



Characterization of Plant Growth Promoting Rhizobacteria Isolated from the Rhizosphere of Peruvian Highlands Native Crops

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Authors' contributions

This work was carried out in collaboration between all authors. Authors KOG, DZD and DA conceived and designed the study and experiments. Authors CCS and KOG performed the experiments. Authors CCS, KOG and DZD analyzed the data. Authors KOG and DZD contributed reagents, materials and analysis tools. Authors KOG and DZD wrote the manuscript. All authors reviewed and approved the final manuscript.

Article Information

DOI: 10.9734/IJPSS/2016/24573

Editor(s):

(1) Susana Rodriguez-Couto, Unit of Environmental Engineering, Paseo Manuel Lardizabal, Donostia-San Sebastián, Spain.

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(4) P. Rama Bhat, Mangalore University, Karnataka, India.

Complete Peer review History: <http://sciencedomain.org/review-history/14511>

Short Communication

Received 26th January 2016

Accepted 15th April 2016

Published 7th May 2016

ABSTRACT

Over the past few decades, research in agricultural microbiology has highlighted the efficacy of plant growth promoting rhizobacteria (PGPR) in inducing seedling emergence, promoting the increase in plant height, weight and overall crop yield. A number of microbial isolates have shown promising antagonistic activity against several soilborne pathogens. In this study, twelve bacterial strains were isolated from root rhizosphere of Peruvian native highlands crops, i.e Goldenberry (*Physalis peruviana* L.), Potato (*Solanum tuberosum* L.) and Maca (*Lepidium meyenii* Walp.). The

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strains were identified using 16S rRNA gene PCR amplification and sequencing and were characterized for their PGPR activities. Among these isolates, all strains were found to be able to produce indol-acetic acid, two strains were able to solubilize hydroxyapatite, bi- and tri-calcium phosphate and the remaining others were able to solubilize at least one source of inorganic phosphate. Of 12 isolates, 10 strains showed antagonistic activity against *Fusarium solani*, 5 showed activity against *Alternaria alternata* and 9 inhibited growth of *Curvularia lunata*. It was also found that out of 12 isolates, three were able to induce tomato seedling emergence by 75% compared to the control in *in-vitro* assays. 16S rRNA gene sequence based analysis of these strains confirmed that, of 12 strains, 4 were members of genus *Pseudomonas*, 3 belong to genus *Bacillus*, 2 were related to genus *Rahnella* and 1 each belong to the genus *Stenotrophomonas*, *Serratia* and *Streptomyces*.

Keywords: Plant growth-promoting rhizobacteria; native crops; antagonists; indolacetic acid; phosphate solubilization; 16S rRNA gene.

1. INTRODUCTION

Peru is an important centre for a number of genetically diverse indigenous crops now cultivated across the world. A number of these native Andean plants form an important part of the routine diet of a significant proportion of population, although their true potential has not been fully realised. In the recent time, these Peruvian native Andean crops are getting recognised more and more across the world not only for their nutritional value, but also for their medicinal properties which has lead to an increase in demand of these crops in the international markets. However, international markets impose high standards for agricultural products concerning to food safety and quality and so restrict import, sales and consumption of products with high content of chemicals pesticide, fungicide and fertilizer residues. In the current scenario, the microbes inhabiting the root rhizosphere of the native crops with antagonistic activity against a variety of phytopathogens and with the ability to promote plant growth provide a better environment and health friendly alternatives to these chemical fertilizers and fungicides. A considerable number of bacterial species from the rhizosphere have been isolated and their efficiency to improve plant growth has been assessed [1]. A few studies have also reported that the efficacy of PGPR depends on the type of crop, climate and soil composition [2]. To expand our knowledge about the useful microbial species associated to Andean crops, the present investigation was directed towards use of microbes isolated from Peruvian highlands soils exhibiting traits associated with plant growth promoting ability, assessed under *in vitro* conditions. Tomato plants are known to be susceptible to several

phytopathogenic fungi, which in turn compel farmers to extensively use chemical products to get rid of them as well as for improving crop yield. Microbial inoculants discussed here are environmentally friendly alternative for chemical fungicides and fertilizers against some of fungal diseases of tomato plants.

