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Characterization and Plant Growth Promoting Aspects of a Novel Phosphate Solubilizing Brown Sarson Endophyte *Pseudomonas fluorescens* Strain smppsap5 Isolated from Northern Himalayas of India

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The present study was conducted to reconnoiter the potential of endophytic bacterium of brown sarson root tissues, *Pseudomonas fluorescens* strain smppsap5 for enhancing the growth of brown sarson plants under organic agriculture of Jammu and Kashmir, India. The novel bacterium strain smppsap5 isolated was identified as *Pseudomonas fluorescens* using 16S ribosomal RNA gene sequencing. The isolated strain was selected among 81 isolates for highest phosphate solubilization activity in terms of the phosphate solubilization index (3.19) and phosphate released (313.58 mg/L) from tri-calcium phosphate in the liquid assay. The optimal phosphate solubilizing activity of the strain was determined with different incubation periods and nitrogen sources. It was observed that maximum phosphate solubilization occurred in presence of the nitrogen source tryptone and incubation period of 72h and phosphate solubilization was minimum among all with nitrogen source $NaNO_3$ and incubation period of 24h. The effect of temperature and PEG on growth of this strain was also observed besides many metabolic, physiological and biochemical attributes. The beneficial effect of *Pseudomonas fluorescens* strain smppsap5 in brown sarson plants was observed in terms of root shoot length, germination percentage, chlorophyll content, oil content and other yield attributes besides its inoculation impact on soil physio-chemical and biological properties. The results from the present study suggested that *Pseudomonas fluorescens* strain smppsap5 enhanced the plant and soil health when compared to control and thus it could be used as a potential bio inoculant under integrated, organic and sustainable farming systems.

Keywords: *Pseudomonas fluorescens*, Brown sarson, phosphate solubilization, endophyte.

The liaison of plants with microbes that do not subdue or even stimulate their growth and development has attracted the attention of scientists, not only as a subject to study with respect to the fundamentals of the simultaneity and interaction of different organisms but because of their probable expanding role in the global sustainable agriculture production systems (Chebotar *et al*., 2015). It is an unwavering fact that bacterial endophytes can offer a growth stimulatory effect on host plants, specifically growth promotion and protection against various pathogens; and that under diverse ecological niches bacterial endophytes are able to communicate and interact with the host more efficiently than the counterpart plant growth promoting bacteria of same abilities (Coutinho *et al.,* 2015). Most of the plant nutrients are abundant in soil but are unavailable to the plants, due to a vast number of reasons like in their complex forms or the poor soil conditions. Same is the case with phosphorus the second most important nutrient of plants next to nitrogen. It exists in soil in the

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form of mineral salts or immobilized into organic compounds. Despite being abundant in soil these phosphorus compounds are not available to the plants due to their insolubility (Miller *et al*., 2010). Plants have need of nearly 30 µmol/L of phosphorus for maximum productivity, but there is only about 1 µmol/L available in most of the soils. Therefore, the non-availability of phosphorus in many soils has been witnessed to be a major growth limiting factor in agricultural and horticultural systems (Daniels *et al*., 2009). This obliges the application of soluble forms of phosphorus in the form of phosphate fertilizers, which too has problem of getting immobilized to insoluble forms rapidly as a result of its reaction with aluminum and iron minerals and as such the efficiency of applied phosphorus rarely exceeds 30 percent due to its fixation in soil (Sharma *et al*., 2013). In addition the phosphorus fertilizers are derived from phosphate rock, which is non-renewable resource and current global reserves are already under threat of getting completely perished in 50-100 years (Cordell *et al.,* 2009). Therefore, exploring the alternative forms of agriculture, where nutrient conservation is key, is of vital importance and with this in mind to explore the native endophytic bacterial microflora for phosphate solubilization in particular and plant growth promotion in general is one of the reasonable alternative. Endophytic bacteria use a number of mechanisms to bring the solubilization of phosphorus that includes the activity of their enzymatic systems like phosphatases or phosphohydrolases with the processes like acidification, chelation, exchange reactions, but main mechanism in operation is the solubilization through the release of metabolites such as organic acids (Young *et al.,* 2013).

