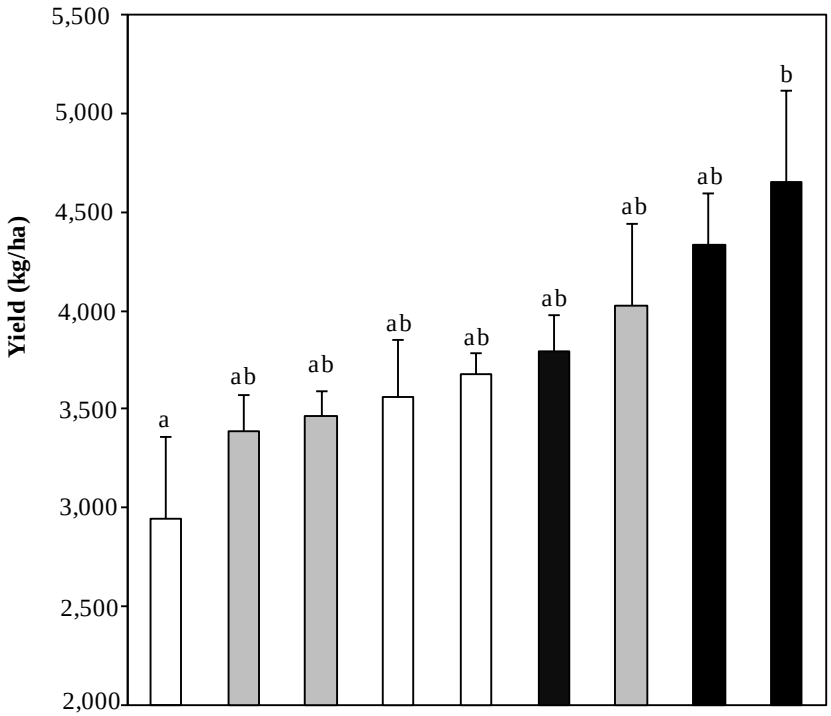

Figure 2: Grain yield of barley plants inoculated with DAP.

The plants were fertilized $(NH_4)_2(HPO_4)$: 0, 35 and 85 kg.h-1. White bar: Control plant without inoculation, grain bar: *Azospirillum brasilense* inoculated plants, black bar: *Enterobacter ludwigii* inoculated plants. Representation of means ± standard deviation (n = 3). Different letters, in each column, indicate significant differences between treatments with $P < 0.05$ (LSD, Fischer test).

3.5 Barley grain size

The quality of barley grains for malting is directly related to their protein content and size. Grain size is defined by the width of the grain and determined by size fractionation with slotted sieves of several sizes (plump grains are >2.5 mm wide). To meet maltsters' quality requirements, barley grains must have a specific protein level and high grain size (i.e. a high proportion of plump grains). These two characteristics related to grain quality are determined during the crop cycle [26].Phosphate plays a major role in the supply of energy for plant processes. The redistribution of stored carbohydrates requires energy, making phosphate nutrition important to achieve good grain size. Thus, phosphate fertilizers can be used to improve the final grain size. The treatments that combined bacteria and DAP allowed to obtain about 83% of plump grains larger than 2.75 mm wide, whereas, at the same Pi dose, only 76% of the grains of the control plants reached that size (Figure 3). Consequently, the inoculation of barley with *E. ludwigii* or *A. brasilense* can lead to commodities that fulfill the consumers' and industrial requirements.