2. MATERIALS AND METHODS

Twelve microbial strains were used in this study. These strains were isolated from the root rhizosphere of three Andean crops, i.e Goldenberry (*Physalis peruviana* L.) and Maca (*Lepidium meyenii* Walp.) from Junin and Potato (*Solanum tuberosum* L.) from Puno [3], located in the highlands of Peru over 3300 meters above sea level. Soils at the collection sites had a high content of organic matter and were moderately acidic, with pH between 4.5 and 5.5. All strains were maintained in nutrient broth (NB) plus 25% (v/v) glycerol at -80°C. Strains were Gram-stained [4]. Colony morphology: size, color, shape, texture, height and edge were recorded after 48 h of growth on nutrient agar plates at 28°C [modified for 5] Phosphate solubilisation assays were performed as described by Nautiyal et al. [6] and formation of a solubilisation halo was observed up to 20 days. Indolacetic acid (IAA) production by these isolates was determined using the method described by Glickmann and Dessaux [7] and appearance of pink colour was considered to be indicative of IAA production. All the strains were also screened for antagonistic activity against phytopathogenic fungi *Fusarium solani*, *Alternaria alternata* and *Curvularia lunata*. Antagonistic activity was tested through the dual culture technique and fungal growth inhibition was quantified as described by Rahman et al.

[8]. The ability of the strains to promote the emergence of seedling after microbial inoculation was also tested. For this assay, seeds of *Solanum lycopersicum* var. Río Grande were used. Bacterial strains were grown to obtain a population of 10^8 cfu/ml. The culture was resuspended in 10 ml of saline solution 0.85%. The surface-sterilized tomato seeds were then soaked in the bacterial suspension for 15 minutes. After that, the seeds were sown in plastic containers using sterile sand as substrate. The plants were irrigated daily with hydroponic solution A and B (provided by Centro de Investigación de Hidroponía y Nutrición Mineral in UNALM, Peru). Inoculated seeds were placed in a greenhouse with controlled temperature (20-25°C) and light cycle (12 hours/day) for 7 days. The assays were performed using 4 replicates per bacterial strain and container. Results were recorded as emergence percentage, when the first two leaves appeared out of the sand. Seeds not inoculated were used as controls. For the molecular identification of the strains isolated, total DNA was extracted from overnight grown cultures broth using AxyPrep Bacterial Genomic DNA Miniprep Kit (Axygen Biosciences, Union city, CA, USA) following the manufacturer's instructions. Primers fD1 and rD1 were used to PCR amplification of the 16S rRNA gene [9]. Phylogenetic neighbours of the isolates were identified using the EzTaxon web server (<http://www.ezbiocloud.net/eztaxon>) [10]. The ClustalX2 software [11] was used to align both studied and selected sequences. Phylogenetic analysis was performed by the neighbor-joining (NJ) method and distances were calculated according to the Kimura-2 method using the MEGA Version 6 [12].

3. RESULTS AND DISCUSSION

Four out of the twelve isolates were Gram-positive rods. One of them (Aa9) showed a filamentous morphology with a branching growth pattern characteristic of actinobacteria [13], while other isolates showed the ability to resist high temperatures (80°C for 30 min). Eight strains were Gram negative. Five of them were diazotrophic bacteria since they were capable to grow in mineral medium (MM) without nitrogen and to acidify it [14]. Other three isolates were classified into pseudomonad group, as they were capable to grow in cetrimide agar and showed fluorescence in Pseudomonas Agar F (DIFCO) [15]. The colony morphology of each group was different. Diazotrophic strains showed translucent colonies with entire edge, sizes between 0.5 to

1.5 mm and convex. As well, pseudomonads showed sizes between 0.5 and 1 mm, umbonated elevation and were cream or iridescent. *Bacillus* colonies were bigger than the other two bacterial groups with sizes between 1 and 3 mm. They appeared opaque, mucoid with entire edge and umbonated elevation. The actinomycete on the other hand, showed punctiform colonies with undulate edges and yellow pigment.