In various studies we have seen a large number of rhizospheric bacteria being used for enhanced crop production and protection but very limited studies on endophytic bacterial isolates are available. Thus the present study carried out to elucidate the effect of *Pseudomonas fluorescens* strain smppsap5 on growth and yield of brown sarson. Various species of *Pseudomonas* isolates have been widely reported to be efficient phosphate solubilizes and thus used in a huge number of sustainable agriculture production systems (Oteino *et al*., 2015). The brown sarson are grown throughout the India and both in tropical and sub-tropical regions of the world besides being major oil seed crop of the Kashmir valley of J&K for the seeds which are used as a spice and for extraction of oil. The reports on *Pseudomonas fluorescens* as a phosphate solubilizer in brown sarson under temperate conditions is not available. The objectives of present investigation were to determine the adaptability of *Pseudomonas fluorescens* strain smppsap5 (isolated from root tissues of brown sarson) in various stress conditions and to evaluate their role on plant growth performance by determining the leaf pigment content, yield attributes and soil physiochemical and biological properties. The ecological impact of *Pseudomonas* inoculants in soil has often been characterized in terms of composition and size of specific microbial groups (Carrol *et al*., 1995).

MATERIALS AND METHODS

Sampling site

The root samples of brown sarson were collected from three districts of Kashmir valley J&K namely; Anantnag, Srinagar and Baramulla. A total of 60 samples were collected and the sample containing *Pseudomonas fluorescens* strain smppsap5 was collected from Hutmara Anantnag with co-ordinates 33^0 46' 49. 693 N, 75^0 13' 50. 953 E and altitude 5467 ft.

Isolation

The isolation of bacterial isolates was done by washing the root samples vigorously in sterile distilled water for five minutes to remove all the adhered soil particles, surface sterilization was done by keeping the washed samples in 1 percent (wt/vol) active chloride (added as sodium hypochlorite [NaOCl] solution) supplemented with one droplet of Tween 80 per 100 ml solution, and rinsed three times in sterile distilled water. Thereafter, the roots were crushed in sterile petri dishes and a loopful of root sap was streaked on tryptic soy/agar (TSA; Merck Co., Germany) medium plates for bacterial culture and incubated at 30 °C for 48 h. The bacterial colonies were differentiated by their morphology and pigmentation and, re-isolated colonies were separately cultured on fresh TSA medium. For long term preservation, isolated bacterial cultures were stored at 4 °C.

Characterization of Bacterial isolates

Based on the highest solubilization index among 81 isolates, endophytic bacterium *Pseudomonas fluorescens* strain smppsap5 was selected and characterized on the basis of colony morphology, biochemical characteristics and molecular phylogeny. The morphological and biochemical characteristics of the isolates were examined according to Bergey's manual of determinative Bacteriology (Kumar *et al*., 2015).

16S rRNA gene amplification and sequencing

Total genomic DNA was isolated by N-Cetyl-N,N-trimethyl-ammonium bromide (CTAB) method (Doyle and Doyle, 1987; Doyle and Dickson, 1987; Cullings, 1992). The forward and reverse primers used for 16S rDNA amplification were: fD1 (5'AGAGTTTGATCCTGGCTCAG 3') and rD1 (5'AAGGAGGTGATCCAGCCGCA 3') (Luckow et al., 2000) were used to amplify 1500 bp region of 16S rRNA gene using a thermal cycler (BioRad, USA). Amplification products were resolved by agarose-gel electrophoresis (1.5%), and visualized using a gel documentation system (Alfa Imager, Alfa Innotech Corporation, USA). The amplicons were purified using Genei Pure™ quick PCR purification kit (GeNei™, Bengaluru, India) and quantified at 260 nm using a spectrophotometer taking calf thymus DNA as control. The purified partial 16S rDNA amplicons were sequenced in an Applied Biosystems 3130 Genetic Analyzer (Applied Biosystems, CA, and USA).

Analysis of 16S rDNA sequences

The partial sequences of nucleotides were compared with available sequences from NCBI database and sequences showing >99% similarity were retrieved by Nucleotide Basic Local Alignment Search Tool (BLAST N) program available at National Center for Biotechnology Information (NCBI) server (www.ncbi.nlm.nih.gov/BLAST).

