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ISOLATION AND CHARACTERISATION OF PHOSPHATE SOLUBILISING BACTERIA FROM *RHIZOSPHERIC* SOIL OF ORANGE TREES OF DARJEELING HILLS, INDIA

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ABSTRACT

Plant growth promoting rhizobacteria (PGPR) influence the plant growth by improvement in the nutrient mobilisation, solubilisation and production of plant growth promoting substances. The *Rhizospheric* soil samples of orange (Citrus reticulata Blanco) trees growing in Mangwa Busty, Darjeeling district was screened for the presence of solubilisation of tricalcium phosphate (TCP) in Pikovskaya's (PKV) agar and two bacterial isolates showing clearing zone around their colonies were selected and subjected to characterisation. The isolates were designated as GCM1 and GCM2. They were further analysed for their capacity to solubilise tricalcium phosphate (TCP) in PKV broth. Phosphate solubilisation was accompanied with a reduction in the pH of the medium from 7.2 to 5.22 (GCM1) after 7 days of incubation. Furthermore these isolates were capable of producing plant growth promoting substance, indole acetic acid (IAA). These isolates (GCM1 & GCM2) were further grown at different glucose concentrations of 5g/l, 10g/l, 15g/l and 20g/l. It was observed that higher concentration of glucose yielded the higher amount of soluble phosphate in the medium. Isolate GCM2 solubilised 200.18 ppm phosphate at 20g/l glucose concentration and pH was reduced to 4.94. Further their survivality were tested at different pH of 5, 7 and 9 and it was found that they could survive well at acidic pH of 5 upto 7 days of incubation. These two isolates GCM1 and GCM2 were identified as Bacillus sp. and Pseudomonas sp. at IMTECH (MTCC), Chandigarh, India. Both the isolates were found to increase the growth of orange seedlings in greenhouse condition. These isolates may be used as field inoculants in the orange plantation and other agricultural systems preceded by detailed agronomical evaluation.

Keywords: phosphate, bacteria, IAA, orange, solubilising

INTRODUCTION

Microorganisms are important in agriculture because of its role in promoting the circulation of plant nutrients and reduce the need for chemical fertilizers as much as possible (Cakmakc *et al*., 2006). Phosphate solubilising microorganisms are capable of solubilising tricalcium, aluminium and iron phosphates, as well as rock phosphate making the phosphorus present in the soil available to the plants (Katznelson *et al*., 1962**).** Soil also contains organic phosphorus, which can be used by crops only if it is mineralized. Organisms that increase plant available phosphate (P) in the soil system belong to a diversified group including bacteria, actinomyces and several groups of fungi. The composition and dynamics of this functional group was influenced greatly by vegetation type, soil texture, soil chemical elements, and pH of soil solution (Lin *et al*., 2000). 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). This conversion is through acidification (Louw and Webley, 1959), chelation and exchange reactions (Gerke, 1992) and presence of strong organic acids (Alexander, 1977), which have become indicators for routine isolation and selection procedures of phosphate solubilising bacteria (Illmer *et al*., 1995). Organic acids produced by PSB solubilize insoluble phosphates by lowering the pH, chelation of cations and competing with phosphate for adsorption sites in the soil (Nahas, 1996). A direct correlation between drop in pH and increase in available P of the culture media has been observed in certain cases (Liu *et al*., 1992). In few others, the degree of solubilisation was not always proportional to the decline in pH (Asea *et al*., 1988).

Phosphate solubilizing microorganisms have an important contribution to overall plant P nutrition and growth, and have increased yields of many crops (Rodriguez and Fraga, 1999). One of the most commonly reported direct plant growth promotion mechanism by bacteria is the production of plant growth substances such as auxins, gibberellins (Chanway, 2002).

Inoculation of phosphate solubilising microorganisms improved growth and increased the yield and P uptake in a variety of crop plants (Hameeda *et al*., 2006).The present investigation was undertaken to isolate and characterize the phosphate solubilising bacteria from the *Rhizospheric* soil of orange plantations of Darjeeling Hills (Subba, 2013). QQA

MATERIALS AND METHODS

Isolation of phosphate solubilizing bacteria

Ten gram (10 g) of *Rhizospheric* 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. Pikovskaya's agar (10g Glucose, 5g tricalcium phosphate, 0.5g ammonium sulphate, 0.2g 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) medium was used for isolation and maintenance of PSB (Pikovskaya, 1948). The serially diluted soil suspensions were spread-plated on Pikovskaya's agar plates and incubated at 37^0C for 7 days. Bacterial colonies causing clear zones by a turbid white background were considered as phosphate solubilisers. The diameter of PSB colony as well as halo zones were measured by using metric scale. Two of the potential phosphate solubilising bacterial isolates screened were selected for further characterisation. All the chemicals, reagents used in this work except otherwise stated were obtained from Hi-Media Laboratories, Mumbai, India.