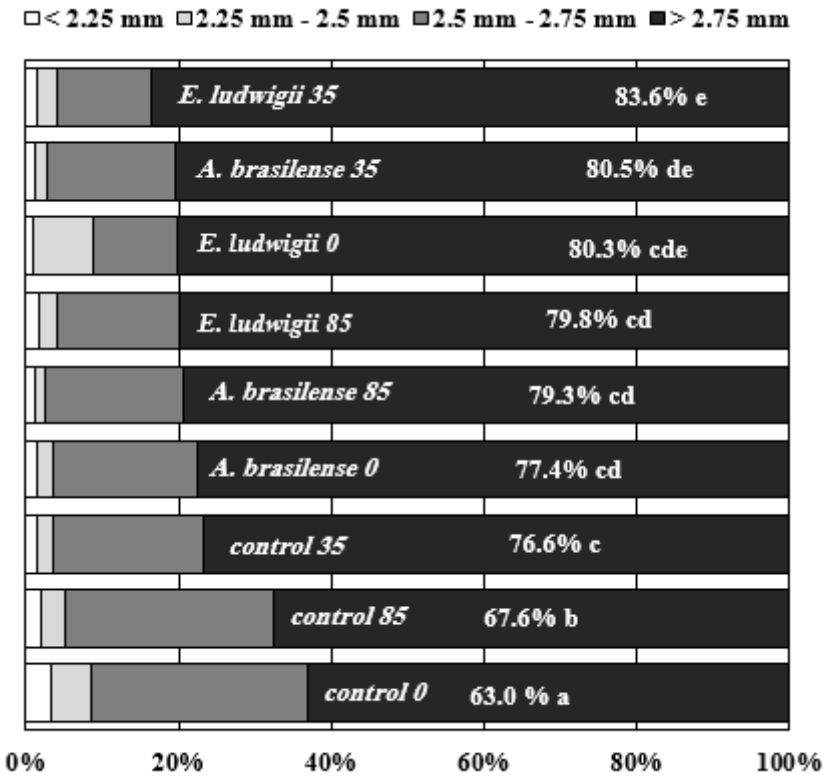

Figure 3: Grain size in *Hordeum vulgare* inoculated with PGPR and fertilized with different Pi doses in field experiment.

C control plants, Ab inoculated with *A. brasilense*, El plants inoculated with *E. ludwigii*.

3.6 P content in whole plants

To evaluate whether the promotion of plant growth was due to a greater supply of P to plants, P content was

measured in barley crop. Ours results showed that the differences in P content in *E. ludwigii* -inoculated plants were significantly higher.

Table 3: Phosphorus content of *Hordeum vulgare*

Treatment	P content in plants (mg P. DW plant^{-1})	P exported to grain (gr P.ha^{-1})
	60 DAS	**120 DAS**
C^{0}	2.93 ± 0.08 ^{ab}	5.90 ± 0.10 ^{a}
C^{35}	4.00 ± 0.05 ^{cd}	6.85 ± 0.05 ^{ab}
C^{85}	2.83 ± 0.32 ^{ab}	6.65 ± 0.15 ^{ab}
Ab^{0}	2.62 ± 0.04 ^{a}	8.45 ± 0.15 ^{d}
Ab^{35}	3.66 ± 0.09 ^{bcd}	7.25 ± 0.25 ^{bc}
Ab^{85}	3.29 ± 0.07 ^{abc}	7.90 ± 0.10 ^{cd}
El^{0}	4.58 ± 0.78 ^{b}	6.20 ± 0.20 ^{a}
El^{35}	3.74 ± 0.08 ^{cd}	9.50 ± 0.20 ^{e}
El^{85}	3.92 ± 0.04 ^{d}	10.95 ± 0.85 ^{f}

Regarding the P content in barley plants at 0 kg/ha of the chemical fertilizer, the *E. ludwigii*-inoculated plants reached a 56% increase in P in the whole plant, at 60 DAS (Table 3). The suboptimal concentration of DAP (35 kg/ha) achieved the same effect as the doses usually used by farmers (85 kg/ha) (Table 3). This response in plant P content in *E. ludwigii*-inoculated plants was higher at all DAP dose evaluated.

3.7 Endophytism

As described in the materials and methods section, to assess the presence of the bacteria in the plant tissues, at 60 DAS, the roots of barley were recovered and the PGPRs were isolated from disinfected roots of inoculated plants. Table 4 summarizes the number of bacteria recorded in dry roots.

Table 4: Bacterial content in *Hordeum vulgare* roots at 60 DAS growing at different DAP level.

