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CHARACTERIZATION OF PHOSPHATE SOLUBILISING BACTERIA ISOLATED FROM THE TEA RHIZOSPHERIC SOIL FROM DARJEELING HILLS

[1,3]Rashi Subba, [1,4]Binod C Sharma and [2]Aniruddha Saha
[1]Postgraduate Department of Botany, Darjeeling Govt. College, Darjeeling-734101, WB, India
[2]Department of Botany, University of North Bengal, Rajarammohunpur, Dt. Darjeeling, WB, India
[3]Present address: Dept. of Botany, Vidyasagar College for Women, Kolkata-700006, WB, India
[4] Present address: Department of Botany, ABN Seal College, Coochbehar-736101, WB, India
[3]Corresponding author: rashis1402@gmail.com

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ABSTRACT

Most soils contain insoluble inorganic phosphates but they are of no use to crops unless it is solubilised. Hence phosphate solubilising microorganisms play a key role in solubilising the tricalcium phosphate (TCP) and make it available to the plants. The present study was aimed to isolate and characterize the selected phosphate solubilizing bacteria from rhizospheric soil of tea from Singla Tea Estate, Darjeeling. Bacteria which showed clear zones in Pikovskaya's agar were selected and screened for further characterization. The two isolates were found to solubilise TCP in Pikovskaya agar and were designated as GCS1 and GCS2. *In vitro* phosphate solubilisation ability of these isolates was determined and it was observed that phosphate solubilisation was associated with the reduction in the pH of the medium. These isolates were also found to produce growth promoting substance IAA. These isolates were found to survive well at different pH levels of 5, 7 and 9 and at two different temperatures (room temperature and 37^{0}C). Germination of fenugreek seeds were augmented by these isolates. The isolates were identified as *Kurthia sp.* (GCS1) *and Bacillus cereus* (GCS2) *at* IMTECH, Chandigarh, India. This study revealed the presence of potent phosphate solubilising bacteria from rhizosphere tea plants which may be used as bioinoculants after detailed on-farm as well as off-farm investigations.

Keywords: Darjeeling, Phosphate solubilising bacteria (PSB), TCP, IAA, tea.

INTRODUCTION

Phosphorus is a major essential macronutrient required for plant growth and development. A greater part of soil phosphorus, approximately 95-99% is present in the form of insoluble phosphates and hence cannot be utilized by the plants (Vassileva *et al.,* 1998). To increase the availability of phosphorus for plants, large amounts of fertilizer are used on a regular basis. But after application, a considerable proportion of the applied phosphorus is quickly transferred to the insoluble form (Omar, 1998). In this way, very little percentage of the applied phosphorus is used, making continuous application necessary (Abd Alla, 1994). Therefore, soil microorganisms play a key role in soil P dynamics and subsequent availability of phosphate to plants (Richardson, 2001). Phosphate solubilising bacteria are common in the rhizosphere and secretion of organic acids and phosphatases are common method of facilitating conversion of insoluble forms of phosphate to plant-available forms (Kim *et al.,* 1998). PSB application has promoted P-uptake as well as the yields in several crops (Khalid *et al.,* 2004). They are capable of producing phytohormones and growth promoting substances. The production of Indole Acetic Acid (IAA), gibberellins and cytokinins by PSB has been reported earlier by several workers (Khalid *et al.,* 2004). This research article describes the isolation and characterization of PSB from tea rhizospheric soil of Darjeeling hills (Subba, 2013).

MATERIALS AND METHODS

Isolation of phosphate solubilizing bacteria

Ten gram (10 g) of soil sample was suspended in 90 ml of sterile distilled water and 10^{-1} dilution was obtained. Serial dilutions were prepared by mixing 1 ml of the suspension made into 9 ml sterile water blanks, until the 10^{-7} dilution was obtained. The Pikovskaya's agar medium (10 g Glucose, 5 g tricalcium phosphate, 0.5 g ammonium sulphate, 0.2 g potassium sulphate, 0.1 g magnesium sulphate, 0.5 g yeast extract, trace amount of manganese sulphate and ferrous sulphate, 20 g agar, 1000 ml distilled water) was used for isolation, enumeration and maintenance of PSB (Pikovskaya, 1948). The serially diluted soil suspensions were spread-plated on Pikovskaya's agar plates and incubated at 37^{0}C for 7 days. Bacterial colonies causing clear zones by a turbid white background were selected and purified for further study. The colony diameter of PSB colony (halo zones) was measured by using metric scale. Two phosphate solubilising

bacterial strains thus screened were selected for further analysis.

