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article

Screening of plant growth promoting bacteria associated with barley plants (*Hordeum vulgare* L.) cultivated in South Brazil

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Abstract: The occurrence of associations between bacteria and plant roots may be beneficial, neutral or detrimental. Plant growth promoting (PGP) bacteria form a heterogeneous group of beneficial microorganisms that can be found in the rhizosphere, the root surfaces or in association with host plant. The aim of this study was to isolate and characterize PGP bacteria associated to barley plants (*Hordeum vulgare* L.) aiming a future application as agricultural inoculant. One hundred and sixty bacterial strains were isolated from roots or rhizospheric soil of barley based on their growth in nitrogen-free selective media. They were evaluated for their ability to produce indolic compounds (ICs) and siderophores, and to solubilize tricalcium phosphate in *in vitro* assays. Most of them (74%) were able to synthesize ICs in the presence of the precursor L-tryptophan, while 57% of the isolates produced siderophores in Fe-limited liquid medium, and 17% were able to solubilize tricalcium phosphate. Thirty-two isolates possessing different PGP characteristics were identified by partial sequencing of their 16S rRNA gene. Strains belonging to *Cedecea* and *Microbacterium* genera promoted the growth of barley plants in insoluble phosphate conditions, indicating that these bacteria could be used as bioinoculants contributing to decrease the amount of fertilizers applied in barley crops.
Keywords: *bacterial 16S rRNA gene, inoculant, barley, phosphate solubilization.*

PONTES, A.P., SOUZA, R., GRANADA, C.E., PASSAGLIA, L.M.P. **Seleção de bactérias promotoras de crescimento vegetal associadas com plantas de cevada (*Hordeum vulgare* L.) cultivadas no Sul do Brasil.** Biota Neotropica. 15(2): e20140105. http://dx.doi.org/10.1590/1676-06032015010514

Resumo: A ocorrência de associações entre bactérias e raízes de plantas pode ser benéfica, neutra ou prejudicial. Bactérias promotoras de crescimento vegetal (BPCV) formam um grupo heterogêneo de micro-organismos benéficos que pode ser encontrado na rizosfera, superfícies de raízes ou em associação com plantas hospedeiras. O objetivo deste estudo foi isolar e caracterizar bactérias promotoras do crescimento vegetal (PCV) associadas a plantas de cevada (*Hordeum vulgare* L.), visando uma futura aplicação como inoculante agrícola. Cento e sessenta linhagens bacterianas foram isoladas a partir de raízes ou solo rizosférico de cevada com base na sua multiplicação em meios seletivos sem nitrogênio. Todos os isolados foram avaliados quanto à sua capacidade de produzir compostos indólicos (CIs), sideróforos e solubilizar fosfato tricálcio, em ensaios *in vitro*. A maioria dos isolados (74%) foi capaz de sintetizar CIs na presença do precursor L-triptofano, enquanto que 57% produziram sideróforos em meio líquido com deficiência de Fe e 17% foram capazes de solubilizar fosfato tricálcio. Trinta e dois isolados que apresentaram diferentes características PCV foram identificados pelo sequenciamento parcial do gene 16S rRNA. Linhagens pertencentes aos gêneros *Cedecea* e *Microbacterium* promoveram o crescimento de plantas de cevada em condições de fosfato insolúvel, indicando que estas bactérias podem ser utilizadas como inoculantes, contribuindo para a redução da quantidade de fertilizantes aplicados no cultivo da cevada.
Palavras-chave: *gene bacteriano 16S rRNA, inoculante, cevada, solubilização de fosfato.*

1. Introduction

The management of microbial soil-plant interactions has emerged as a power agricultural tool evidenced by an increase in crop productivity, by the reduction of production costs through the reduction of the amount of chemical fertilizers and by a better conservation of environmental resources (Shridhar 2012). One of the most promising techniques to achieve these benefits is the use of bioinoculants- also called biofertilizers, which are composed by beneficial bacteria.

