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Tomato Growth Promotion Induced by Stress Tolerant Phosphate Solubilising *Pseudomonas simiae* in Arid trans-Himalaya

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ABSTRACT

Pseudomonas simiae isolated from Seabuckthorn rhizosphere solubilised insoluble phosphate at 4-40 ºC, pH 4-12 and in presence of 1-5 per cent salt concentration. The optimum condition was observed at 28 ºC, pH 6 and devoid of any salt stress. $Ca_3(PO_4)_2$ was solubilised to a great extent than $FePO_4$ and $AlPO_4$. The isolate possess plant growth promoting attributes such as IAA (32 mg l^{-1}), siderophore (78 percent) and HCN (0.1 OD at A_{625}) production. Seed bacterisation resulted in 30 per cent and 51 per cent enhanced shoot and root length, respectively in tomato seedling. Pot experiments revealed enhanced plant growth in *P. simiae* treated plants in both green shade net and open field conditions. Fruit yield was 9.8 per cent and 19.8 per cent higher over control in open and shade net condition, respectively.

Keywords: Abiotic stress; *Hippophae*; Ladakh; *Lycopersicon esculentum*; PGPR

1. INTRODUCTION

Phosphate solubilising microbes play key role in mediating between inorganic and organic soil phosphorus fraction. Plant require only 30 µmol l^{-1} of phosphorus for maximum productivity, but only 1 µmol l^{-1} of phosphorus is available in many soil. Hence, the unavailability of phosphorus in various soils has been known as a major limiting factor for plant growth[1]. The soil beneficial microbes in the microbiome plant roots endow plant with vital services as they provide nourishment and fortification against plant pathogens[2,3]. The microorganism inhabiting higher altitude of Himalayan regions are able to endure, flourish and become accustomed to changing extreme environmental condition with remarkable diversity[4]. The ability of these cold adapted psychrophiles and psychrotrophs have been reported to possess plant growth promoting ability[5]. The present work focus on investigation of *Pseudomonas simiae* PS2 to solubilize phosphate under temperature, pH and salt stress; and its ability to promote tomato growth in trans-Himalayan Ladakh.

2. MATERIALS AND METHODS

2.1 Isolation and Characterisation of Phosphate Solubilising Bacteria

Seabuckthorn (*Hippophae rhamnoides* L.) rhizospheric soil was sampled from experimental field of Defence Institute of High Altitude Research (34º34.7′N, 77º29.9′E, elevation 3060 m amsl) in trans-Himalaya. Soil adhering the root was collected in plastic bag and store at 4ºC until analysis. Ten-fold dilutions were made in phosphate buffer and 10^{-4} to 10^{-6} dilution was plated on Pikovskaya agar medium[6] containing tricalcium phosphate as a sole source of phosphorus. Isolate PS2, which had a marked phosphate solubilising activity on Pikovskaya agar, was identified as *Pseudomonas simiae* using polyphasic approach by Microbial Type Culture Collection (MTCC), Institute of Microbial Technology, India.

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2.2 *In Vitro* Insoluble Inorganic Phosphate Solubilization under Stress Conditions

P. simiae PS2 was screened in Pikovskaya liquid medium for insoluble phosphate solubilization potential at 4-44 ºC; pH 4-12; 1-5 percent KCl, NaCl, and $CaCl_2$ salt concentration; and PEG 10-50 percent. One variable was changed at a time while maintaining the other factors as constant. The isolate was also tested for its capacity to solubilise two other common types of insoluble phosphates viz. $FePO_4$ and $AlPO_4$ with the concentration range of 0.2-1.2 percent. The assay was done in 50 ml of Pikovskaya broth medium inoculated with 1 ml of culture (10^9 cells ml^{-1}). Uninoculated flasks served as a control. The flasks were incubated with shaking (200 rpm) on a rotary shaker. The culture was harvested by centrifugation at 11,400 rpm for 30 min. Supernatant was used to assess phosphate released into the solution.

