



Full Length Article

First Report of Phosphate-Solubilizing Bacteria Associated with *Agave angustifolia*

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Abstract

In this study, we isolated, characterized, and evaluated the tricalcium phosphate solubilization ability of phosphate-solubilizing bacteria (PSB) from the rhizosphere of maguey espadín (*Agave angustifolia* Haw.) plants cropped in mountain, hill and valley soils in Oaxaca, Mexico. Strains with the greatest phosphate-solubilizing capacity were identified biochemically using the API 20 NE and API 20 E kits (bioMerieux, USA). Forty-four PSB strains were isolated, and 24 were selected based on their phosphate solubilization efficiency and solubilization indices. Of these 24, nine (PSBVa, PSBVb, PSBVf, PSBHa, PSBHc, PSBHd, PSBMg, PSBMh and PSBMi) were selected as the most efficient phosphate solubilizers based on criteria including the amounts of solubilized phosphorus in culture filtrate and organic acids produced and the culture filtrate pH change. The highest PSB population was found in mountain soils. All selected strains were able to produce indole-3-acetic acid and gibberellic acid. Biochemical and morphological tests revealed genetic diversity among the strains studied. PSBVb, PSBMg, and PSBMh were identified as *Pseudomonas luteola*; PSBHc and PSBHd as *Burkholderia cepacia*; PSBVa as *Enterobacter* spp.; PSBVf as *Burkholderia gladioli*; PSBHa as *Sphingomonas paucimobilis*; and PSBMi as *Aeromonas hydrophila*. These PSB could serve as potential biofertilizers to improve the phosphorus nutrition of maguey espadín plants as has been demonstrated in previous studies. © 2018 Friends Science Publishers

Keywords: Maguey espadín; Mountain soils; Solubilized phosphorus; Tricalcium phosphate

Introduction

The concentration of soluble phosphorus in soil is usually very low, at levels of 1 ppm or less (Goldstein, 1994). The availability of phosphorus is highly dependent on the chemical composition and the biological processes existing in the soil (Neumann and Römheld, 2002). In the rhizosphere, there are a considerable number of phosphate-solubilizing bacteria (PSB) that include aerobic and well as anaerobic microorganisms (Rodríguez and Fraga, 1999). PSB intervene in the mineralization and/or solubilization of organic and inorganic phosphorus compounds, releasing phosphorus in quantities greater than what is required for plant growth and development (Vance *et al.*, 2003). In semiarid areas of the state of Oaxaca in Southern Mexico, approximately 8,422.7 ha owned by small landholders are cropped with maguey espadín (*Agave angustifolia* Haw.) (Oficina Estatal de Información para el Desarrollo Rural

Sustentable de Oaxaca, 2011). Maguey espadín is a basic ingredient in the production of “mezcal,” a traditional alcoholic beverage (Bautista-Cruz *et al.*, 2007). The soils where maguey is grown are predominately calcareous, with high soil pH values and low levels of plant-available phosphorus (Bautista-Cruz *et al.*, 2007). Phosphorus precipitation and fixation in the soil is highly dependent on pH and the soil type. The characteristics of the soils cropped with maguey espadín suggest that the phosphorus is probably fixed by calcium ions, forming insoluble calcium phosphates (Alia *et al.*, 2013). One option with the potential to increase phosphorus availability for maguey espadín crops, increase yields and counteract the collateral effects caused by mineral fertilizers, could be the use of PSB. We hypothesized that the rhizosphere of maguey espadín supports a diverse group of PSB. This work aimed to isolate, characterize, and evaluate the phosphate-solubilizing ability of PSB in the rhizosphere of maguey espadín. The

strains with the greatest phosphate-solubilizing capacity were identified through biochemical testing using API 20 NE and API 20 E kits (bioMérieux, USA).