Treatment	CFU/g root dry weight DAP (kg.ha^{-1})		
	0	**35**	**85**
A. brasilense	6.34 x 10^{3}	7.15 x 10^{3}	3.23 x 10^{4}
E. ludwigii	1.79 x 10^{4}	1.10 x 10^{4}	1.15 x 10^{4}

We isolated the inoculated bacteria present in the barley tissues at 60 DAS. Table 4 shows the number of CFU present in the dry roots at the different Pi doses used. We found an efficient establishment of *E. ludwigii* as an endophyte, reaching populations with values of 1.79 × 10^{4} CFU/g in the barley roots, thus evidencing the ability

of this microorganism to colonize the internal tissues of the barley plant and to remain stable throughout the experiment. Thereby, these were able to colonize and persist inside tissues until 60 DAS.

The non-inoculated controls showed no growth of microorganisms when macerated barley roots were plated on Congo red medium.

4. Discussion

Rhizobacteria have growth-promoting abilities, which have been correlated with several mechanisms that have been extensively reviewed. Among these bacteria, the genus *Azospirillum* is the most extensively studied and a high number of reports have shown the successful use of this bacterium to improve growth, development, and yield of crops throughout the world [5; 25]. However, the genus *Enterobacter* is one of the most common genera of bacteria isolated from the plant endorhizosphere of crops such as maize, rice, cotton, cucumber, common bean, broccoli and sweet potato [27, 3, 13]. Some of the *E. ludwigii* strains isolated earlier have been characterized as human pathogens [28]. However, more recently reported strains have been isolated from environmental sources and have been studied for their various beneficial activities. All these reports have improved the knowledge of *E. ludwigii*, but few have studied this bacterium as a P-solubilizing organism. The authors in [29] found that both *E. ludwigii* and *E. hormaechei* increase the available P content in the bacterial culture medium, being *E. ludwigii* better than *E. hormaechei*, but did not perform in vivo experiments. Based on the above, the aim of the present study was to investigate the role of *E. ludwigii* in the growth promotion and contribution to the P content in *Hordeum vulgare* (barley) in field experiments. Previously, we visually detected and semiquantitatively estimated the phosphate-solubilizing ability of this bacterium by using plate screening methods [11]. These methods can be regarded as generally reliable for isolation and preliminary characterization of phosphate-solubilizing microorganisms. However, as suggested by other researchers, the ability to solubilize P is not necessarily correlated with the higher P content in plant and with the ability to promote plant growth [30], because the promotion of growth, even by PSB, can be the outcome of other mechanisms, as commented earlier in [31]. In our study, we first characterized *E. ludwigii* BNM0357 as PGPR with a plethora of benefits but not as PSB and did not study its direct contribution to P plant nutrition in winter grasses. Thus, to access the P release capacity of *E. ludwigii* and that of soluble and insoluble P fertilizers, we conducted a full crop cycle barley study under field experiments. This allowed us to establish, beyond doubt, a positive role of this bacterium in the nutrition and growth of barley (Table 1). *A. brasilense*, on the other hand, produces plant growth-promoting substances, which may have also contributed to the increased growth observed, but lacks the ability to solubilize phosphate in vitro and in vivo [10]. Thus, we attributed the increased grass biomass and barley yield observed in plants inoculated with *A. brasilense* to the nitrogen fixation and enhanced uptake of $N0_3^-$ and Pi due the greater development of the root surface [32; 33]. Enterobacter has the same or even better attributes than *A. brasilense* and is also a PSB, which may have led to the increased phosphate availability found in PSB-inoculated soils. The release of P from DAP was investigated in barley field experiments that involved the same combinations between PGPR and barley. The efficiency of DAP in promoting growth and yield characteristics of barley was significantly lower in control plants than in inoculated ones for most of the parameters studied. The P release capacity of the PSB studied was evident for *E. ludwigii* (Table 1) and P accumulation in plants inoculated with this bacterium was more significant. Our results showed that the