Quantification of P solubilization

The phosphorus solubilizing potential of PSB strains was tested *in vitro* by estimating available phosphorus in the Pikovskaya's broth amended with known amount of tricalcium phosphate as a substrate. A control without any inoculation was also maintained. The organisms were allowed to grow for 7, 14, 21 and 28 days at 37^0C and centrifuged at 10,000 rpm for 10 min in a cooling centrifuge (REMI-C30BL, Remi, India). Soluble phosphorus was determined in supernatant following the standard protocol (Fiske and Subbarow, 1925).

Measurement of pH

A change in pH of the medium due to the growth of PSB was measured with a pH meter (Elico, India) after 7, 14, 21, 28 days of incubation.

IAA Production

The production of IAA was determined following the standard protocol (Bano and Mussarat, 2003). The tested bacterial strains were grown in LC medium in the presence of tryptophan (100mg/l) and incubated at 30^0C. The IAA production by bacterial strains was measured after 3 days of incubation. A 2 ml culture was removed from each tube and centrifuged at 10,000 rpm for 15 min in a cooling centrifuge (REMI-C30BL, Remi, India). One milliliter of supernatant fluid was transferred to fresh tube to which 100 µl of 10 mM orthophosphoric acid and 2 ml of reagent consisting of 1 ml of 0.5 $FeCl_3$ in 50 ml of 35% $HClO_4$ were added sequentially. The absorbance of the developed pink color was read at 530 nm after 25 min. The IAA concentration in the culture was determined by using a calibration curve of pure IAA as a standard, following linear regression analysis.

Effect of different pH on the survivability of selected PSB isolates

A loopful of bacteria from overnight grown broth cultures of two PSB isolates were inoculated to PKV broth with different pH i.e., 5, 7, 9 and incubated at 37^0C. At each day interval 0.1 ml of the suspension was spread plated on nutrient agar plates and colony forming units (cfu/ml) were calculated up to 7 days.

Survivability pattern of PSB isolates in sterilised soil at different temperatures

Perforated polypacks of 2 kg capacity were filled with soil collected from actively engaged agricultural field and sterilised in an autoclave at 15 psi for 1 hour each day for 3 days. Each pot in duplicate was inoculated with broth cultures of PSB isolates GCS1 and GCS2 and incubated at two temperature regimes i.e., room temperature and 37^0C and cfu/g of soil was determined by serial dilution spreadplating technique on nutrient agar after one day and thereafter every fifteen days upto 60 days.

Effect of PSB isolates on seed germination of fenugreek *(Trigonella foenicum)*

Seeds of fenugreek were surface sterilized with 0.1% $HgCl_2$ (3 minutes) followed by successive washings with sterile distilled water (Shende *et al.,* 1977) and treated with 4 days old liquid cultures of these PSB strains GCS1 and GCS2. The control seeds were treated with the sterilized medium alone. They were placed (50 each) on water soaked sterile filter paper in petridishes and incubated at room temperature. Germination was recorded each day upto 7 days.

RESULTS

Two colonies which showed clear zone on Pikovskaya's agar were selected for further characterization and designated as GCS1 and GCS2. They were sent to IMTEH (MTCC), Chandigarh for the identification and were identified as *Kurthia sp.* (GCS1) and *Bacillus cereus* (GCS2).

Table 1: Diameter of halozone of PSB isolates on PKV agar plates after 7 days of incubation

PSB isolates	Diameter of bacterial colony (mm)	Diameter of halo zone (mm)
GCS1	10	12
GCS2	10	11

Diameters of colony as well as clearing zones were measured. It was found that the halozone around the colony of GCS1 measured 12mm and GCS2 as 11mm whereas the diameters of both the colonies were 10mm. Isolate GCS1 solubilise more phosphates than GCS2 in the Pikovskaya's agar.

Table 2: Final pH and soluble phosphate (SP) in Pikovskaya's broth inoculated with PSB isolates after 7, 14, 21 and 28 days of incubation.

PSB Isolates	No. of days (Incubation time)							
	7		14		21		28	
	pH	**SP (ppm) (mean±sd)	pH	SP (ppm) (mean±sd)	pH	SP (ppm) (mean±sd)	pH	SP (ppm) (mean±sd)
GCS1	5.60	40.62 ± 1.13	5.51	44.30 ± 0.80	5.32	47.51 ± 1.56	5.23	49.57 ± 1.56
GCS2	4.69	136.72 ± 1.70	4.57	146.56 ± 1.77	4.41	166.75 ± 1.15	4.07	193.29 ± 1.52

The above data shows that *in vitro* solubilisation of phosphate by two isolates GCS1 and GCS2 in PKV broth after 7, 14, 21 and 28 days of incubation and corresponding change in pH of the medium. In case of isolate GCS1, it was observed that there was increase in available phosphate from 40.6 to 49.5 with the decrease in pH from 5.6 to 5.2 after 28 days of incubation where the initial pH was 7. Similarly with respect to isolate GCS2 there was increase in available phosphate from 136.7 to 193.2 with the decrease in pH from 4.6 to 4 where the initial pH was adjusted to 7.