The plant rhizosphere can be defined as the region of the soil where the processes mediated by microorganisms are specifically influenced by the root system (Gray & Smith 2005). Root-colonizing plant beneficial bacteria, commonly referred to as plant growth-promoting bacteria (PGPB), are rhizospheric bacteria that can enhance plant growth using a wide variety of direct and indirect mechanisms (Glick 2012). The favorable effects of PGPB inoculation on plant growth have been widely studied (Ambrosini et al. 2012; Glick 2012; Granada et al. 2013; Souza et al. 2013).

PGPBs are able to accelerate seed germination, improve seedling emergence, protect plants from disease, and promote root growth (Lugtenberg et al. 2002). PGPB can also exert biocontrol of pathogenic fungi through the production of antibiotics, competition for nutrients or by the induction of systemic resistance (Glick 2012). Moreover, such bacteria may improve the nutritional status of plants through biological nitrogen fixation (Saikia & Jain 2007), phosphate solubilization through the production of organic acids and phosphatases (Chen et al. 2006; Rodríguez et al. 2006), and siderophores production (Lemanceau et al. 2009). In addition, the hormonal effects that occurred when PGPB produce chemical compounds, such as auxins, cytokinins, and gibberellins, directly impact the plant growth by stimulating the uptake of nutrients (Vessey 2003; Jha & Saraf 2012). Thus, PGPB are able to use different pathways to promote plant growth at various stages during the plant life cycle. The manner in which each PGPB can influence plant growth differs from species to species as well as by strain (Glick et al. 1999).

Barley (*Hordeum vulgare* L.) is a fast growing annual grain crop that could be used as forage or cover crop to improve soil fertility (Ghanbari et al. 2012). Moreover, it is often grown for many purposes, including as a source of protein for animal or human consumption as well as for malting. This study was undertaken in order to: (i) isolate and identify putative PGPB associated with rhizospheric soil and roots of barley plants cropped in different areas of southern Brazil; (ii) evaluate several plant growth promotion (PGP) activities of the bacterial isolates; (iii) and test their PGP abilities to promote plant growth in a growth chamber experiment.

2. Materials and Methods

2.1. Sampling and isolation of putative plant growth promoting bacteria

Samples were collected from three different barley producing regions in the state of Rio Grande do Sul (RS), Brazil: Júlio de Castilhos (JC; 29° 13' 37'' S, 53° 40' 54'' W), São Borja (SB; 28°39' 39'' S, 56° 00' 14'' W), and Vacaria (VA; 28°30' 43'' S, 50° 56' 02'' W).

Rhizospheric and root-associated bacteria were isolated from five independent plants collected from each sampling region with adhering (rhizospheric) soil that were spaced at least 2 m away from each other. Samples were randomly taken and bulked to obtain a representative composite sample. To isolate root-associated bacteria, root samples were first sterilized by surface disinfection performed by washing the roots in running tap water, followed by a 70% ethanol wash for 1 min, a sodium hypochlorite solution (4%, v/v) wash for 2 min, and five serial rinses in sterilized distilled water. After disinfection, 10 g of roots from each sampling region was sliced with a sterile scalpel and placed into 250 ml Erlenmeyer flasks containing 90 ml of sterile saline solution (0.85% NaCl). Rhizospheric bacteria were isolated from 10 g of rhizospheric soil from each sampling region that was also placed in 250 ml Erlenmeyer flasks containing 90 ml of sterile saline solution. Both rhizospheric soil and sliced roots samples were incubated at 4°C with agitation (125 rpm) for 4-6 h.

Putative diazotrophic bacteria were isolated according to Döbereiner (1988), using the nitrogen-free semi-solid NFb, LGI, and LGI-P media, with the modifications described in Ambrosini et al. (2012) and Souza et al. (2013). After incubation, distinct colonies were randomly selected and grown in liquid LB medium (Sambrook & Russel 2001) at 28°C under agitation (200 rpm). From each sampled region, 30 colonies of root-associated bacteria and 30 colonies of rhizospheric soil bacteria (totaling approximately 60 colonies from each region) were isolated. These bacterial isolates were individually analyzed by Gram-staining and immediately stored in sterile glycerol solution (50%) at -20°C.