Materials and Methods

The study area is located in the District of Tlacolula, Oaxaca, Mexico, in the communities of Magdalena Teitipac (16° 54' N and 96° 33' W), San Baltazar Guelavila (19° 80' N and 96° 29' W), and San Juan del Río (16° 53' N and 96° 09' W). The soil types in this area include Regosols and Leptosols, the altitude ranges from 1,060–1,700 m, the average annual precipitation is 726 mm, and the average annual temperature is between 28 and 32°C (National Commission of Biodiversity, 2004). The native vegetation is dry deciduous lowland forest (Lorence and García Mendoza, 1989). Three tillage systems are utilized in maguey espadín cultivation (Sánchez-López, 2005). Minimum tillage is predominant in mountainous zones (San Juan del Río) with slopes of 35–45%, animal-drawn plowing is used in hilly zones (San Baltazar Guelavila) with slopes of 15–30%, and disk plowing is carried out in valleys (Magdalena Teitipac), with slopes of 3–10%. Three plots of land were delimited in each topographic area. The plots had a surface of approximately 4,000 m² with maguey plants of approximately 1.5–3.5-years-old. In each plot five maguey plants including the roots and the soil attached to a depth of 20 cm, were extracted and maintained at a temperature of 4–5°C until PSB isolation. The soil from the rhizosphere of each plant was mixed homogeneously to form one composite sample for each plot.

PSB were isolated from each sample by serial dilution of the soil solution onto Sundara Rao and Sinha (SRS) agar medium (Sundara-Rao and Sinha, 1963) using Ca₃(PO₄)₂ as an insoluble source of phosphorus (Sundara-Rao and Sinha, 1963; Carrillo *et al.*, 1998). PSB were visually identified by the formation of solubilization halos around the colonies. To preselect strains with the greatest phosphate-solubilizing ability, criteria based on the phosphate solubilization efficiency (PSE) and the solubilization index (SI) was employed (Nguyen *et al.*, 1992; Kumar and Narula, 1999).

$$PSE = \text{halo zone diameter} / \text{colony diameter} \times 100$$

$$SI = \text{colony diameter} + \text{halo zone diameter} / \text{colony diameter}$$

Plates with < 40 isolated colonies were selected, and the repetition average was calculated and multiplied by the correction factor and by the corresponding dilution factor, with results reported in colony-forming units (CFU) g⁻¹ of soil (Collins, 1989). Isolated colonies were restreaked using the depletion method on SRS plates, which were incubated at 30°C for 48 h with the objective of obtaining pure strains (Dubey, 2001). The colony morphological characteristics were recorded in accordance with Goenadi *et al.* (2000). Slides of purified bacterial isolates were prepared for Gram-staining reactions in accordance with Breed *et al.* (1957).

The most effective PSB strains were identified by the production of organic acids, the pH change of culture filtrate, and the solubilized phosphorus in the culture filtrate. The pH change of culture filtrate due to the growth of PSB was measured after 7 days of growth. The titratable acidity of the culture filtrate, 7-day-old culture filtrates was estimated according to Balamurugan *et al.* (2010). The phosphorus solubilization by PSB strains was estimated in accordance with the method of Olsen *et al.* (1954).

The production of indole-3-acetic acid (IAA) and gibberellic acid (GA) by the most effective PSB strains was determined. Production of IAA was detected by a modified colorimetric method using Salkowski reagent (Lara-Mantilla *et al.*, 2011). GA production was determined according to the method described by Graham and Henderson (1960). Strains with the greatest phosphate-solubilizing capacity were identified through biochemical testing using API 20 NE and the API 20 E kits (bioMérieux, USA), and identification was carried out with the APIWEBTM software.

Results

The PSB population density in maguey espadín rhizosphere soils was highest in mountain soils (Table 1). A total of 44 PSB strains were isolated from the rhizosphere soils of maguey espadín cropped under different topographic conditions. These strains demonstrated tricalcium phosphate-solubilizing activity 48 h after their inoculation onto the SRS medium. The diameter of the solubilization halos around the colonies ranged from 2 to 7 mm. Of the 44 PSB strains isolated, 24 strains were selected based on their PSE and SI. Of these 24 strains, the PSBHg strain had the highest PSE, with the SI of the 24 strains ranging from 1.4 to 4.5 (Table 1).