Quantification of P solubilization

The phosphate solubilizing potential of PSB strains were 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 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 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% $HClO_4$. 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 glucose concentration on phosphate solubilisation by selected PSB isolates

Selected PSB isolates were assessed for their capacity to release soluble phosphate in PKV broth with varying concentration of glucose i.e. 5, 10, 15 and 20 g/l. The soluble phosphate was read after 7 days of incubation at 37°C following the protocol described in section 2.2.

2.6. Effect of different pH on the survivality 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.

Effect of PSB isolates on growth of orange *(Citrus reticulata* Blanco*)* seedlings

The study was conducted at Green house of Darjeeling Govt. College. Seeds of orange (*Citrus reticulata*) were collected from the healthy fruits of same plant and germinated in sterilized soil contained in a polypack. Seedlings of uniform size (40 mm height) were selected, roots freed of soil, washed with sterile distilled water and treated with PKV broth having confluent growth of PSB isolates for 2 hours. Garden soil actively engaged in agriculture was filled in 2 kg capacity perforated black polybags and sterilized at 15 psi for 1 hour in an autoclave for three consecutive days. The treated seedlings of orange were transplanted in the polypacks containing sterilized soil in April 2011 and kept in green house condition. Weeding and watering was done regularly. All the measurements were performed after one year of growth i.e. in April, 2012. Root length and plant height measured in mm scale.

Statistical analysis: All the *in vitro* studies were replicated thrice and data represented as mean±standard deviation (mean±sd).

RESULTS AND DISCUSSION

Two colonies which showed clear zone on Pikovskaya's agar were selected for characterization and designated as GCM1 and GCM2. They were sent to IMTECH (MTCC), Chandigarh for the identification and were identified as *Bacillus sp*. (GCM1) and *Pseudomonas sp*. (GCM2)

Table 1

Diameter of colony as well as clearing zone were measured and subjected for calculation of SI and SE and the results are presented in Table 1. Diameter of halo zone around the colonies of both the selected isolates was 12 mm. Solubilisation index and the solubilisation efficiency of these isolates on solid media ranged from 2.2 to 4 and 120% to 300% respectively. GCM2 (*Pseudomonas* sp.) showed higher solubilisation efficiency than GCM1 (*Bacillus sp.*).

Table 2. depicts the data of *in vitro* phosphate solubilising ability of PSB isolates in PKV broth after 7 days of incubation and corresponding change in pH of the medium. Increase in available phosphate of 50.49 ppm with respect to pH decrease of 5.22 was observed with strain GCM1 (*Bacillus* sp.), similar type of relationship between pH and available P was observed with strain GCM2 (*Pseudomonas* sp.).

These two PSB isolates were grown in tryptophan

Table 1: SI & SE of PSB isolates on PKV agar plates after 7 days of incubation

PSB isolates	Diameter of bacterial colony (mm)	Diameter of halo zone (mm)	Solubilisation Index (SI)	Solubilisation Efficiency (SE) (%)
GCM1: *Bacillus sp.*	10	12	2.2	120
GCM2: *Pseudomonas sp*	4	12	4	300

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

PSB isolates	pH	SP ppm (mean±sd)
GCM1	5.22	50.49 ± 1.04
GCM2	5.29	32.19 ± 1.37

Table 3: In vitro production of IAA by PSB isolates

PSB isolates	IAA (ppm)
GCM1	40.00
GCM2	35.00

Table 4: Final pH and soluble phosphate (SP) of Pikovskaya's broth containing different concentrations of glucose and inoculatedwithPSBisolates,after7daysofincubation