2.2. Evaluation of the characteristics that promote plant growth

The putative PGP capacity of the bacterial isolates was evaluated by *in vitro* tests. Bacterial suspensions (10 µl of 10^8 CFU ml^{-1}) of each isolate grown in LB medium at 28°C with agitation (125 rpm) for 48 h were used as inocula for the PGP experiments. Analysis of production of indolic compounds (ICs) and siderophores, and tricalcium phosphate solubilization activities were carried out for all bacterial isolates.

The *in vitro* ICs production assay was performed in King B medium with tryptophan (Glickmann & Dessaux 1995) by incubation the isolates at 28°C under agitation (200 rpm) for 72 h. As described in Ambrosini et al. (2012) the supernatant (500 µl) was mixed with an equal volume of Salkowski reagent (12 g l^{-1} $FeCl_3$ + 7.9 M H_2SO_4) in test tubes, and the mixture was kept in the dark for 30 min to allow for color development. The pink to red color produced after exposure to Salkowski reagent was considered to be indicative of bacterial production of ICs. The samples were measured spectrophotometrically at 550 nm using a standard curve for calibration.

Siderophores production was assayed according to Schwyn & Neilands (1987) using King B medium (Glickmann & Dessaux 1995) without tryptophan. The isolates were spot inoculated onto Chrome azurol S agar plates and incubated at 28°C for 48-72 h. Development of a yellow, orange or violet halo around the bacterial colony was considered to be positive for siderophores production.

To identify isolates able to solubilize tricalcium phosphate bacteria were grown in glucose yeast medium (GY). Two other solutions were prepared separately, one containing 5 g K_2HPO_4 in 50 ml of distilled water, and the other containing 10 g $CaCl_2$ in 100 ml of distilled water. These solutions were added to one liter of GY medium just before pouring onto Petri dishes, and together they formed an insoluble layer of calcium phosphate that made the medium opaque. The plates were inoculated with the bacterial isolates, and then incubated for seven days at 28°C. Those isolates that formed visibly clear halos around their colonies were considered to be tricalcium phosphate solubilizers.

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2.3. Extraction of bacterial DNA, PCR amplification and partial sequencing of the 16S rRNA gene

Bacterial DNA extraction was performed according to Ambrosini et al. (2012). Phenol-chloroform extraction and ethanol precipitation were performed as described by Sambrook & Russel (2001). The quality and integrity of the DNA were determined by electrophoresis in 0.8% agarose gels containing ethidium bromide and visualized under UV light. Fifty nanograms of bacterial DNA were used as a template for PCR procedures.

Partial sequences of the 16S rRNA gene (roughly 450 bp) from each isolate were amplified using the primers U968 (AACGCGAAGAACCTTAC) and L1401 (CGGTGTGTA CAAGACCC) (Felske et al. 1997), which cover the region between nucleotides 968 and 1401 of the *Escherichia coli* 16S rRNA gene, and using the conditions described in Souza et al. (2013). Thermal cycling was performed according to Garbeva et al. (2003). PCR products were analyzed by electrophoresis in 1% agarose gels containing ethidium bromide and visualized under UV light.

Sequences were trimmed to exclude low quality sequenced nucleotides. DNA sequences were compared with sequences from the EzTaxon Server version 2.1 (http://eztaxon-e.ezbiocloud.net/) and the GenBank using BLASTN software (http://blast.ncbi.nlm.nih.gov/). The nucleotide sequences of the 32 partial 16S rRNA gene segments determined in this study have been deposited in GenBank (accession numbers KM068182 to KM068213).