2.3 Analytical Method

The cell growth was determined by measuring absorbance at 600 nm recorded in a micro-plate reader (SpectroMax M2e, Molecular Devices, Sunnyvale, CA,

United States). Samples from culture grown in the medium with insoluble phosphate were diluted with 1N HCl (1:1 v/v) to dissolve the residual insoluble phosphate and measured against a blank identically treated[7]. Solubilised phosphate content of the culture filtrate was determined by the molybdenum blue method[8]. Change in pH of the culture broth was recorded using a pH meter (SensION$^+$ PH3, HACH, Barcelona) equipped with a glass electrode in supernatant after centrifugation. IAA production was studied using Salkowski reagent according to Patten & Glick[9]. Siderophore production was estimated by the chrome azurol-S assay[10] and HCN production was determined by the qualitative method of Bakker & Schipper[11].

2.4 Plant Growth Promotion Bioassay

Plant growth promotion ability of the isolate PS2 was determined by paper towel method. Tomato seed were surface sterilised with 1 percent sodium hypochlorite and coated with peat based inoculums (10^8 - 10^9 CFU ml^{-1}) using 1 percent carboxylmethylcellulose as adhesive[12] and air dried. The cell count was 10^7 - 10^8 CFU per seed. After bacterization, 20 seeds were placed in each germination paper towel and incubate in a plant growth chamber (Adaptis A1000, Conviron, Canada) at 25 °C and 60 percent relative humidity. Seeds treated only with peat served as control. Five replications for each treatment were maintained and repeated thrice. Shoot and root length were recorded after 10 days.

Pot culture assay for plant growth promotion ability of the isolate PS2 was determined in non-sterile soil mixed with farm yard manure (10:1) in a completely randomised design in open and green shade net conditions. A pre-planting soil test using a soil testing kit (Lamotte Combination Soil Outfit 5010-01, Maryland, USA) revealed pH 7.8, high humus, nitrate nitrogen 100 lb/a, phosphorus 100 lb/a and potassium 120 lb/a. Fifteen pots each with PS2 as well as control were maintained. Urea (0.2 g/ pot) was applied in two split doses at vegetative and reproductive growth stage. Root of 30 days old tomato (*var. Tolstoi*) seedlings were washed with sterile distilled water and soaked in peat based bacterial suspension at the concentration of 10^9 cells ml^{-1} about 30 min prior to transplanting. Plant growth was recorded at 30 days, 60 days, and 90 days after transplant (DAT). At the end of the experimental period, plants were uprooted, washed under running water, and root/shoot biomass and fruit yield were determined. The mean maximum and minimum temperature during the crop growing period was 21.0±2.6 ºC and 8.3±2.0 ºC, respectively.

2.5 Statistical Analysis

The data was analysed using one way analysis of variance (ANOVA) and Student's *t* –test using statistical package of social science (SPSS Inc.) determined at $p \leq 0.05$.

3. RESULTS AND DISCUSSION

3.1 Phosphate Solubilisation under Stress Conditions

Isolate PS2 tolerance to incubation temperatures for $Ca_3(PO_4)_2$ solubilisation, concomitant microbial growth and media pH are showed in Table 1. The highest growth and phosphate solubilising activity was obtained at 28 °C at which soluble phosphate concentration was 224 mgl^{-1}, with maximum fall in media pH after 5 days of incubation. Comparable outcome have been described during the course of phosphate solubilisation by *Rhodotorula* with optimal activity at 30 °C[13] and *Pseudomonas* sp. showing both psychrotophic (4 °C) to mesophilic (28 °C) range and improves plant growth[14] Phosphate solubilising ability at lower temperature may be because of the cold adapted nature of the isolate in cold arid conditions of trans-Himalaya. Significant drop in media pH may be because of release of organic acid during the course of phosphate solubilisation. Effect of initial pH on phosphate solubilisation and growth of PS2 are shown in Table 2. The isolate was able to solubilise $Ca_3(PO_4)_2$ at pH 4-12 with optimal activity (223 mgl^{-1}) at pH 6. Wide pH tolerance exhibited by the isolate may be due to its isolation from dry and salt affected ecosystem. A pH tolerant PSB has been reported from alkaline soil[15]. *Pantoea agglomerans* and *Rhodotorula* sp have also been reported to solubilize phosphate at wide pH and temperature range[13,16].