The phosphate solubilizing ability of PSB isolates indicated that all preselected strains were able to solubilize tricalcium phosphate effectively (Table 1). Of the 24 isolates, three PSB strains were selected from each topography as the most effective. The selected PSB strains included PSBVa, PSBVb, and PSBVf from valley soil; PSBHc and PSBHd from hill soil; and PSBMg, PSBMh, and PSBmi from mountain soil. All the selected strains reduced the initial pH of the culture filtrate (Table 1). An estimation of titratable acidity indicated that organic acid production was highest for strain PSBHc. Of the nine selected strains, PSBmi was the most effective phosphate solubilizer (Table 1). All the selected PSB strains were able to produce IAA and GA. The PSBMh, PSBmi, PSBHc, and PSBVa strains exhibited the highest production of IAA. PSBMg demonstrated highest production of GA (Table 2).

Morphologically, all the bacterial isolates were Gram-negative bacilli (75%) and cocci (25%) (Table 3). Based on the biochemical and morphological tests, PSB was identified at the genus level.

Table 1: Quantitative estimation of tricalcium phosphate solubilization in liquid medium and population density of phosphate-solubilizing bacteria (PSB) isolated from the rhizosphere of maguey espadín (*Agave angustifolia* Haw.) cropped in three different topographies in Tlacolula, Oaxaca, Mexico

Strain	Solubilized phosphorus in culture filtrate ($\mu\text{g mL}^{-1}$)	Solubilization index	Phosphate solubilization efficiency	pH of culture filtrate	Titrateable acidity of culture filtrate (mL of 0.01 N NaOH consumed)	Halozone diameter (mm)	Colony-forming units (g^{-1} of soil)
-----Valley soils-----							
PSBVa	60.6	2.3	133	4.5	24.4	6	2×10^6
PSBVb	61.2	2.3	133	4.5	23.0	6	2×10^6
PSBVc	48.9	2.3	133	4.6	11.4	4	7×10^7
PSBVd	44.8	2.25	125	4.8	8.5	5	1×10^8
PSBVe	53.2	1.7	75	4.8	13.6	3	2.5×10^6
PSBVf	58.7	2	100	4.9	16.3	2	2.5×10^6
PSBVg	14.7	2	100	4.5	6.7	4	1×10^8
PSBVh	49.2	2	100	4.5	10.2	2	3×10^6
-----Hill soils-----							
PSBHa	39.5	1.5	57	4.5	13.7	3	1×10^7
PSBHb	53.7	1.6	60	4.8	7.9	4	1×10^7
PSBHc	54.0	2	100	4.4	38.3	3	4.5×10^5
PSBHd	37.4	1.4	42.8	4.6	12.3	3	6×10^7
PSBHe	9.3	1.6	60	6.8	5.8	3	6×10^7
PSBHf	30.7	2	100	5.6	3.0	3	2×10^8
PSBHg	12.5	4.5	350	6.1	0.72	7	3×10^7
-----Mountain soils-----							
PSBMa	16.0	2	100	6.5	0.84	2	7×10^6
PSBMb	36.5	2	100	4.6	5.9	2	7×10^6
PSBMc	19.2	2	100	6.4	0.45	2	4×10^7
PSBMd	9.2	2	100	6.4	0.45	3	4×10^7
PSBMe	56.6	2	100	4.7	5.4	2	1×10^8
PSBMf	55.6	2	100	5.3	6.2	2	6.5×10^5
PSBMg	57.0	2	100	4.5	8.6	2	1×10^7
PSBMh	55.1	2.3	133.3	5.2	13.9	4	1×10^8
PSBMi	67.0	2.5	150	4.6	27.0	3	6×10^6

Table 2: Indole-3-acetic-acid (IAA) and gibberellic acid (GA) produced by phosphate-solubilizing bacteria (PSB) isolated from the rhizosphere of maguey espadín (*Agave angustifolia* Haw.)

Strain	Colorimetric test IAA ($\mu\text{g mL}^{-1}$)	Colorimetric test GA ($\mu\text{g mL}^{-1}$)
PSBVa	$6.5 \pm 0.4\text{abc}$	$0.1 \pm 0.0\text{c}$
PSBVb	$1.3 \pm 0.5\text{e}$	$0.1 \pm 0.0\text{c}$
PSBVf	$6.2 \pm 0.8\text{bc}$	$3.6 \pm 0.6\text{b}$
PSBHa	$2.8 \pm 0.4\text{de}$	$1.7 \pm 0.2\text{bc}$
PSBHc	$7.7 \pm 0.6\text{ab}$	$0.1 \pm 0.0\text{c}$
PSBHd	$4.5 \pm 0.4\text{cd}$	$0.7 \pm 0.1\text{c}$
PSBMg	$4.3 \pm 0.4\text{cd}$	$9.2 \pm 1.0\text{a}$
PSBMh	$8.8 \pm 0.7\text{a}$	$0.08 \pm 0.0\text{c}$
PSBMi	$8.1 \pm 0.4\text{ab}$	$1.2 \pm 0.1\text{c}$