The majority of the isolates showed one or more PGPR attribute. All strains were able to produce auxin in the range of 8.1 – 67.5 ppm in the presence of IAA precursor tryptophan. Strain Ba60 produced the maximum IAA level (67.5 ppm) followed by two diazotrophic isolates, Azo16M2 and LMTZ064-66 (Table 1). However, most of the pseudomonads produced low amounts of IAA compared to the other bacterial groups, showing values between 7 and 10 ppm. Agaras *et al* (2015) [16] reported similar values of this phytohormone in different species of pseudomonads isolates and their positive control *P. putida* GR12-22. *Bacillus* sp. and *Streptomyces* strains were documented with a production up to 76 ppm [17] and 5 ppm [18], respectively. IAA production by PGPR can vary among different species and it depends on culture conditions, growth stage and substrate availability [19]. Auxins produced by the PGPR strains play an important role in increasing the overall surface area of the plant root. The larger surface area of the roots enable the plant to absorb the water and other important nutrients more efficiently, which in turn leads to a better growth and development of the plant PGPR. All the isolates were able to solubilize at least one inorganic phosphate source tested in the assays. Two isolates (Da29 and Azo16M2) were capable of solubilizing hydroxyapatite, bi- and tri-calcium phosphate. Nine strains were capable to solubilize bicalcium phosphate with maximum halo sizes varying between one and 17.5 mm and two isolates (Ba60 and Ps42) could solubilize only hydroxyapatite (Table 1). It is well documented the fact that a number of microorganisms can make insoluble soil phosphorous available to plants by solubilizing mineral phosphates through the production of organic acids or phosphatases. The results obtained in the present study showed that most of the bacterial strains tested could solubilize bicalcium phosphate better than tricalcium phosphate and hydroxyapatite. These results are similar to those obtained by Ben Farhat *et al.* [20], who report tricalcium phosphate and hydroxyapatite as bacterial inorganic phosphate

sources, with a low rate of conversion than the bicalcium phosphate.

In antagonistic assays, strain Ba60 could inhibit the growth of *Alternaria alternata* only, while strains LMZ064-66 and Pa86 could inhibit the growth of only *Fusarium solani*. Azo16M2 and Ba8 were able to restrict the growth of both *Fusarium solani* and *Alternaria alternata*. The percentages fungal growth inhibition for all the strains was greater than 20%. Azo16M2 and Ba8 isolates were also able to inhibit the growth of *Curvularia lunata* with percentages inhibition 0.7 and 5.2% respectively. *Bacillus* isolates and the diazotrophic bacteria Azo16M2 showed greater antagonistic activity against *Alternaria alternata* (Fig. 1). Ba8 have shown antagonistic activity against all the phytopathogenic fungi tested in our study. A number of species from the genus *Bacillus* are widely known to produce various antifungal and antibacterial secondary metabolites effective against a range of phytopathogenic fungi [21]. Some species like *B. subtilis*, *B. amyloliquefaciens*, *B. pumilus* and *B. cereus* are already being used as biological control agents against many soil-borne plant pathogens [8].

Based upon the previous results, five of the twelve isolates were further tested for their ability to induce tomato seedling emergence. The results obtained in the test clearly indicated a

positive effect of the strains on the seedling emergence of tomato plants. Three out of 5 chosen strains (Ps42, Da29 and Aa25) significantly increased the seedling emergence of tomato (75%) compared to the non-inoculated control, that showed a level of 50% seedling emergence. Increase in the seed emergence due to the production and secretion of some phytohormones and enzymes by microbes can be used as an attribute for a primary rapid screening of PGPR strains [22]. An increase in the seedlings emergence may favor an early development of the efficient root system of the plant which is in itself an indicative of better plant health and growth.

The NJ phylogenetic tree (Fig. 2) showed that all the studied isolates were clustered in 6 groups and showed close affiliation with the genus *Pseudomonas*, *Rhanelia*, *Serratia*, *Stenotrophomonas*, *Bacillus* and *Streptomyces*. Within *Pseudomonas* genus, the isolates Pa86 and LMTZ064-90 were clustered with *P. azotoformans* group, while Ps42 and Pa82 with *P. syringae* and *P. putida* groups respectively. The comparison of the 16S rRNA gene sequence of all the isolates against type strains of bacterial species recorded in the EzTaxon database showed that LMTZ06466 [KU750792] is closely related with *Serratia proteamaculans* DSM4543^T, LMTZ064-90 [KU750791] with *Pseudomonas yamanorum* 8HI1^T,

