

CHARACTERIZATION OF *BACILLUS* ISOLATES OF POTATO RHIZOSPHERE FROM ANDEAN SOILS OF PERU AND THEIR POTENTIAL PGPR CHARACTERISTICS

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ABSTRACT

Bacillus spp. are well known rhizosphere residents of many crops and usually show plant growth promoting (PGP) activities that include biocontrol capacity against some phytopathogenic fungi. Potato crops in the Andean Highlands of Peru face many nutritional and phytopathogenic problems that have a significant impact on production. In this context is important to investigate the natural presence of these microorganisms in the potato rhizosphere and propose a selective screening to find promising PGP strains. In this study, sixty three *Bacillus* strains isolated from the rhizosphere of native potato varieties growing in the Andean highlands of Peru were screened for *in vitro* antagonism against *Rhizoctonia solani* and *Fusarium solani*. A high prevalence (68%) of antagonists against *R. solani* was found. Ninety one percent of those strains also inhibited the growth of *F. solani*. The antagonistic strains were also tested for other plant growth promotion activities. Eighty one percent produced some level of the auxin indole-3-acetic acid, and 58% solubilized tricalcium phosphate. Phylogenetic analysis revealed that the majority of the strains belonged to the *B. amyloliquefaciens* species, while strains Bac17M11, Bac20M1 and Bac20M2 may correspond to a putative new *Bacillus* species. The results suggested that the rhizosphere of native potatoes growing in their natural habitat in the Andes is a rich source of *Bacillus* fungal antagonists, which have a potential to be used in the future as PGP inoculants to improve potato crop.

Key words: *Bacillus*; plant growth-promoting rhizobacteria; potato; antagonism; phosphate solubilization; 16S rRNA gene.

INTRODUCTION

Potato is a staple crop in 130 countries worldwide, ranking fourth in production after rice, maize, and wheat. This important crop was domesticated by pre-Columbian civilizations in the Andean highlands of Peru and Bolivia. Nowadays, the yield levels of potato crop in subsistence

agriculture in the Andes are low to medium, ranging between 5 to 8 t ha⁻¹ for low input systems and 15 to 20 t ha⁻¹ for high input systems, indicating a low soil fertility and a low nutrient availability (11). Also, fungal diseases affect crop yield and tubers quality. Black scurf and dry rot caused by *Rhizoctonia solani* and *Fusarium solani*, respectively, are among the most common potato fungal diseases in the Andes (31).

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The use of beneficial microorganisms could be an environmentally sound option to increase crop yields and reduce disease incidence. Plant growth-promoting rhizobacteria (PGPR) colonize plant roots and induce an increase in plant growth (33). Among the mechanisms by which PGPR exert beneficial effects on plants are facilitating the uptake of nutrients such as phosphorus via phosphate solubilization, synthesizing stimulatory phytohormones like indole-3-acetic acid (IAA) (33), or aiding in the control of the deleterious effects of pathogens by producing inhibitory substances, excluding them from the roots by competition or by inducing systemic resistance (7).

Strains of the genus *Bacillus* are among the most commonly reported PGPR (7, 33). The secondary metabolites produced by certain species and strains of *Bacillus* have antibacterial or antifungal activity against phytopathogenic microorganisms (2, 7). Products for plant disease biocontrol containing *B. subtilis* and other *Bacillus* species have been used over the past years as seed dressings in several crops (26). *Bacillus* strains have the advantage of being able to form endospores which confers them high stability as biofungicides or biofertilizers (26).

Exotic strains from commercial inoculants may not survive in local soils due to different edaphic or climatic conditions, or be out-competed by better adapted native bacteria during plant colonization resulting in poor performance of PGPR (4, 18). Therefore, isolation and screening of native strains is justified. *Bacillus* strains have been previously isolated from potato rhizospheres (5, 28) but nothing is known about the *Bacillus* strains associated with potato growing in its domestication Andean areas. The aims of this study were (i) to screen for native rhizospheric *Bacillus* showing *in vitro* antagonism against *R. solani* and *F. solani* and other plant growth promoting activities, and (iii) to determine the phylogenetic affiliation of some representative selected isolates.