PSB Isolates	Concentration of glucose in Pikovskaya's broth (g/l)							
	5		10		15		20	
	pH	SP ppm (mean±sd)	pH	SP ppm (mean±sd)	pH	SP ppm (mean±sd)	pH	SP ppm (mean±sd)
GCM1	4.84	45.22±1.14	5.22	50. 49±1.04	5.46	68.53±1.36	4.53	145.15±1.70
GCM2	6.69	29.20±1.61	5.19	32.19±1.37	5.44	49.87±1.24	4.94	200.18±1.28

Table 5: Survivality pattern of selected PSB isolates at different pH upto 7 days of incubation

PSB isolates	pH	(cfu×105 /ml) Incubation period (days)						
		1	2	3	4	5	6	7
GCM1	5	537.9	441.7	423.4	146.9	22.2	2	1.6
	7	501.2	197.7	189.7	24.6	17.2	1.6	1.3
	9	242	166.4	155.6	18.4	14.3	1.2	1.0
GCM2	5	206.9	174.4	159	82.2	12.2	5.5	2.6
	7	181.5	169.8	85.83	54.7	7.2	2.7	2.2
	9	122.0	118.7	58.6	32.8	3.5	2.7	1.7

Table 6: Growth parameters of PSB isolates treated Orange (Citrus reticulata Blanco) seedlings and percentage of increase over control (in parenthesis)

PSB Isolates	Plant Height (mm)	Length of Main root (mm)	No. of leaves per plant	No. of Branches per plant
GCM1	180 (25%)	135 (42.10%)	34 (61.90%)	2
GCM2	175 (21.52%)	130 (36.84%)	32 (52.38%)	2
CONTROL	144	95	21	1

supplemented medium and IAA content in the media was estimated after 3 days of incubation. They were able to produce IAA in the presence of a physiological precursor, tryptophan in the culture medium. Isolate GCM1 (*Bacillus* sp.) produced higher amount of IAA (40 ppm) than GCM2 (*Pseudomonas* sp.) which produced 35 ppm.

Table 4:

These two strains were further allowed to grow in Pikovskaya's broth with varying concentration of glucose and observe for phosphate solubilisation and change in pH after 7days of incubation. Both the strains solubilised phosphate in increasing order with increasing glucose concentration and showed maximum solubilisation at 20g/l glucose concentration. Simultaneously, reduction in pH was observed from 7.2 to 4.84 at the lowest level of glucose concentration 5g/l. by GCM1 whereas GCM2 showed the reduction in pH from 7.2 to 4.94 at 20g/l glucose concentration.

Table 5

PKV broth was adjusted to three different pH with NaOH/ HCl and tested for growth response of PSB isolates upto 7 days of incubation. Population (cfu/ml) of PSB isolates in PKV broth at pH 5, 7 and 9 were determined on daily basis upto 7 days of incubation. Both the isolates GCM1 and GCM2 showed maximum cfu/ml at acidic pH (5) throughout the period of study.

Table 6

The treatment of orange seedlings with PSB isolates was beneficial as there was increase in the plant height, length of the main root, the number of leaves per plant and the number of branches per plant as compared to control as observed in all cases. Inoculation with strain *Bacillus* sp. (GCM1) resulted in 25%, 42.10% and 61.90% increased in plant height, length of main root and the number of leaves respectively. Similarly, treatment with *Pseudomonas* sp. (GCM2), there was increase in a plant height (21.52%), length of main root (36.84%), no. of leaves (52.38%) as well as no. of branches as compared to control.

Discussion

The present study indicated the presence of phosphate solubilising bacteria in the *Rhizospheric* soil of orange plants. Many researchers have selected the phosphate solubilising bacteria on the basis of their ability to solubilize tricalcium phosphate in the solid media and the same method was followed in the study as well. Bacteria were screened according to their ability to solubilize tricalcium phosphate in the solid media and subsequent evaluation in liquid media

Earlier studies (Chen *et. al.,* 2005) discussed that the solubilisation of TCP in the liquid medium by different strains was accompanied by a significant drop in pH (to 4.9 and 6.0) from an initial pH of 6.8–7.0 after 72 hours of incubation. The soluble-phosphate concentration in the medium ranged between 31.5 and 519.7 mg/l with variations among different isolates. According to Rodriguez *et. al.,* 2004; the decrease in pH values clearly indicates the production of acids, which is considered the main mechanism responsible for phosphate solubilisation. Nautiyal *et.al.,* (2000) suggested that microorganisms, which decrease the medium pH during growth are efficient phosphate solubilisers. These findings were found to be true in this present study also where range of pH of the isolates were 5.22 and 5.29 with corresponding available phosphate 50.49mg/l and 32.19 mg/l after seven days of incubation. It may be because of the production of organic acids which has already been mentioned by the earlier researchers.