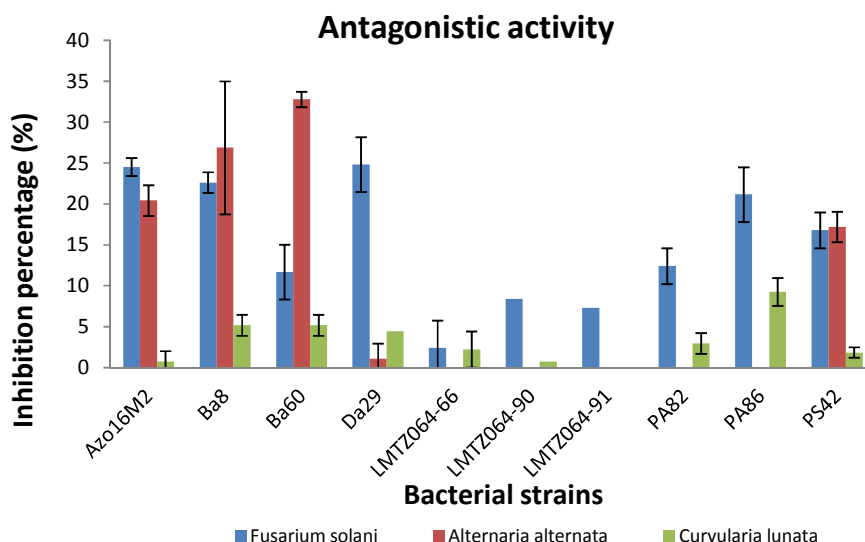


Fig. 1. Antagonistic activity of the isolates against three phytopathogen fungi of tomato

Table 1. Plant growth promoting features of isolated strains from golden berry, maca and potato rhizosphere

Isolates	Sampling place	Crop	Gram stain	Colony				[IAA] ppm	Bi-calcium phosphate halo (mm)	Tri-calcium phosphate halo (mm)	Hydroxyapatite halo (mm)
				Form	Color	Edge	Elevation				
Aa25	Concepcion*	goldenberry	G+	punctiform	yellow opaque	undulate	slightly convex	21.7	11.8	0	0
Ba8	Concepcion*	goldenberry	G+	irregular	cream shiny	round	umbonate	12.9	1	0	0
Ba51	Concepcion*	goldenberry	G+	circular	cream shiny	entire	umbonate	17.9	0	8.5	0
Ba60	Concepcion*	goldenberry	G+	circular	cream shiny	entire	umbonate	67.5	0	0	1.3
Da29	Concepcion*	goldenberry	G-	circular	colorless opaque	entire	convex	47	9.8	1.3	8.8
Pa82	Concepcion*	goldenberry	G-	circular	cream shiny	entire	umbonate	7.4	1	1	0
Pa86	Concepción*	goldenberry	G-	irregular	cream shiny	undulate	umbonate	10	2	3	0
Azo16M2	Thunco**	potato	G-	circular	colorless shiny	entire	convex	67.4	13	2.1	10.1
LMTZ064-66	Acomachay*	maca	G-	circular	cream opaque	entire	convex	66.8	8	7.5	0
LMTZ064-90	Galpon Condorin*	maca	G-	circular	colorless shiny	entire	convex	32.7	17.5	5	0
LMTZ064-91	Galpon Condorin*	maca	G-	circular	colorless shiny	entire	convex	39.7	14	6	0
Ps42	Condorin*	maca	G-	circular	iridescent shiny	entire	convex	8.1	0	0	0.7