2.4. Growth chamber assay

Bacterial isolates demonstrating different PGP characteristics were tested in experiments with barley (*Hordeum vulgare* L.) in a growth chamber. The growth chamber experiment was conducted with a photoperiod cycle of 14 h light at 28°C and 10 h dark at 20°C. The experimental units consisted of pots (15 X 20 cm) sterilized with 0.7% sodium hypochlorite solution before seeding. Barley seeds were surface-disinfected as described by Souza et al. (2013) and were planted in sterile vermiculite, 2 cm below the surface. Three bacterial isolates (JC57, SB41, and VA7) were grown in LB medium with agitation (125 rpm) for 48 h at 28°C. Pure bacterial cultures were centrifuged and diluted to a final concentration of 10^9 CFU ml^{-1} in sterile saline solution. Seeds were inoculated with 5 ml aliquots of the cell suspensions by direct irrigation of the substrate. The treatments were as follows: seeds inoculated with JC57, SB41 or VA7 isolates and a non-inoculated control. The experiment consisted of five replicates per treatment and a completely randomized design. A 50 ml volume of Hoagland's nutrient solution (Hoagland & Snyder 1933) diluted to 25% was added to each pot every 15 days. All treatments were divided into two conditions: one received soluble phosphate (complete Hoagland's nutrient solution), while the other received insoluble phosphate (Hoagland's nutrient solution without KH_2PO_4 but supplemented with 0.2 g of rock phosphate per pot in total). The experiment was maintained for 40 days, after which plants were harvested and length data were recorded. Shoots and roots were dried at 65°C to constant weight to evaluate dry matter.

Data from the pot trials were statistically analyzed using ANOVA, and the means were compared using Tukey test ($p = 0.05\%$). Homoscedasticity was verified using Levene's test and normality by histogram analysis.

3. Results and Discussion

3.1. Isolation, screening of plant growth-promoting (PGP) traits, and identification of bacterial isolates

In this work, bacteria possessing different PGP characteristics were isolated from rhizospheric soil and roots of barley plants collected from three different barley-producing regions of the state of Rio Grande do Sul, Brazil. In total, 160 bacterial strains were selectively isolated based on their growth in three selective semi-solid nitrogen free media, NFb, LGI, and LGI-P. These selective media were used as a discriminating strategy to select putative nitrogen-fixing and plant growth-promoting rhizobacteria. After the isolation, the production of ICs and siderophores and the ability to solubilize tricalcium phosphate were analyzed for the 160 bacterial strains (Table 1).

According to Table 1, one of the most evident characteristic among the isolates was their ability to produce ICs: 118 (74%) isolates were able to synthesize ICs in the presence of the precursor L-tryptophan, which can act as a phytohormone. Of these, four bacterial isolates produced among 51-100 µg of ICs ml^{-1} after 72 h of incubation. Our results agreed with those of Ahmad et al. (2006), who also reported that ICs production was the most prevalent plant growth promoting characteristic in the majority of their isolates. High numbers of ICs-producing bacteria have also been documented by other studies (Ambrosini et al. 2012; Costa et al. 2013; Granada et al. 2013; Souza et al. 2013).

Table 1. Number of isolates, siderophores production, tricalcium phosphate solubilization, and indolic compound (ICs) production by bacterial isolates at each sampling site.

Site		Number of isolates	Siderophores production	Phosphate solubilization	ICs production (µg ml^{-1})		
					0.1-50	51-100	>100
São Borja	Root	27	20	8	19	1	1
	Soil	28	12	10	14	1	0
Vacaria	Root	26	16	0	20	2	0
	Soil	28	11	3	26	0	0
Júlio de Castilhos	Root	27	15	4	21	0	0
	Soil	24	17	3	13	0	0
Total		160	91	28	113	4	1

Table 2. Identification and PGP attributes of selected isolates.

Isolates[a]	16S rRNA gene sequence[b]	Phosphate solubilization	Siderophores production	ICs production (μg ml^{-1})
SB41	*Cedecea* sp. (100%)	+	+	55
VA7	*Microbacterium* sp. (99%)	-	-	84
JC57	*Ochrobactrum* sp. (100%)	-	+	12

[a]Bacteria isolated from: SB41 (São Borja); VA7(Vacaria); JC57 (Júlio de Castilhos).
[b]Identities are based on comparison with the GenBank database using the BLASTN program.

Among the phytohormones, the auxin (indole-3-acetic acid, IAA) is widely distributed among bacteria associated with plants (Spaepen et al. 2007), and approximately 80% of bacterial isolates from the rhizosphere are capable of produce IAA (Patten & Glick 2002).