Both these bacterial isolates were able to produce IAA *in-vitro* in the presence of physiological precursor tryptophan in the culture medium. The amount of IAA produced was higher (GCM1:- *Bacillus* sp. - 40 ppm; GCM2:- *Pseudomonas* sp. - 35 ppm) than that have been reported by Husen (2003) which ranged from 2.09 to 33.28 ppm. Phosphate solubilising bacteria which are capable of producing physiologically active auxins may have pronounced effects on plant growth. An increase of IAA production was observed in the presence of tryptophan (Ponmurugan and Gopi, 2006; Suresh *et. al*., 2010).

Many researchers have studied the effect of carbon sources of phosphate solubilisation (Narsian and Patel, 2000). PVK broth containing glucose as a carbon source showed maximum solubilisation of phosphorus in broth culture with resulting low pH (Yadav *et. al.,* 2011).

Glucose concentration directly influenced the level of soluble phosphate in PKV broth in the present study. Out of the four glucose concentration used in the media (5, 10, 15 and 20g/l), highest soluble phosphate was observed at 20 g/l glucose. Jung *et. al*., (2002) reported that increase in glucose concentration to 20g/l enhanced solubilisation of phosphate to 349-1675 ppm with abrupt drop in pH.

Nautiyal (1999) also found that not only was glucose necessary, but its concentration was important for bacterial phosphate solubilisation in liquid. Soluble phosphate concentration increased with an increase in glucose by *Pseudomonas* sp. (Nautiyal, 1999).

In a study by Jung *et. al*., (2002) it was found that there was decrease in pH from 7.24 to 5.49, but at the same time the population of PSB remained constant. *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. Similar to this observation PSB isolate GCM2 (*Pseudomonas* sp.) in our study showed highest population at pH 5 throughout the period of incubation (upto 7 days). However they were able to grow well at pH 7 and 9 showing wide pH tolerance. The form in which phosphate exists also changes according to soil pH. Below pH 6.0, most phosphate will be present as monovalent H_2PO_4 species. The plant uptake is also high at the pH range of 5.0-6.0, which indicates that phosphate is taken up as monovalent form (Furihata *et. al.,* 1992).

Acidic pH of the *Rhizospheric* soils from which the strains were isolated may be one of the factors for the optimum growth and survival of these isolates in low pH. This property of selected PSB isolates would be advantageous as their use as biofertiliser organisms preferably in hill agriculture where soil is generally acidic.

Egamberdiyeva *et. al.,* (2004) reported that PGPR in combination with phosphorit significantly increased shoot, root length of wheat and maize. There were stimulatory effects of *Pseudomonas, Bacillus, Arthrobacter and Rhizobium* on growth of wheat, maize and cotton growth, yield, N, P-uptake, soil phosphate content.

The effect of these PSB isolates on orange plants was determined by a pot trial. *Bacillus* sp. (GCM1) was found to be more effective strain in improving plant height, root length and number of leaves and branches of orange plants. This enhancement represented 25%, 42.10% and 61.90% over the control, respectively (Table 6).

The PSB strain *Pseudomonas sp*. (GCM2) which solubilised more phosphate, showed less impact on growth of orange plants than *Bacillus* sp. (GCM1) which solubilised least

P. This effect may be supplemented by the production of plant growth promoting substance as *Bacillus* sp. (GCM1) produced the highest IAA (40 ppm) than GCM2 strain.

CONCLUSION

In conclusion, result of this study have shown that GCM1 (*Bacillus* sp.) and GCM2 (*Pseudomonas* sp.) isolated from the *Rhizospheric* soil of orange tree are capable of solubilising tricalcium phosphate both in solid and liquid media thereby decreasing the pH of the medium, capable of producing growth promoting substance IAA, solubilize high amount of insoluble phosphate in the presence of highest glucose concentration and able to survive at different pH. Treatment of orange seedlings by these isolates showed good growth rate. We can conclude that these two bacterial isolates showed very good result *in vitro* and need to do further investigations on these before field application as bioinoculants.

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