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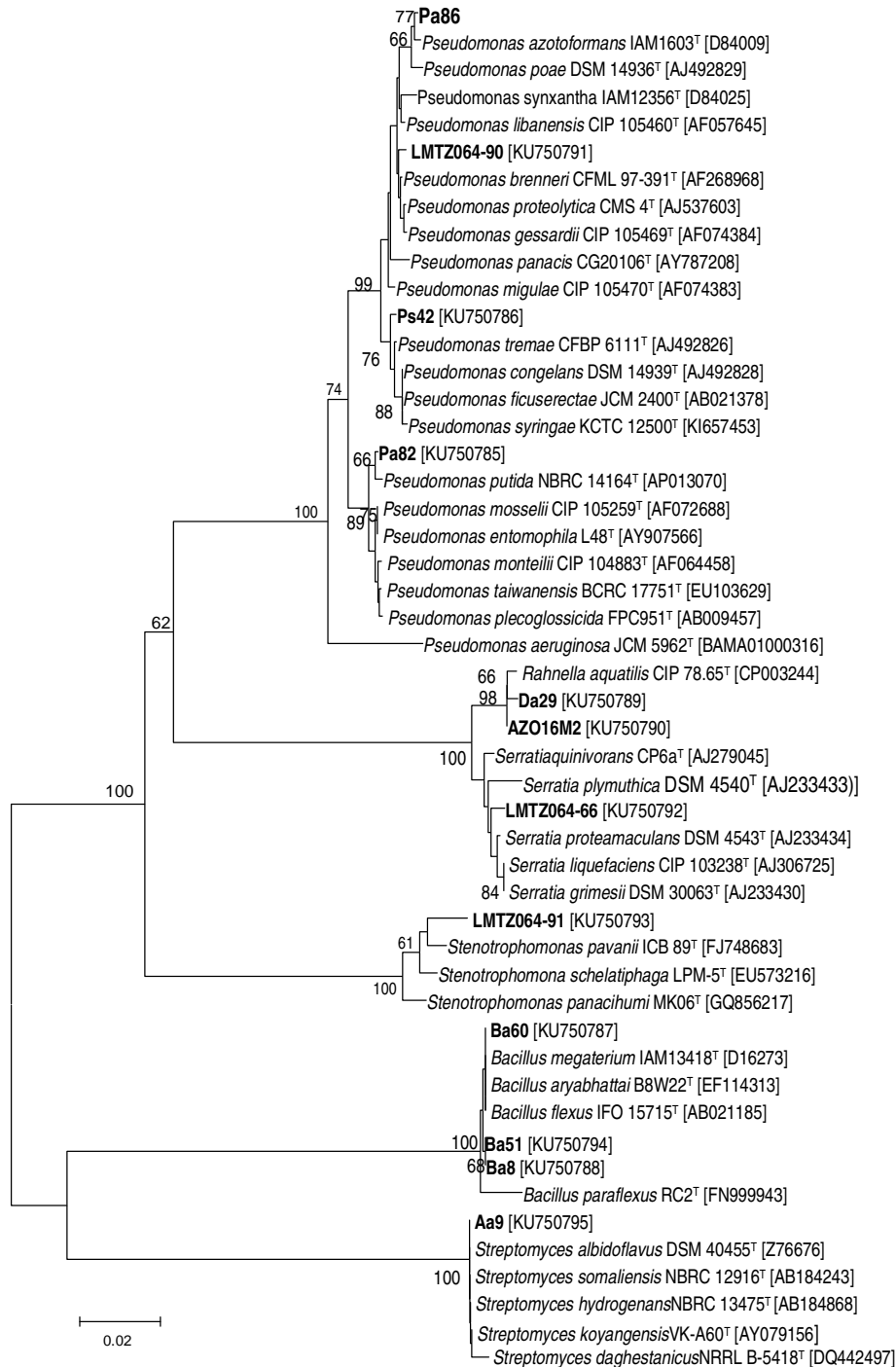


Fig. 2. Phylogenetic analysis of 16S rRNA sequences from isolates obtained in this study. The isolates using in these study are indicated in bold. Only bootstrap values greater than 60 % are shown (1,000 pseudoreplicates)

LMTZ06491 [KU750793] with *Stenotrophomonas panacihumi* MK06^T, Pa86 [KU750785] with *Pseudomonas libanensis* CIP105460^T, Pa82 [KU750796] with *Pseudomonas plecoglossicida* FPC951^T, Ps42 [KU750786] with *Pseudomonas ficuserectae* JCM 2400^T, Ba8 [KU750788], Ba51 [KU750794] and Ba60 [KU750787] with *Bacillus aryabhatai* B8W22^T, Azo16M2 [KU750790] and Da29 [KU750789] with *Rahnella aquatilis* CIP 78.65^T and Aa9 [KU750795] with *Streptomyces hydrogenans* NBRC 13475^T. Similarity values were over 99%. Further up to species level details could be inferred from the tree provided (Fig. 2) though the accurate identification for some of the strains in this study require phylogenetic analysis using a few more marker genes sequences.

4. CONCLUSIONS

The results obtained in this study clearly illustrate the potential of these isolates as PGPR under in vitro conditions. Though, it cannot be also ruled out that the interaction between PGPR and plants could have different results under field conditions. However, the present work is among the first few which provides primary information about the microbial strains with antagonistic and plant growth promoting activity from these Peruvian Andes region which has not been exploited yet up to its true potential. The study also creates a platform for the future field trial using the strains described in the present study as potential PGPRs.

ACKNOWLEDGEMENTS

This research was supported by PROCYT N°325 - 2011 - CONCYTEC, FONDECYT-CONCYTEC-145 – Project - 2013. We thank E. Ormeño-Orrillo, M. Finetti-Sialer and Prashant Kumar Pandey for their suggestions and critical editing of the manuscript.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
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