MATERIALS AND METHODS

Bacillus strains

Sixty three *Bacillus* strains previously isolated from the rhizosphere of native varieties of potato (*Solanum tuberosum*) growing in Huancavelica and Puno, two Andean regions of Peru, were used (6). Strains were grown in TGE agar (composition per liter: triptone 5 g, glucose 10 g, beef extract 3 g, agar 15 g) at 28 °C. Pure cultures were kept at 4 °C in TGE agar slants and at -80 °C in 15% glycerol. *B. amyloliquefaciens* FZB24, a known antagonist of phytopathogenic fungi and IAA producer, was used as control in several tests (17). The lecithinase test was used to discard any strains putatively belonging to the potentially pathogenic *Bacillus cereus* species. The production of lecithinase was assayed by observing the formation of a white precipitate around colonies after streaking on MEYP (Mannitol-Egg Yolk-Polymixin) agar, where the majority of strains of *B. cereus* give a positive reaction (14).

In vitro screening for antagonism

One pathogenic strain of *Rhizoctonia solani* and one of *Fusarium solani* were isolated in this work from sick tubers obtained in a potato field located in Huancavelica. For *R. solani*, the tubers were disinfected with sodium hypochlorite (1,5%) for one minute, the sclerotia were retired and placed in water agar and PDA (Potato Dextrose Agar) plates. For *F. solani*, the white characteristic mycelium of affected tubers was retired and placed on water agar and PDA plates. The plates were incubated at 20 °C for 7-10 d. After purification, the fungi were identified in the International Potato Center's Phytopathology Laboratory by microscopic observation of characteristic structures (3), namely, the anatomy of the septal pore and the cellular nuclear number for *R. solani* (1), and the macroconidia and microconidia produced in the aerial mycelium for *F. solani* (23).

Antagonistic activity was tested using the dual culture technique (16). Briefly, three 5 µl drops of each bacterial culture (10^8 cfu ml⁻¹) were equidistantly placed on the margins of potato dextrose agar plates adjusted to pH 7. A one cm agar disc from a fresh culture of *R. solani* or *F. solani* was placed at the center of the plate. Control plates not inoculated with bacteria were also prepared. Plates were incubated at 20 °C for

5 d (*R. solani*) or 7 d (*F. solani*). The fungal growth inhibition was quantified by using the percentage inhibition formula: $((R-r) \times R^{-1} \times 100)$, where, r is the radius of the fungal colony that grew towards the bacterial colony and R is the maximum radius of the fungal colony away from the bacterial colony (the maximum growth that the fungi can have in the Petri dish) (16). Two independent experiments with each bacterial isolate replicated two times were performed.

Production of IAA

The Salkowski reagent (0.01 M FeCl₃ in 36% H₂SO₄) was used to colorimetrically assay the production of IAA (12, 13). Isolates were grown in TGE supplemented with 5 mM of L-tryptophan with agitation (150 rpm) at 28 °C for 4 d. 300 µl of the Salkowski reagent was added to 100 µl of cultures in a microplate. After 15 minutes in the dark, color reaction was visually scored. The results were expressed in an arbitrary scale of color intensity. Non inoculated and untreated controls were kept for comparison. The experiment was independently performed twice with two replicates of the bacterial strains each time.

Assay for phosphate solubilization

Phosphate solubilization test was conducted by plating 5 µl of bacterial cultures (10⁸ ufc ml⁻¹) in NBRIP (National Botanical Research Institute's phosphate growth medium) agar that contains insoluble tricalcium phosphate making the medium opaque (22). The solubilization halo was calculated by subtracting colony diameter from the total diameter. Measures were taken every 2 d during a 30 d period. Two independent experiments with each bacterial isolate replicated two times were performed.

Correlation analysis

The correlation between the different plant growth promoting characteristics was determined by calculating Pearson product moment correlation coefficients (36). The correlations were considered significant if $P < 0.05$.

DNA techniques and phylogenetic analysis

Total DNA was extracted from liquid cultures with the GenomicPrep kit (Amersham) using the manufacturer's instructions. BOX genomic fingerprints were generated as described by Versalovic *et al.* (32). DNA fragments were separated in a 3% agarose gel, photographed after ethidium bromide staining, and the bands visually recorded. Different BOX profiles were assigned to strains having at least one different band. 16S rRNA genes were PCR amplified using primers fD1 and rD1 (35) and sequenced. The sequences of type- and reference strains of related *Bacillus* species (34) were identified by BLASTN searches. Phylogenetic analysis was performed by the neighbor joining (NJ) method with genetic distances computed with the Kimura's two-parameter model using Mega4 (30, 34). The sequences determined in this study have been deposited in the GenBank database under accession numbers FJ889050 - FJ889057.