Growth and phosphate solubilization of isolate PS2

Table 1. Effect of incubation temperatures on $Ca_3(PO_4)_2$ solubilization by *Pseudomonas simiae* PS2 and concomitant microbial growth and final media pH

Temp (°C)	*Periods (days)	Soluble P (mg l^{-1})	Growth (A_{600})	Final pH
4	9	155.6±2.9^d	0.83±0.003^d	4.4±0.02^g
8	8	175.2±1.7^e	0.94±0.003^e	4.2±0.02^f
12	8	182.9±0.5^e	0.99±0.004^e	3.7±0.02^e
16	7	196.3±3.3^e	1.00±0.002^e	3.5±0.01^d
20	7	206.8±1.7^g	1.16±0.059^f	3.4±0.01^c
24	6	212.2±0.2^g	1.31±0.004^g	3.2±0.03^b
28	5	224.1±1.8^h	1.44±0.013^h	3.1±0.03^a
32	3	105.2±1.9^c	0.74±0.004^c	5.5±0.03^h
36	2	42.7±1.8^b	0.35±0.001^b	5.6±0.01^i
40	2	12.0±0.8^a	0.12±0.001^a	5.7±0.02^j

Different superscripts in a column differ significantly (P<0.05)

*Incubation period (days) at which maximum phosphate solubilization was observed

Table 2. Effect of initial pH on $Ca_3(PO_4)_2$ solubilization by Pseudomonas simiae PS2 at 28°C on 5th day after incubation

pH	Soluble P (mg l^{-1})	Growth (A_{600})
4.0	134.3±0.4^{cd}	1.16±0.002^g
5.0	147.6±4.8^{de}	1.23±0.004^h
6.0	223.7±2.3^f	1.27±0.003^i
7.0	214.2±2.9^f	1.04±0.002^f
8.0	154.1±2.7^e	0.98±0.003^e
9.0	152.1±1.2^e	0.95±0.002^d
10.0	138.4±7.2^{cd}	0.77±0.002^c
11.0	125.2±0.8^{ab}	0.66±0.006^b
12.0	121.8±1.5^a	0.50±0.002^a

Different superscripts in a column differ significantly (P<0.05)

under different salts with varying concentrations (1-5 percent) are presented in Table 3. Amount of soluble phosphate production were significantly higher in case of 1 percent NaCl with 227 mg l^{-1} followed by 218 mg l^{-1} at 1 percent KCl. The lowest phosphate solubilising activity was observed in presence of $CaCl_2$. In comparison, *Pseudomonas* PDMZnCd2003 has been reported to tolerant 8 percent NaCl[17]. Solubilising potential of different insoluble phosphate sources viz $Ca_3(PO_4)_2$, $AlPO_4$ and $FePO_4$ ranging from 0.2-1.2 percent (w/v) are shown in Table 4. The isolate solubilize $Ca_3(PO_4)_2$ to a greater extent than $AlPO_4$ and $FePO_4$. Similar results were reported earlier with rhizospheric microbes *Rhodotorula* PS4[13] and *Pantoea agglomerans* R-42[16]. Effect of different PEG concentration (10-50 percent) on phosphate solubilising activity is presented in Table 5. A decreasing trend in growth and phosphate solubilising activity was observed with increasing PEG concentrations. Ability of a microbes to grow in presence of PEG is an indication for drought tolerance as reported in *P. simiae*[18], *Pseudomonas* sp.[19] and *P. putida*[20]. Introduction of drought tolerant microorganisms can alleviate drought stress in plant by deamination of ACC via production of ACC deaminase thereby lowering ethylene level in plant and improving plant growth and development[21].