Another ability displayed by most of the isolates was siderophores production (91 out of 160; Table 1). Moreover, the barley roots sampled from São Borja locality showed the highest number of siderophores-producing strains (22%) when compared to the other regions. Many microorganisms can produce siderophores which are involved in the sequestration of iron as Fe^{3+} that displays low solubility in aerobic conditions (Masalha et al. 2000). Souza et al. (2013), for example, evaluating the diversity of cultivable siderophores-producing bacteria associate to rice (*Oryza sativa* L.) found that 84% of the 336 isolates examined produced siderophores in Fe-limited liquid medium. According to Wei Jin et al. (2010) plants submitted to iron deficiency may alter the community of associated siderophores-producing bacteria. These authors submitted plants of clover (*Trifolium pratense*) to the treatment with deficiency of iron and found a highest number of bacteria that secreted siderophores within the first 24 h of growth when compared with the iron control condition. Besides high affinity for iron, siderophores may have affinity for other metals. In a study with *Azotobacter vinelandii*, Kraepiel et al. (2009) showed that this bacterium excretes catecholate compounds previously identified as siderophores, which bind to metal cofactors of the nitrogenase (Mo, V and Fe) enzyme.

In addition to the above characteristics, 28 of 160 isolates were able to solubilize tricalcium phosphate (Table 1); the rhizospheric soil and barley roots obtained from São Borja locality showed the highest number of phosphate-solubilizing strains (65%) when compared to the other regions. The sources of phosphorus (P) in soil are available from organic phosphate compounds (Richardson & Simpson 2011) and inorganic phosphate compounds, mainly in the form of insoluble mineral complexes (Rodriguez et al. 2006). Phosphate-solubilizing bacteria are able to solubilize phosphate inorganic compounds, for example, tricalcium phosphate, by the production of organic acids (Chen et al. 2006). Similarly low numbers of phosphate-solubilizing bacteria have been documented by other studies (Beneduzi et al. 2008; Ambrosini et al. 2012; Costa et al. 2013; Granada et al. 2013; Souza et al. 2013).

Many of the 160 bacterial strains isolated in this study presented more than one PGP attribute: 70 (45%) isolates were capable to produce ICs and siderophores; 19 (12%) isolates were able to produce siderophores and solubilize tricalcium phosphate, 16 (10%) were able to produce ICs and siderophore, and 13 (8.1%) were able to produce ICs and siderophores and to solubilize tricalcium phosphate at the same time. According to Glick et al. (1999) rhizospheric bacteria are able to use different pathways to promote plant growth at various stages during the plant life cycle and the manner in which PGPB can influence plant growth differs from species to species as well as by strain.

Of those 160 bacterial strains, a total of thirty-two isolates possessing different PGP characteristics were selected and identified by PCR amplification and partial sequencing of the 16S rRNA gene. According to the partial sequences of the 16S rRNA gene, the main bacterial strains identified belonged predominantly to *Achromobacter* (8), *Burkholderia* (4), *Cedecea* (1), *Devosia* (1), *Enterobacter* (1), *Herbaspirillum* (1), *Leclercia* (2), *Pseudomonas* (1), *Microbacterium* (1), *Ochrobactrum* (1), *Rhizobium* (7), *Salmonella* (1), *Staphylococcus* (2) and *Stenotrophomonas* (1) genera. Of the identified bacteria, three strains were further selected for the growth chamber experiment. The selection was based on their PGP characteristics and their taxonomic identification (Table 2). According to Bhromsiri & Bhromsiri (2010) PGPB constitute a heterogeneous and beneficial group of microorganisms that may be found in the rhizosphere or in association with the host plant. Moreover, different strains belonging to *Burkholderia, Cedecea, Rhizobium, Enterobacter*, and *Stenotrophomonas* genera were also found in the roots and rhizospheric soil of different plants (Lim et al. 2008; Magnani et al. 2010; Santi Ferrara et al. 2011; Ambrosini et al. 2012; Farina et al. 2012; Costa et al. 2013; Granada et al. 2013; Souza et al. 2013).

3.2. Efficiency of growth promotion by bacterial inoculation of barley plants

To test the interaction between PGPB and barley, an *in vivo* experiment was conducted with three selected isolates in a growth chamber. Table 2 shows the results of the PGP activities evaluated for these isolates. The bacterial isolates used to inoculate the seeds of barley were identified as JC57 (*Ochrobactrum* sp.), SB41 (*Cedecea* sp.), and VA7 (*Microbacterium* sp.).