RESULTS

Antagonistic activity

Forty three of the 63 *Bacillus* strains (68%) isolated from potato rhizosphere (6) were able to reduce the growth of *R. solani* with percentages of inhibition ranging from 69% to 91% (Table 1). Thirty nine of the 43 antagonistic strains (91%) were also able to control the growth of *F. solani* with percentages of inhibition ranging from 56% to 86% (Table 1). The non antagonistic strains grew normally and were not inhibited by the fungi (data not shown). There was not a significant correlation between the control capacity against the two pathogens ($P > 0.05$), yet some strains, like BacNe2c and Bac20M1, showed high inhibition against both fungi. Mainly saprophytic *Bacillus* species have been reported in potato rhizospheres but the potentially pathogenic *B. cereus* has also been obtained (5). All our antagonistic strains showed a negative reaction in the lecithinase test, suggesting that they did not belong to *B. cereus*.

Table1. Geographical origin and plant growth promoting activities of the antagonistic *Bacillus* strains isolated from potato rhizospheres.

Strain	Geographical origin	Growth inhibition (%) against		PO ₄ solubilization halo (mm)	IAA ^a	BOX profile ^b
		<i>R. solani</i>	<i>F. solani</i>			
Bac3M3	Huancavelica	75.0	59.8	0	+	
Bac3M4	Huancavelica	69.1	56.7	3	+	
Bac3M5	Huancavelica	71.4	65.9	0	+	A
Bac3M6	Huancavelica	59.5	0	7	+	
Bac3M8	Huancavelica	83.3	57.9	0	+	
Bac3M9	Huancavelica	82.1	60.1	0	+	
Bac5M1	Huancavelica	70.2	65.9	0	-	
Bac6M1	Huancavelica	79.8	69.9	2	+	
Bac7M1	Huancavelica	84.5	75.6	0	++	
Bac7M3	Huancavelica	77.4	74.7	0	+	B
BacC7M1	Huancavelica	77.4	77.0	2	+++	
Bac8M7(1)	Huancavelica	77.4	79.3	3	-	C
Bac8M2	Huancavelica	83.3	0	1	-	
Bac13M3	Puno	89.3	0	9	+	
Bac14M1a	Puno	75.0	65.5	0	+	C
Bac14M1b	Puno	84.5	74.7	0	+	
Bac14M1c	Puno	81.0	81.7	0	+	
Bac15Ma	Puno	86.9	78.6	0	+	
Bac15Mb	Puno	82.1	77.9	4	+	C
Bac17M7	Puno	84.5	63.2	0	+	
Bac17M8	Puno	84.5	70.1	5	+	
Bac17M9	Puno	79.8	63.2	0	++++	D
Bac17M10	Puno	81.0	63.2	3	+++	
Bac17M11	Puno	86.9	63.2	5	+	E
Bac17M12	Puno	76.2	77.0	0	-	
Bac17M13	Puno	85.7	72.4	0	++	H
Bac17M8+	Puno	89.3	77.9	3	++	
Bac17M21a	Puno	77.4	54.9	2	-	
Bac17M22b	Puno	88.1	60.2	2	+++	
Bac19M1	Puno	85.7	0	3	+++	
Bac20M1	Puno	91.7	83.1	5	++	E
Bac20M2	Puno	89.3	80.5	2	-	E
BacYU1	Puno	76.2	79.8	0	-	H
Bacbla1a	Huancavelica	75.0	72.4	3	+	H
Bacbla1b	Huancavelica	86.9	72.4	4	+	
Bacbla1c	Huancavelica	79.8	77.0	2	-	H
BacNeIB1	Huancavelica	86.9	63.2	0	++	
BacNeIB1a	Huancavelica	84.5	70.1	3	+	
BacNeIB1b	Huancavelica	79.8	65.5	0	+	H
BacNe2a	Huancavelica	78.6	72.4	3	+	C
BacNe2b	Huancavelica	82.1	72.4	4	+	
BacNe2c	Huancavelica	86.9	86.9	2	+	
BacNe2d	Huancavelica	81.0	77.8	2	+	C
Bac3M2	Huancavelica	0	0	0	+	H
Bac3M7	Huancavelica	0	0	0	-	G
FZB24 ^c		72.6	60.8	0	+	

^a Arbitrary colorimetric scale of IAA production from - (no production) to ++++ (high production).^b Only a subset of strains were analyzed.^c Commercially used *Bacillus amyloliquefaciens* strain.