3.2 Plant Growth Promoting Traits

P. simiae PS2 was tested for plant growth promoting traits at 28°C for 5 days and results are presented in Table 6. IAA production was maximum (32.3±0.5 mg l^{-1}) at 60 h and then showed a decline trend. In comparison, 11.66 mg l^{-1} was reported for *Pseudomonas* sp.[22]. Production of phytohormone stimulates plant growth and facilitates plant to become tolerant against environmental stress[23].

Table 3. Effect of different salts concentrations on $Ca_3(PO_4)_2$ solubilisation by *Pseudomonas simiae* PS2

Salt (percent)	Growth (A_{600})			Soluble phosphate (mg l^{-1})		
	NaCl	KCl	$CaCl_2$	NaCl	KCl	$CaCl_2$
1	$1.20\pm0.002^{e}_{z}$	$1.02\pm0.002^{e}_{y}$	$0.27\pm0.004^{c}_{x}$	$227.2\pm0.5^{e}_{z}$	$218.9\pm0.2^{d}_{y}$	$58.5\pm1.0^{d}_{x}$
2	$0.90\pm0.002^{d}_{y}$	$0.92\pm0.003^{d}_{z}$	$0.34\pm0.004^{d}_{x}$	$167.4\pm3.2^{d}_{y}$	$175.4\pm2.8^{b}_{y}$	$84.4\pm0.7^{e}_{x}$
3	$0.76\pm0.002^{c}_{y}$	$0.84\pm0.018^{c}_{z}$	$0.13\pm0.003^{b}_{x}$	$142.1\pm3.2^{c}_{y}$	$185.1\pm0.5^{c}_{z}$	$48.5\pm0.9^{c}_{x}$
4	$0.62\pm0.004^{b}_{y}$	$0.76\pm0.004^{b}_{z}$	$0.12\pm0.002^{b}_{x}$	$118.9\pm1.0^{b}_{y}$	$186.4\pm2.5^{c}_{z}$	$35.2\pm0.6^{b}_{x}$
5	$0.38\pm0.003^{d}_{y}$	$0.43\pm0.004^{a}_{z}$	$0.10\pm0.002^{a}_{x}$	$94.2\pm2.2^{a}_{y}$	$121.3\pm0.5^{a}_{z}$	$28.8\pm0.6^{a}_{x}$

[a, b, c, d] different superscripts in a column differ significantly ($P<0.05$)
[x, y, z] different subscripts in a row differ significantly (P<0.05)

Table 4. Growth and phosphate solubilization by *Pseudomonas simiae* PS2 in Pikovskaya broth media containing various insoluble phosphate sources

Phosphate source conc. (percent)	Growth (A_{600})			Soluble phosphate (mg l^{-1})		
	$Ca_3(PO_4)_2$	$AlPO_4$	$FePO_4$	$Ca_3(PO_4)_2$	$AlPO_4$	$FePO_4$
0.2	$1.48\pm0.043^{b}_{z}$	$1.03\pm0.002^{c}_{y}$	$0.53\pm0.003^{d}_{x}$	$221.4\pm1.6^{b}_{y}$	$214.1\pm5.2^{c}_{y}$	$91.9\pm2.0^{c}_{x}$
0.4	$1.41\pm0.005^{b}_{z}$	$0.72\pm0.004^{b}_{y}$	$0.49\pm0.003^{c}_{x}$	$233.9\pm1.9^{c}_{z}$	$185.6\pm0.6^{b}_{y}$	$88.9\pm2.0^{c}_{x}$
0.6	$1.39\pm0.013^{b}_{z}$	$0.71\pm0.004^{b}_{y}$	$0.31\pm0.001^{b}_{x}$	213.5 ± 2.2^{b}	126.2 ± 2.6^{a}	79.5 ± 1.4^{b}
1.2	$1.04\pm0.033^{a}_{z}$	$0.65\pm0.005^{a}_{y}$	$0.27\pm0.004^{a}_{x}$	$187.0\pm3.4^{a}_{z}$	$111.3\pm2.2^{a}_{y}$	$64.7\pm1.2^{a}_{x}$