application of DAP, which is commonly used in crops at the higher dose (85 kg/ha), had a lower effect on aerial biomass development, grain/ear, one thousand weight, yield, P content in plants, and P exported to grains than the lower P dose in combination with the PSB *E. ludwigii.* A further increase in the grain and dry matter yield and nutrient uptake in barley due to the combined inoculation and DAP fertilization over the inoculation without DAP seems quite obvious as the plants are well supplied with P, thus resulting in better growth and yield of barley plants. Seed bacterization with this PGPR along with 35 kg DAP/ha produced a yield almost similar to that produced by 85 kg DAP/ha in barley, indicating a net saving of 50 kg DAP/ha due to the bacterial inoculation (Figure 3). In [34] the authors also reported an increase in the grain yield of wheat when rock phosphate was applied to the soil and seeds were inoculated with *Pseudomonas*. In this case, the response of the crop to the bacterial inoculation was equivalent to 50 kg P_20_5/ha as superphosphate. Grain size is a term used to describe a morphological character of barley grain. This parameter was positively affected by the inoculation treatment. More than 80% of the grains of the barley plants inoculated with the PGPRs studied had a size grain larger than 2.75 mm. This is an important characteristic, especially considering that a large, plump and uniform grain size is required by various international grain handling, malting and brewing industries. The introduced bacteria were able to actively colonize the roots of the barley. The data summarized in Tables 4 demonstrate that bacteria were present in natural soil for up to 60 days, and that the fertilizer treatment significantly increased the number of bacteria in this grass inoculated with *E. ludwigii*. Our experimental data indicate that the inoculation with the N_2-fixing and P-solubilizing *E. ludwigii* bacterium increased with higher Pi fertilization. This beneficial effect of the fertilizer on the microbial population may have been due to an increase in the supply of available P and, considering that endophytic colonization allows the bacterium to establish a more direct interaction with the plant, it may have facilitated an efficient transfer of nutrients [35]. The favorable effect of the bacterial inoculation on plant growth, especially with reference to the increase in root biomass, P content, and bacterial count, may have been due to the production of growth-promoting substances by this PGPR as was previously showed by ours in [11]. *E. ludwigii* improved root growth and morphology and increased root surface (Zaballa, personal communication), and this can influence the density of microorganisms. Comparatively, the number of bacteria was lower under field conditions than under greenhouse conditions because these must compete with other bacteria commonly established in the rhizosphere. After seed inoculation, *E. ludwigii* was able to efficiently colonize internal root tissues of barley plants. *E. ludwigii* was isolated from *Lolium perenne* and to date, there are no data on the interaction of *E. ludwigii* with barley plants. Although certain publications have described associations of barley with different PGPR [36,37,38,39,40] to our knowledge, the present report is the first involving barley colonized by *E. ludwigii.* These results show a favorable plant response to inoculation with this P-solubilizing rhizobacterium in soils with low or intermediate phosphate availability and support the idea that a better understanding of the bacterial mechanisms that lead to P availability will open the way to increased P use efficiency by crops. The use of biofertilizers possessing bacteria with P-solubilizing activities in agricultural soils is considered a biotechnological environmentally friendly alternative to the use of further applications of chemical-based P fertilizers. Characterizing and developing innovative rhizobacterial formulations may help in the selection of potential candidates as biofertilizers, and the combination of PSB with an intermediate dose of DAP or a lower dose of Ca_3PO_4 holds a lot of promise as an efficient alternative to the use of conventional P fertilizers, especially regarding its effectiveness in the utilization of insoluble Pi.

4.1 Conclusions

Enterobacter ludwigii promoted plant growth and P uptake in barley. These results indicate that the application of *E. ludwigii* in the absence of any chemical phosphorus fertilizer has an appropriate performance, increasing the crop production to an acceptable level. Thus, this bacterium could be considered as a suitable substitute or supplement of chemical phosphorous fertilizers in agricultural systems in both normal and poor soils, and has great potential to be developed as a bio-fertilizer which could enhance soil fertility, minimizing chemical fertilization.

4.2 Recommendations

1. The *Enterobacter ludwigii* application over barley plants showed favorable responses in all parameters registered and should be evaluated over others crops and under different soils conditions.

2. The use of *Enterobacter ludwigii* as biofertilizer should be considered as a useful option to decrease or even to eliminate the chemical fertilization over barley plant.