Table 3: *In vitro* production of IAA by PSB isolates after 3 days of incubation

PSB isolates	IAA production (ppm)
GCS1	17.50
GCS2	30.00

Both the isolates were grown in tryptophan supplemented medium for the production of IAA and it was estimated after 3 days of incubation. Isolate GCS2 produced higher amount of IAA i.e. 30ppm than GCS1 which produced 17.5 ppm.

PKV broth was adjusted to three different levels pH 5, 7 and 9 using NaOH/HCl. Population (cfu/ml) of PSB isolates in PKV broth at pH 5,7 and 9 were determined on daily basis up to 7 days of incubation. Both the isolates GCS1 and GCS2 showed maximum cfu/ml at acidic pH 5 throughout the period of study. However both the PSB isolates showed their survivability at pH 7 and 9 also.

Two PSB strains inoculated in the sterile garden soil, incubated at two different temperatures i.e., room temperature and 37°C. Population (cfu/ml) of PSB was observed after 1, 15, 30, 45 and 60 days of incubation. It was observed that population of both the isolates GCS1 and GCS2 decreased with respect to days of incubation but both the strains found to survive till the end of study (60 days).

Surface sterilised fenugreek seeds treated with PSB isolates were placed on moistened filter paper for germination and observed up to 7 days and percentage of germination was calculated. The treatment of fenugreek seeds with these two PSB isolates was found to be beneficial. Percent increase in germination over control was found to be 10.0% by GCS1 to 12.5% by GCS2.

Table 4: Survivability pattern of selected PSB isolates at different pH upto 7 days of incubation

PSB isolates	pH	(cfu×10^5 /ml)						
		Incubation period (days)						
		1	2	3	4	5	6	7
GCS1	5	588.2	437.1	95	82.4	67.3	5	3.4
	7	77.8	54.9	52.6	29.8	9.2	3	2.7
	9	58.4	51.5	35.7	5.5	2.5	2.3	1
GCS2	5	307.4	291.1	268.7	25.2	23.9	22.9	5.9
	7	292	285.2	242.1	20.9	20.4	10.4	2.2
	9	235.3	232.5	209.2	17.9	12.7	2	2

Table 5: Survivability pattern of selected PSB isolates in sterilised soil at different incubation temperatures

Incubation period	Population (cfu×10^5/g)									
	1 day		15 days		30 days		45 days		60 days	
PSB isolates	Incubation temp									
	Room temp	37^0C	Room temp	37^0C	Room temp	37^0C	Room temp	37^0C	Room temp	37^0C
GCS1	60.0	82.2	31.4	69.6	21.2	27.6	8.4	7.6	3.0	1.04
GCS2	51.0	53.6	35.2	40.8	16.0	26.4	10.8	14.6	1.28	1.12

Table 6: Effect of selected PSB isolates on seed germination of fenugreek (*Trigonella foenum*)

PSB isolates	Germination (%)	Increase over control (%)
GCS1	88	10.0
GCS2	90	12.5

DISCUSSION

In the present study it was observed that there was the presence of phosphate solubilising bacteria in the rhizospheric soil of tea plants of Darjeeling hills. Bacteria were selected on the basis of their ability to solubilise insoluble tricalcium phosphates in Pikovskaya's agar media as most of the researchers follow this method. They were screened in both the solid and liquid media for the phosphate solubilisation.

In this study both the isolates showed halozone of 11 and 12 mm which is almost similar to the findings of Murumkar *et al*. (2012) who reported that 47 isolates of *Bacillus megaterium* showed zone of clearance on PKV agar with the halozone diameter ranging from 5 to 14 mm.

According to Gupta *et al*. (1994) that some isolates with little clear zone on solid medium exhibited high efficiency for dissolving insoluble phosphates in liquid medium and some showed large clearance zones on agar but low phosphate solubilisation in liquid medium. Similar type of observations were noticed with the two isolates GCS1 and GCS2, where GCS1 solubilise more insoluble tricalcium phosphate in PKV agar than GCS2 whereas in PKV broth GCS2 was found to be efficient phosphate solubiliser than GCS1. In both the cases it was noticed that there was decrease in pH with the increase in phosphate solubilisation. This shows that there was the release of organic acids due to which there was reduction in the pH. This shows that the plate technique is insufficient to detect all phosphate solubiliers as commented by Nautiyal (1999) that some microbes should be screened in broth cultures for the identification of most efficient solubilisers.