[a, b, c, d] different superscripts in a column differ significantly ($P<0.05$)
[x, y, z] different subscripts in a row differ significantly ($p \leq 0.05$)

Table 5. Growth and phosphate solubilisation by *Pseudomonas simiae* PS2 in modified Pikovskaya broth media containing PEG

PEG (percent)	Growth (A_{600})	Soluble P (mg l^{-1})
10	0.47 ± 0.009^{d}	121.5 ± 5.2^{d}
20	0.25 ± 0.004^{c}	77.3 ± 0.7^{c}
30	0.21 ± 0.003^{b}	53.2 ± 1.0^{b}
40	0.12 ± 0.001^{a}	44.8 ± 1.3^{ab}
50	0.11 ± 0.001^{a}	40.6 ± 0.8^{a}

[a, b, c, d] different superscripts in a column differ significantly ($p \leq 0.05$)

Table 6. Plant growth promoting traits of *Pseudomonas simiae* PS2 at 28°C

Time interval after incubation (h)	Plant growth promoting traits		
	IAA (mg l^{-1})	Siderophore (percent)	HCN (A_{625})
0	0.2 ± 0.1^{a}	0.7 ± 0.2^{a}	0.01 ± 0.00^{a}
12	4.8 ± 0.5^{b}	14.5 ± 0.1^{b}	0.01 ± 0.00^{ab}
24	8.8 ± 0.7^{c}	22.9 ± 0.7^{d}	0.02 ± 0.00^{cd}
36	11.7 ± 0.4^{e}	32.9 ± 0.1^{e}	0.03 ± 0.00^{de}
48	17.7 ± 0.8^{g}	57.2 ± 0.2^{g}	0.05 ± 0.01^{g}
60	32.3 ± 0.1^{i}	77.7 ± 0.5^{h}	0.10 ± 0.01^{h}
72	22.7 ± 0.2^{h}	41.8 ± 0.6^{f}	0.04 ± 0.00^{f}
84	17.0 ± 0.3^{g}	24.3 ± 2.0^{d}	0.03 ± 0.00^{ef}
90	13.5 ± 0.6^{f}	19.9 ± 0.2^{c}	0.02 ± 0.00^{cde}
108	10.3 ± 0.7^{d}	13.4 ± 0.1^{b}	0.02 ± 0.00^{bc}

Different superscripts in a column differ significantly ($p \leq 0.05$)

Siderophore production ranged from 0.7 percent to 78 percent at different time interval and maximum value was recorded at 60 h. Siderophore producing bacteria stimulate plant growth via several mechanisms such as providing Fe to plant, production of organic acid[24] and protection against plant pathogen[25]. Over 43 percent of PSB from Seabuckthorn rhizosphere are known to produce Siderophore[26]. PS2 also showed HCN production with increasing trend up to 60 h followed by a declining trend. Studies on HCN producing isolate from Seabuckthorn rhizosphere is reported to have antagonistic activity against *Fusarium oxysporium* and *Alternaria*[26].

3.3 Plant Growth Promotion Bioassay

PS2 showed plant growth promotion activity as determined by paper towel method (Table 7). The mean shoot and root length of tomato seedling germinated from bacterised seed after 10 days was 4.7±0.9 cm and 8.6±1.2 cm, respectively as compare to 3.6±0.4 cm and 5.7±0.8 cm, respectively in case of untreated seed. Therefore, increased in shoot and root length due to PS2 treatment was 30 percent and 51 percent, respectively over control. *P. simiae* WCS417r has been reported to improve root formation and shoot growth via volatile organic compound secretion[27]. Plant growth promotion via bacterial plant interaction is an intricate phenomenon to which more than one plant growth promotion trait can be attributed.