3. In order to add knowledge about the effect of *E. ludwigii* inoculation on barley seed physiology and development would be of great importance to evaluate possible changes in seed chemical composition such as protein content that results required by various brewing industries.

Ours results have shown that interaction between *E. ludwigii* and barley plants has been positive and stable but it was limited to cereal plants (as even we reported in [8,10,11]). In different plant species, such as horticultural species, the effect of this bacterium as a P-solubilizing organism could give different results in the available P content to plants.

References

[1] A. Abril, L. Roca. “Impact of nitrogen fertilization on soil and aquifers in the Humid Pampa, Argentina”. The Open Agriculture Journal, vol. 2, pp 22-27, May. 2008.

[2] D.M. Sylvia, P.G Hartel, J. Furhmann, D. Zuberer. “Principles and applications of soil microbiology”. 2nd Edn., Upper Saddle River, New Jersey, Prentice Hall Inc Jul, 2004, pp 672.

[3] S. Mundra, R. Arora, T. Stobdan. “Solubilization of insoluble inorganic phosphates by a novel temperature, pH, and salt tolerant yeast, Rhodotorula sp. PS4, isolated from seabuckthorn rhizosphere, growing in cold desert of Ladakh, India”. World J. Microbiol. Technol., vol. 27 pp 2387-2396, Oct. 2011.

[4] S. Compant, C. Clément, A. Sessitscha. “Plant growth-promoting bacteria in the rhizo-and endosphere of plants: role, colonization, mechanisms involved and prospects for utilization”. Soil Biol Biochem, vol. 42 pp 669-678, Nov. 2010.

[5] S. Gouda, R.G. Kerry, G. Das, S. Paramithiotis, H.S. Shin, J.K. Patra. “Revitalization of plant growth promoting rhizobacteria for sustainable development in agriculture”. Microbiol Res, vol. 206 pp 131-140, Jan. 2018.

[6] G. Santoyo, G. Moreno-Hagelsiebb, M.C. Orozco-Mosqueda, B. Glick. "Plant growth-promoting bacterial endophytes". Microbiological Research, vol. 183 pp 92–99, Feb. 2016.

[7] M. Bucher. "Functional biology of plant phosphate uptake at root and mycorrhiza interface". New Phytol., vol. 173 pp 11-26, Feb. 2007.

[8] M. Schoebitz, C. Ceballos, L. Ciampi. "Effect of immobilized phosphate solubilizing bacteria on wheat growth and phosphate uptake". J. Soil Sci. Plant Nutr., vol. 13 pp 1-10, Mar. 2013.

[9] H.A. Alikhani, N. Saleh-Rastin, H. Antoun. "Phosphate solubilization of rhizobia native to Iranian soils". P. Soil, vol. 287 pp 35-41, May 2006.

[10] M. Nico, C.M. Ribaudo, J.I. Gori, M.L Cantore, J.A Curá. "Uptake of phosphate and promotion of vegetative growth in glucose-exuding rice plants (Oryza sativa) inoculated with plant growth-promoting bacteria". Applied soil ecology, vol. 61 pp 190-195, Oct. 2011.

[11] M. Schoebitz, C.M. Ribaudo, M. Pardo, M.L Cantore, L. Ciampi, J. A. Cura. "Plant growth promoting properties of a strain of Enterobacter ludwigii isolated from Lolium perenne rhizosphere". Soil Biol Biochem. vol. 41, pp 1768-1774, Dec. 2007.

[12] Y.E. Morales-García, D.A. Juárez-Hernández C. Aragón-Hernández, M.A. Mascarua-Esparza M.R., Bustillos-Cristales, L.E. Fuentes-Ramírez, R.D. Martínez Contreras, J. Muñoz-Rojas, "Growth Response of Maize Plantlets Inoculated with Enterobacter sp., as a Model for Alternative Agriculture". Rev Argent Microbiol., vol. 43 pp 287-293, Dec. 2011.

[13] F. Ogbo, J. Okonkwo, "Some Characteristics of a Plant Growth Promoting Enterobacter sp. Isolated from the roots of maize" Adv Microbiol., vol, 2 pp 368-374, Aug. 2012.