IAA production and phosphate solubilization

The majority of the antagonistic strains (81%) produced IAA to various extents (Table 1). Twenty five of the positive strains (71%) produced it to the same level as the commercially-used *B. amyloliquefaciens* FZB24 control strain, while the remaining showed higher production (Table 1). The phosphate solubilization process, observed as growing solubilization haloes in a 30 d period, was progressive and reaches its maximum between the 10 and 15 d of incubation for all the isolates (data not shown). Twenty five out of the 43 antagonistic strains (58%) were able to solubilize tricalcium phosphate with maximum halo sizes varying between 1 and 9 mm (Table 1). There was not significant correlations ($P > 0.05$) between any pair of plant growth promoting activities except for a slight negative correlation between phosphate solubilization and the antagonism against *F. solani* ($r = -0.39$, $P = 0.0105$). We did not find any relationship between the plant growth promoting abilities of the strains and their geographical origin.

Phylogenetic affiliation

Eight strains showing antagonism against both fungi were randomly chosen from each geographical region to be subjected to phylogenetic analysis. Two non-antagonistic strains (Bac3M2 and Bac3M7) were also included. Genomic fingerprinting of these 18 strains revealed eight distinct BOX profiles (Table 1). Eight strains representing seven of the BOX profiles were selected for 16S rRNA gene sequencing. The phylogram presented in Fig. 1 showed that the potato strains belonged to the '*B. subtilis* group' phylogenetic branch (34). The Bac17M11 strain occupied an independent position indicating that it belong to a not yet described *Bacillus* species, while the remaining strains were intermingled with *B. amyloliquefaciens*, indicating that they belong to this species. The non-antagonistic strains were closely related to the antagonistic strains by BOX fingerprinting (Table 1) or 16S rRNA gene sequence (Fig. 1) indicating that strains possessing similar genomic backgrounds or 16S phylogeny may widely vary in their antagonistic abilities.