Alikhani *et al*. (2006) reported that significant drops in pH accompanied with the release of soluble phosphate from

TCP in the culture supernatants. This confirms the implication of organic acid production in P solubilisation by PSB like rhizobia also (Halder & Chakrabarty, 1993).

In the present study isolates GCS1 produced 17.5 ppm and GCS2 30ppm IAA in the presence of physiological precursor tryptophan which was found to be higher than the findings of Boro *et al*. (2004) who reported that the *Azospirillum* sp. and PSB isolates produced varying quantity of IAA from tryptophan which ranged from 2.0 to 10.5 ppm and 7.6 to 12.7 ppm respectively.

According to Shahab *et al*. (2009), the range of IAA production in PSB isolates with tryptophan was 57-288 µg/ml which was higher than our findings.

The variation in the production of IAA may be because that IAA production by bacteria can vary among different species and strains, and it is also influenced by culture condition, growth stage and substrate availability as reported by Mutluru and Konada (2007).

This large variation may be because of inherent properties of the individual bacteria and environmental factors prevailing in the bioassay, which has been reported for analyses of auxins in different soils (Sarwar *et al*., 1992).

In the present study it was found that population of PSB isolates in acidic pH (pH 5) was higher throughout the period of study i.e. for 7 days of incubation. However they were able to grow well at pH 7 and 9 showing wide pH tolerance. It may be that as they were isolated from acidic soil as reported by Subba *et al*. (2021) and could survive at the acidic pH well. This characteristic of these PSB isolates may be considered for universal use as bioinoculants for hilly region where the soil is acidic as well as other regions with higher pH. In a study by Jung *et al*. (2002) it was found that decrease in pH from 7.24 to 5.49 the population of PSB remained constant. Malakooti and Nafisi (1995) declared that the best pH for phosphorus uptake by plants is 6.5. The most efficient use of phosphate in neutral and calcareous soils occurs between pH 6 to 7 (Sharpley, 2006). *Pseudomonas corrugata* isolated from Sikkim himalaya (Pandey and Palni, 1998) gave moderate growth at pH 6-8 and showed wide range of pH tolerance i.e., from 4-11.

It was observed that there was reduction in the population of these PSB isolates GCS1 and GCS2 after every 15 days of incubation, till up to 60 days (duration of observation) both at room temperature and at 37˚C. These results are in line with the earlier findings of Anandham *et al*. (2006) who reported that the incubation period also resulted in a significant decrease in the population. This may be attributed to the depletion of nutrients, moisture and autolysis of cells as pointed out by Gaind and Gaur, 1990. In both the cases it was observed that the population of GCS1 and GCS2 was found to be more in the room temperature than that of 37˚C and it may be because that these isolates are from the rhizospheric soil of hilly region where the optimum temperature remains 20˚C to 35˚C during summer season.

Seed germination of Fenugreek seeds by these PSB isolates GCS1 and GCS2 was found to be 88% and 90% i.e. 10% and 12.5% higher over the control respectively. GCS2 identified as *Bacillus cereus* was found to be most potent in the enhancement of seed germination of fenugreek which may be attributed to the production of higher amount of IAA i.e. 30 ppm than GCS1 (17.5ppm). Sindhu *et al.* (2002) reported that this may be due to their rapid colonization and stimulation of plant growth and also because they offer unique characteristics (e.g. stress-resistant spores) which may be appropriate for seed inoculants (De Freitas *et al.,* 1997). These findings have been further supported by Fulchieri and Frioni (1994) who have stated that the effects on yield due to bacterial inoculations depend on the bacterial strain, plant cultivar and environmental conditions.

CONCLUSION

The results in this study have shown that there was the presence of PSB in the tea rhizospheric soil of Darjeeling hills such as GCS1 (*Kurthia sp.*) and GCS2 (*Bacillus cereus*) and are capable of solubilising insoluble tricalcium phosphates in the Pikovskaya's media both in the solid and in the liquid thereby decreasing the pH of the medium. These two isolates were able of produce growth promoting substance IAA in the presence of physiological precursor tryptophan. They are able to survive at three different pH levels 5, 7 and 9. Both the isolates survived at room temperature and 37^0C even after 60 days of incubation. They even showed good effect in the seed germination of fenugreek. Darjeeling hill soil is the habitat of good population of phosphate sulubilising bacteria and more investigations are warranted before their use as bioinoculants in the organic system of farming.

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