[14] Q. Pan, I. Shikano, K. Hoover, T-X Liu, G.W. Felton. "Enterobacter ludwigii, isolated from the gut microbiota of Helicoverpa zea, promotes tomato plant growth and yield without compromising anti-herbivore defenses." Arthropod-Plant Interactions, vol. 13, pp. 271-278, Apr. 2019.

[15] K.K.I.U. Arunakumara, B.Ch. Walpola, J.-S. Song, M.J. Shin, Ch-J. Lee, M-H. Yoon. "Phytoextraction of Heavy Metals Induced by Bioaugmentation of a Phosphate Solubilizing Bacterium. Korean" J. Environ. Agriculture, vol. 33 pp 220-230, Jun. 2014.

[16] E. Egidi, J.L. Wood, S. Aracic, R. Kannan, L. McDonald, C.A Bell, E.M. Fox, W. Liu, A.E Franks. "Draft Genome Sequence of Enterobacter ludwigii NCR3, a Heavy Metal-Resistant Rhizobacterium". Genome Announc., vol. 6 pp 4, Oct. 2016.

[17] O. Psakia, S. Mainaa, A. Vlysidisb, S. Papanikolaoua, A. Machado de Castro, D.M.G. Freired, E. Dheskalie, I. Kookose, A. Koutinasa. "Optimisation of 2,3-butanediol production by Enterobacter ludwigii using sugarcane molasses" Biochemical Engineering Journal, vol. 152, Dec. 2019.

[18] J. Döbereiner. "Isolation and identification of aerobic nitrogen fixing bacteria from soil and plants" In Methods in Applied Soil microbiology and Biochemistry. K Alef and P Nannipieri (eds.) Academic Press. Harcourt Brace y Company Publishers. London. pp. 134-141, 1995.

[19] A. Walkley, I.A. Black. "An examination of the Degtjareff method for determining soil organic matter, and a proposed modification of the chromic acid titration method". Soil Sci, vol. 37, pp 29–38, Jan. 1934.

[20] R.H. Bray, L.T. Kurtz. "Determination of total, organic, and available forms of phosphorus in soils". Soil Sci., vol.59, pp 39-45, Jan. 1945.

[21] A.J, Pieters, M.J., Paul, D.W. Lawlor. "Low sink demand limits photosynthesis under Pi deficiency". J Exp Bot, vol. 52, pp 1083-1091, May. 2001.

[22] E. Rodríguez-Cáceres. "Improved medium for isolation of Azospirillum spp". Appl. Environ. Microbiol., vol.44 pp, 990-991, Oct. 1982.

[23] W. Jarecki, J. Buczek, D. Bobrecka-Jamro. "Response of spring wheat to different soil and foliar fertilization". Journal of Central European Agriculture, vol. 18, pp. 460-476, Jun. 2017.

[24] G. Kalayu. "Phosphate Solubilizing Microorganisms: Promising Approach as Biofertilizers" International Journal of Agronomy, vol. 2019, pps 7, May 2019.

[25] A.L. Oliveira, O.J. Santos, P.R. Marcelino, K.M. Milani, M.Y. Zuluaga, C. Zucareli, L.S Gonçalves. "Maize Inoculation with Azospirillum brasilense Ab-V5 cells enriched with exopolysaccharides and polyhydroxybutyrate results in high productivity under low N fertilizer input". Front Microbiol., vol. 8 pp, 1873, Sept. 2017.

[26] P. Magliano, P. Prystupa, F. Gutiérrez-Boem. "Protein content of grains of different size fractions in malting barley". J. Inst. Brew. Vol. 120, pp 347-352, Sept. 2014.

[27] M. Zakria, A. Ohsako, Y. Saeki, A. Yamamoto, S. Akao. "Colonization and Growth Promotion Characteristics of Enterobacter sp. and Herbaspirillum sp. on Brassica oleracea". Soil Sc. Plant Nutr., vol. 54, pp 507-516, Jul. 2008.

[28] G. Li, Z. Hu, P. Zeng, B. Zhu, L.Wu. "Whole genome sequence of Enterobacter ludwigii type strain EN-119T, isolated from clinical specimens". FEMS Microbiol. Let. vol., 362, pp 1-3, Feb. 2015.