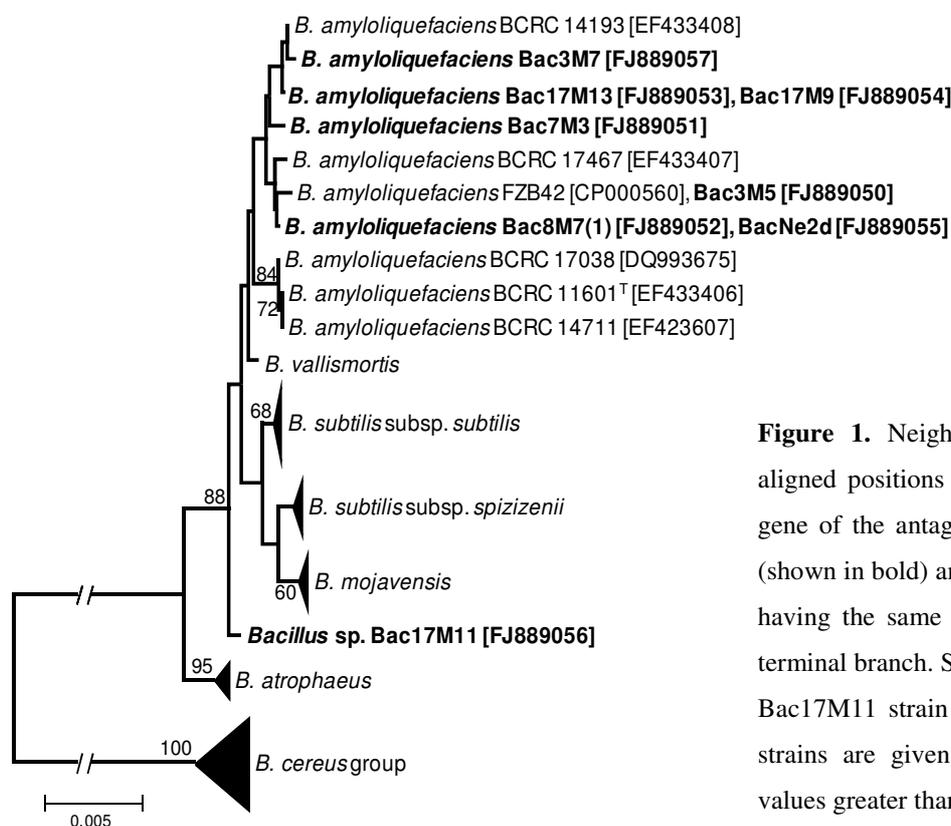


Figure 1. Neighbor joining phylogeny of 1390 aligned positions (without gaps) of the 16S rRNA gene of the antagonistic potato-associated *Bacillus* (shown in bold) and related *Bacillus* species. Strains having the same sequence are shown in the same terminal branch. Sequence accession numbers of the Bac17M11 strain and all the *B. amyloliquefaciens* strains are given within brackets. Only bootstrap values greater than 60% are shown.

DISCUSSION

The results of this research evidenced a high prevalence of antagonistic *Bacillus* towards *R. solani* and *F. solani* in the rhizosphere of potatoes growing in their natural habitat in the Andes. In other studies where a collection of *Bacillus* strains has been challenged against *R. solani*, only 9.5 to 36% were antagonistic to this pathogen (9, 20). *In vitro* antagonism towards *R. solani* has been previously found in *Bacillus* spp. isolated from several sources (9, 10, 19, 20, 24), including potato rhizosphere (5). The reported growth inhibitions are generally lower than those obtained here, ranging from 28 to 74% (19-21, 24). In contrast to the ample literature involving *R. solani*, only in few studies *Bacillus* spp. has been challenged against *F. solani* (10, 19, 29). The behavior reported ranged from no inhibition (29) to 10-64% growth reduction (10, 19).

A relatively wide range of antagonistic performances among *Bacillus* strains was observed here and has also been noted in other studies involving the same or different fungi (16). Several mechanisms have been proposed to explain the inhibition of fungal pathogens by *Bacillus* spp., including production of antimicrobials, secretion of hydrolytic enzymes, competition for nutrients, or a combination of mechanisms (7). Given that more than one mechanism may be involved, a complex response with a range of antagonistic effects among *Bacillus* strains and distinct responses by different pathogens could be expected as was observed here.

The capacity to produce phytohormones, like auxins, is a desirable characteristic of a PGPR (33). Several species of *Bacillus* have been reported to produce auxins. Idris *et al.* (15) have shown that mutants of *B. amyloliquefaciens* FZB42 with diminished levels of IAA production were less efficient in promoting plant growth. Although low IAA production scores were assigned to the majority of our strains, it has been shown that even low concentrations can induce an increase in the radical length and the number of secondary roots, and not always high concentrations results in a better growth promotion (27).

Several microorganisms are able to make insoluble soil phosphorous available to plants by solubilizing mineral

phosphates through the production of organic acids or phosphatases. *Pseudomonas* and *Azotobacter* are two of the most reported phosphate solubilization genera; however *Bacillus* strains also have this capacity (8, 22, 33). The phosphate solubilization abilities of our strains were similar to those reported for other *Bacillus* tested under similar assay conditions (8, 22).

Some of the strains characterized by BOX fingerprinting and 16S rRNA gene phylogeny seemed to belong to a novel *Bacillus* species but the majority of the strains were ascribed to *B. amyloliquefaciens*, a species with several reported PGPR representatives like FZB24. Reva *et al.* (25) proposed the existence of two ecotypes within *B. amyloliquefaciens*, one including the type strain and other including strains that seems to be well adapted to plant colonization. Interestingly, the *B. amyloliquefaciens* strains isolated here formed a subcluster separated from the type strain (Fig. 1), that subcluster may correspond to the plant-associated ecotype proposed by Reva *et al.* (25). It is worth to note that strains belonging to *B. amyloliquefaciens* are considered safe for use in biotechnological applications.

The results obtained indicate that the rhizosphere of potato is a rich source of potential PGPR strains of *Bacillus*. Some of the strains isolated here are currently being tested for plant growth promoting effects on potato in greenhouse experiments.

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