Among the nine PSB strains, three strains (PSBVb, PSBMg and PSBMh) were identified as *Pseudomonas luteola*, two strains (PSBHc and PSBHd) as *Burkholderia cepacia*, one strain (PSBVa) as *Enterobacter* spp., one strain (PSBVf) as *Burkholderia gladioli*, one strain (PSBHa) as *Sphingomonas paucimobilis*, and one strain (PSBMi) as *Aeromonas hydrophila*.

Discussion

The variation in the population of PSB under the different topographic conditions where maguey espadín is cropped

may be attributed to many soil factors, such as nutrient status, pH, moisture content, organic matter, and enzyme activities (Alia *et al.*, 2013).

The presence of solubilization halos around the colonies is due to the production of phosphorus solubilizing organic acids. However, many PSB that do not produce such halos can dissolve elevated concentrations of phosphorus since the production of acid in a solid medium is not the only method for the solubilization of inorganic, insoluble phosphates (Khan *et al.*, 2009).

PSBHg strain had the highest value of PSE, although this value was lower than that reported by Ramachandran *et al.* (2003) of 412.5 by some bacterial isolates from rhizosphere soil of *Piper nigrum* L. with tricalcium phosphate as phosphorus source. Ponmurugan and Gopi (2006b) reported PSE values between 44 and 75 in PSB isolated from cultivated soils. The SI of the 24 preselected strains ranged from 1.4 to 4.5.

Similar results have been previously reported by Rashid *et al.* (2004). Alia *et al.* (2013) reported an SI of 1.8 to 5 in PSB associated with vegetable roots of different ecologies. In addition, a study of the *Pseudomonas* (54RB) strain showed it to have an SI of 4.1 (Afzal *et al.*, 2010). The SI in the present work is greater than those observed by Alia *et al.* (2013). The present data reflects that highly efficient P-solubilizers inhabit the rhizosphere soil of maguey espadín.

All the selected strains reduced the initial pH value of

Table 3: Cultural characteristics and cell morphologies of bacterial strains isolated from the rhizosphere of maguey espadín (*Agave angustifolia* Haw.) cropped in three different topographies in Tlacolula, Oaxaca, Mexico

Strain	Colony color	Colony shape	Colony margin	Consistency	Bacterial form	Gram staining
-----Valley soils-----						
PSBVa	Yellow	Irregular	Wavy	Greasy	Bacillus	-
PSBVb	Brown	Irregular	Wavy	Friable	Bacillus	-
PSBVc	Gray	Circular	Entire	Greasy	Bacillus	-
PSBVd	Brown	Irregular	Wavy	Friable	Long Bacillus	+
PSBVe	Brown	Irregular	Wavy	Greasy	Long Bacillus	+
PSBVf	Yellow	Irregular	Wavy	Viscose	Bacillus	-
PSBVg	Brown	Irregular	Wavy	Greasy	Long Bacillus	-
PSBVh	Yellow	Circular	Entire	Viscose	Cocci	+
-----Hill soils-----						
PSBHa	Brown	Irregular	Lobate	Greasy	Long Bacillus	-
PSBHb	Beige	Irregular	Lobate	Greasy	Bacillus	-
PSBHc	Yellow	Circular	Entire	Greasy	Bacillus	-
PSBHd	Yellow	Irregular	Lobate	Greasy	Short Bacillus	-
PSBHe	Yellow	Irregular	Lobate	Greasy	Bacillus	-
PSBHf	Brown	Irregular	Wavy	Greasy	Short Bacillus	-
PSBHg	Yellow	Irregular	Wavy	Greasy	Short Bacillus	-
-----Mountain soils-----						
PSBMa	Brown	Irregular	Wavy	Greasy	Bacillus	-
PSBMb	Yellow	Irregular	Wavy	Greasy	Short Bacillus	-
PSBMc	Yellow	Irregular	Wavy	Greasy	Short Bacillus	-
PSBMd	Yellow	Circular	Wavy	Greasy	Bacillus	-
PSBMe	Yellow	Irregular	Wavy	Greasy	Short Bacillus	-
PSBMf	Yellow	Irregular	Wavy	Greasy	Short Bacillus	-
PSBMg	Yellow	Irregular	Wavy	Greasy	Bacillus	-
PSBMh	Yellow	Circular	Entire	Greasy	Bacillus	-
PSBMi	Gray	Circular	Entire	Greasy	Bacillus	-