Results of pot culture assay for plant growth promotion ability of the isolate PS2 is presented in Table 8. Significantly higher vegetative growth (at 30 DAT, 60 DAT, and 90 DAT) and fruit yield (at 90 DAT) was observed in PS2 treated seedlings in open field condition. However, under green shade net condition, enhanced vegetative growth in PS2 treated plants was more conspicuous at 60 DAT and 90 DAT. Increase in fruit yield due to PS2 treatment was 9.8 percent and 19.8 percent in open and green shade net conditions, respectively. Inoculation of mung bean seedling with *Pseudomonas* and *Rhizobium* is reported to improve total dry matter over control[22]. Psychrotolerant *P. fragi* improve wheat seedling shoot and root biomass significantly over control[28]. Psychrotorophic *Pseudomonas* strains isolated from different plant rhizosphere of Himalaya possessing phosphate solubilising activity, IAA, siderophore and HCN production are reported to effectively improve shoot and length, shoot and root dry weight and number of nodule over control in lentil after 40 days of seed bacterization[29].

Table 7. Effect of seed bacterization with *Pseudomonas simiae* PS2 on growth of tomato seedlings

Treatment	Shoot length (cm)	Root length (cm)
PS2	4.7±0.9*	8.6±1.2**
Control	3.6±0.4	5.7±0.8

*Value significantly higher than that of control at $p \leq 0.05$; **value significantly higher than that of control at $p \leq 0.01$ by Student's t-test

4. CONCLUSION

Stress tolerant *Pseudomonas simiae* PS2 was isolated from Seabuckthorn rhizosphere. Insoluble phosphate solubilisation was maximum at 28 ºC, pH 6 and devoid of any salt stress. $Ca_3(PO_4)_2$ was solubilise to a great extent than $FePO_4$ and $AlPO_4$. The isolate possess plant growth promoting attributes such as IAA, siderophore and HCN production. Seed bacterisation resulted in 30 percent and 51 percent enhanced shoot and root length, respectively in tomato seedling. Pot experiments revealed enhanced growth in PS2 treated plants in both green shade net and open field conditions. Fruit yield was 9.8 percent and 19.8 percent higher over control in open and shade net condition, respectively.

Conflict of Interest: None

Table 8. Effect of inoculation of *Pseudomonas simiae* PS2 on growth and fruit yield of tomato (var. Tolstoi)

DAT	Plant growth and yield	Open field		Green shade net	
		PS2	Control	PS2	Control
30	Plant height (cm)	16.1±1.7**	13.2±2.5	15.6±3.1*	13.5±1.8
	Stem dia. (mm)	3.9±0.8	3.7±1.0	4.5±1.4*	3.2±1.1
	Chlorophyll (SPAD Unit)	44.7±6.2*	38.0±6.3	45.3±3.2	46.9±6.6
60	Plant height (cm)	57.8±6.0*	51.2±6.9	57.0±5.5*	49.9±9.7
	Stem dia. (mm)	15.7±3.2 *	12.8±2.6	14.9±4.3	12.4±2.7
	No. of leaf	56.4±8.3**	46.6±7.2	47.1±10.0***	29.6±7.4
	No. of fruit	15.6±9.7	15.2±6.7	10.1±4.6**	4.6±5.1
	No. of bud	37.2±10.1*	29.1±7.0	34.3±8.2**	22.5±10.0
90	Plant height (cm)	60.1±8.3*	53.3±5.1	59.6±3.2*	55.8±4.5
	Stem dia. (mm)	15.3±2.9	14.0±1.9	13.7±1.4*	12.1±2.2
	No. of branch	4.2±0.8*	3.4±0.9	3.6±1.2	4.0±1.1
	Yield (gm plant^{-1})	863.5±71.3*	786.3±124.8	873.7±128.7*	729.1±226.2

*value significantly higher than that of control at $p \leq 0.05$;
**value significantly higher than that of control at $p \leq 0.01$;
***value significantly higher than that of control at $p \leq 0.001$ by Student's t-test

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