[29] B.C. Walpola, K.K.I.U Arunakumara. "Assement of phosphate solubilization and indole acetic acid production in plant growth promoting bacteria isolated from greenhouse soils of Gonju-Gun, South Korea". Tropical Agricultural Research and Extension, vol. 18 pp. 31–39, Nov. 2016.

[30] M.M. Collavino, P.A. Sansberro, L.A. Mroginski, O.M. "Comparison of in vitro solubilization activity of diverse phosphate-solubilizing bacteria native to acid soil and their ability to promote Phaseolus vulgaris growth". Biol Fertil Soils, vol. 46 pp. 727-738, Jul. 2010.

[31] Y. Bashan, A. Kamnev, L.E Bashan. "Tricalcium phosphate is inappropriate as a universal selection factor for isolating and testing phosphate-solubilizing bacteria that enhance plant growth: a proposal for an alternative procedure". Biol Fertil Soils, vol. 49, pp. 465-479, Jul. 2013.

[32] C.M. Ribaudo, A.N. Paccusse, J.A. Curá, A.A. Fraschina. "Azospirillum-maize association: effects on yield dry matter content and the nitrate reductase activity". Agricultura Tropica et Subtropica, vol. 31, pp. 61-70, May 1998.

[33] C.M. Ribaudo, E. M. Krumpholz, F.D. Cassán, R. Bottini, M L. Cantore, JA Curá. "Azospirillum sp. Promotes Root Hair Development in Tomato Plants through a Mechanism that Involves Ethylene". J Plant Growth Regul., vol. 25, pp. 175–185, Jun. 2006.

[34] B.S. Kundu, A.C Gaur."Establishment of nitrogen fixing and phosphate dissolving bacteria in rhizosphere and their effect on yield and nutrient uptake of wheat crop". Pl. Soil, vol. 57, pp 223–230, Jun. 1980.

[35] R.P. Ryan, K. Germaine, A. Franks, D.J. Ryan, D.N Dowling."Bacterial endophytes: recent developments and applications". FEMS Microbiol Lett., vol. 278, pp. 1–9, Jan. 2008.

[36] M.Y. Canbolat, S. Bilen, R, Çakmak, F. Şahin, A. Aydın. "Effect of plant growth-promoting bacteria

and soil compaction on barley seedling growth, nutrient uptake, soil properties and rhizosphere microflora". Biol Fertil Soils., vol. 42, pp. 350–357, Aug. 2005.

[37] P. K. Chang, K.E. Gerhardt, X.D Huang, X.M Yu, B.R. Glick, P.D, Gerwing, B.M. Greenberg. "Plant growth-promoting bacteria facilitate the growth of barley and oats in salt-impacted soil: implications for phytoremediation of saline soils". Int J Phytoremediation, vol. 16, pp. 1133-1147, Feb. 2014.

[38] A.R.S. Santos, R.M. Etto, R.W. Furmam, D.L. Freitas, K.F.D.N. Santos, E.M. Souza, F.O. Pedrosa, R.A. Ayub, M.B.R. Steffens, C.W. Galvão. "Labeled Azospirillum brasilense wild type and excretion-ammonium strains in association with barley roots". Plant Physiol Biochem., vol. 118 pp. 422-426, Sept. 2017.

[39] M. Cardinale, S. Ratering, C. Suarez, A.M. Zapata Montoya, R. Geissler-Plaum, S. Schnell. "Paradox of plant growth promotion potential of rhizobacteria and their actual promotion effect on growth of barley (Hordeum vulgare L.) under salt stress". Microbiol Res., vol. 181 pp. 22-32, Dec. 2015.

[40] M.M. Rahman, E. Flory, H.W. Koyro, Z. Abideen, A. Schikora, C. Suarez, S. Schnell, M. Cardinale. "Consistent associations with beneficial bacteria in the seed endosphere of barley (Hordeum vulgare L.)". Syst Appl Microbiol., vol. 41 pp. 386-398, Jul. 2018.

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