According to Table 3, in the treatment with soluble phosphate, the growth of plants inoculated with bacterial isolates was statistically equivalent to those of plants without inoculation regarding all parameters of plant growth analyzed.

In relation to the treatment with insoluble phosphate two selected isolates used for the inoculation of barley seeds resulted in satisfactory effects on plant growth compared with the non-inoculated plants (Table 3). Barley plants inoculated with SB41 (*Cedecea* sp.) and VA7 (*Microbacterium* sp.) isolates presented significantly higher results than the non-inoculated control plants in terms of dry shoot biomass. Moreover, plants inoculated with VA7 strain presented a significant increase in the dry root weight as well in the root length when compared with the non-inoculated plants (Table 3). Barley plants inoculated with P solubilizing bacteria showed significantly enhanced growth when fertilized with rock phosphate (insoluble phosphate) (Belimov et al. 1995). Similar results were

Table 3. The effect of the inoculation of native PGPB on the promotion of barley growth under growth chamber conditions in the presence of soluble phosphate.

Treatment[a]	Soluble phosphate				Insoluble phosphate			
	Shoot growth		Root growth		Shoot growth		Root growth	
	Lenght (mm)	Dry matter (mg)	Lenght (mm)	Dry matter (mg)	Lenght (mm)	Dry matter (mg)	Lenght (mm)	Dry matter (mg)
Non-inoculated	189.5 a	143.7 a	450.2 a	198.4 a	457.0 a	113.9 b	164.2 b	110.9 c
SB41	189.5 a	119.2 b	477.5 a	192.6 a	463.5 a	155.5 a	171.2 b	120.6 bc
VA7	198.5 a	143.4 a	459.0 a	198.9 a	456.2 a	157.6 a	212.5 a	148.6 a
JC57	185.5 a	156.4 a	460.0 a	201.1 a	478.5 a	148.3 ab	195.7 ab	139.0 ab

Data represent the means of 5 replicates of plants grown in vermiculite in a photoperiod chamber.
Values in the same column followed by the same letter did not differ significantly at $P > 0.05$ (Tukey Test).
[a]Bacteria isolated from: SB41 (São Borja); VA7(Vacaria); JC57 (Júlio de Castilhos).

showed by Granada et al. (2013) in an experiment conducted with *Lupinus albescens* plants, involving soluble and insoluble phosphate. In this work, the authors demonstrated that the inoculation of *L. albescens* with the 103R (*Ochrobactrum* sp.), 22R (*Sphingomonas* sp.), and 230S (*Burkholderia* sp.) isolates improved plant growth, with the best results obtained under the insoluble P condition. The favorable effects of PGPB inoculation on plant growth have also been extensively reported in others works (Beneduzi et al. 2008; Sasaki et al. 2010; Santi Ferrara et al. 2011; Ambrosini et al. 2012; Glick 2012; Arruda et al. 2013; Costa et al. 2013; Souza et al. 2013).

Plant growth-promoting bacteria can affect host plants directly or indirectly. The SB41 and VA7 isolates displayed different PGP attributes in our *in vitro* assays. These observations indicated that these growth promotion mechanisms could have influenced the plant development and growth. The hormone auxin is an important regulator that directly influences plant development and growth (Jaillais & Chory 2010). Several studies show that the excretion of siderophores by rhizospheric bacteria may stimulate plant growth, improving plant nutrition (Masalha et al. 2000). On other hand, phosphate solubilizing bacteria can facilitate the conversion of insoluble forms of P making it available to plants (Chen et al. 2006).

4. Conclusion

Bacterial strains associated with roots and rhizospheric soil of barley display different plant growth-promoting traits that can be used to promote plant growth. The growth chamber experiment showed that bacterial isolates from the rhizosphere of barley presented a plant growth-promoting effect in insoluble phosphate conditions. The present work clearly indicates that SB41 (*Cedecea* sp.) and VA7 (*Microbacterium* sp.) strains may be a good candidates for formulation of a bioinoculant, allowing a reduction in the use of fertilizers and reducing environmental problems.

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