the culture medium. This decrease in pH appears to indicate the production of organic acid by PSB (Vassilev *et al.*, 2006). PSB Mi was the most effective phosphate solubilizer. This isolate appears to be among the most efficient phosphate-solubilizing bacterial strains when compared to other reports of microbial solubilization of phosphate substrates (Hernández-Leal *et al.*, 2011). Chen *et al.* (2006) reported that *Arthrobacter ureafaciens* was able to solubilize 36.8 $\mu\text{g mL}^{-1}$ of phosphate, whereas *Gordonia* spp. solubilized 31.5 $\mu\text{g mL}^{-1}$ of phosphate. According to Samiran *et al.* (2010) *Bacillus* spp. TRSB16 consistently showed high solubilization rates of tricalcium phosphate (144 $\mu\text{g mL}^{-1}$), and similar results have been reported by Ponnurugan and Gopi (2006a).

The production of IAA by the isolated bacteria ranged from 1.38 to 8.86 $\mu\text{g mL}^{-1}$. Similar results were reported for *Brevibacillus* B-I, which produced 4 $\mu\text{g mL}^{-1}$ of IAA (Vivas *et al.*, 2005). *P. fluorescens* AK1 and *P. aeruginosa* AK2 produced 4.0 $\mu\text{g mL}^{-1}$ and 3.9 $\mu\text{g mL}^{-1}$ of IAA, respectively (Pant and Agrawal, 2014). Lara *et al.* (2011) reported a high range of IAA production of 3.0–45 $\mu\text{g mL}^{-1}$ by the plant growth-promoting bacteria isolated from the rhizosphere of banana, yucca, maize, grass, and cotton in the Sinú zone of the Department of Córdoba, Colombia.

All isolated bacteria were able to produce GA at a range of 0.08 to 9.29 $\mu\text{g mL}^{-1}$. These values were higher than those found by UmaMaheswari *et al.* (2013), who reported on endophytic bacteria that produced GA in a range of 0.75 to 2.83 $\mu\text{g mL}^{-1}$. Gonzalez-Lopez *et al.* (1986) found that the production of GA by *Azotobacter vinelandii* ranged from 0.8

to 3.1 $\mu\text{g mL}^{-1}$. From the nine PSB strains described here, PSBMh (*P. luteola*), PSBMg (*P. luteola*), and PSB Mi (*A. hydrophila*) were the most efficient biofertilizers for maguey espadín cultivation under greenhouse conditions as has been demonstrated by Bautista-Cruz *et al.* (2015).

Biochemical and morphological tests revealed taxonomic diversity among the strains studied. Amos and Harwood (1998) noted that many factors could be related to taxonomic diversity in a bacterial population, such as the site of origin of the strains and the association of the bacteria with the crop.

Conclusion

The soils cropped with maguey espadín support a diverse group of potential phosphate-solubilizing bacteria. Mountain soil had a rich microflora population density in which phosphate-solubilizing bacteria were found. Of all the strains isolated from the three different topographies, PSB Mi exhibited the maximum phosphate-solubilizing ability. All the selected PSB strains were able to produce indole-3-acetic-acid and gibberellic acid. Biochemical and morphological tests indicated genetic diversity among the strains studied. According to previous studies, these phosphate-solubilizing bacteria could serve as effective biofertilizers to improve the phosphorus-nutrition of maguey espadín plants. Moreover, inoculation, adaption, and succession in the plant rhizosphere is easier for native strains than for those that have been isolated from a different environment.

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