PGP traits analysis

Phosphate solubilization

All the isolates were grown in TSA broth. Log phase growing cells of each culture (15µL) were spotted on Pikovskaya's medium plates (Pikovskaya, 1948). These plates were incubated at 28+2^{0}C for 3-4 days. Zone of solubilization was measured and colony size was also measured to calculate solubilization index by the formula; SI = colony diameter+ halo-zone diameter/colony diameter (Edi-Premono *et al.,* 1996). To carry out the assay for phosphate estimation the method given by Bray and Kartz (1945) was employed. The soluble phosphorus formed was estimated calorimetrically. The optimization was carried out at different incubation periods and nitrogen sources viz. Tryptone, peptone, $NaNO_3$, casein, urea.

Indole-3-acetic acid (IAA) production

IAA production was estimated at different levels of L-tryptophan (0.05, 0.1, 0.15, 0.2 and 0.25 mg/mL) and different incubation periods (48, 96 and 144 h) by Salkowski's method (Tang and Borner, 1974). The endophytic bacterial isolate *Pseudomonas fluorescens* strain smppsap5 was inoculated in 25 mL nutrient broth and incubated at 28+2 °C in a shaking BOD incubator. After different incubations, 2mL of each culture broth was centrifuged at 7,000 rpm for two minutes and then IAA was determined in culture supernatant by adding Salkowski's reagent (1 mL of 0.05 M $Fecl_3$ in 50 mL of 35 % HCo_4) of equal volume. The contents were shook continuously for 30 minutes for pink color development which was estimated calorimetrically at 500 nm using spectrophotometer.

Production of ammonia and hydrocyanic acid

The freshly grown culture of *Pseudomonas fluorescens* strain smppsap5 was inoculated in 10 mL peptone water broth and incubated for 72 h at 30+2 °C. About 0.5 mL Nessler's reagent was added and appearance of yellow/brown color indicated the ammonia production (Cappuccino and Sherman, 2010). Quantitatively ammonia production was analyzed by inoculating the fresh culture into peptone water broth for 72 h at 30+2 °C and then centrifuged for 15 min. at 1000 rpm. The supernatant was collected and 1 mL Nessler's reagent was added to 1 mL of supernatant collected and then final volume was made up to 10 mL by adding distilled water. Brown/yellow color was developed and absorbance was measured spectrophotometrically at 630 nm. The amount of ammonia was estimated by relating with the standard curve of ammonium sulphate in the range of 0.1-1 mol/mL (Demutskaya and Kalinichenko, 2010). Then nitrogen source peptone was replaced by yeast extract (at 0.5, 1 and 1.5 %) and incubations of 48, 96 and 144 h.

For hydrocyanic acid (HCN), the Baker and Schippers (1987) was followed in Kings B

medium (King *et al.,* 1954), supplemented with 4.4g/L of glycine. HCN production was inferred with change in color of the filter paper previously dipped in 2% sodium carbonate prepared in 0.05% picric acid, it was rated visually depending upon the intensity of color change from yellow to dark brown. For quantitative assay by hanging method (sadasivam *et al.,* 1992) the bacteria was grown in King's B broth amended with glycine (4.4 g/L) and uniform strips of filter paper (10 × 0.5 cm^2) were soaked in alkaline picrate solution and kept hanging inside the conical flask. After incubation at 28+2 °C for 48 h, the sodium picrate in the filter paper was reduced to reddish compound in fraction to the amount of HCN evolved. The color evaluation was done by placing the filter paper in a test tube containing 10 mL distilled water and the absorbance of this colored water was read at 625 nm.

Siderophore estimation using Chrome-azurol-S (CAS) liquid assay method (Schwan and Neilands, 1987)

In this method 0.1mL of cell free extract of supernatant was mixed with 0.5 mL Chrome-azurol-S (CAS) solution along with 10 µL of shuttle solution (0.2 M 5-Sulfosalicylic acid). It was kept at room temperature for ten minutes and absorbance was recorded at 630 nm. The minimal media was used as a blank and the reference (r) was prepared using exactly the same components except the cell free extract of culture supernatant. The siderophore units were calculated using formula:

Percent Siderophore unit = A_r-A_s/A_s × 100

Where A_r is the absorbance at 630 nm reference

A_s is the absorbance at 630 nm of the test

Antifungal activity

In order to test the efficacy of *Pseudomonas fluorescens* strain smppsap5 as a potential antagonist (*in vitro*) against various root rot pathogens viz. *Fusarium oxysporum, Fusarium solani, Fusarium amphidermatum, Dematophora necatrix*. The assay for antagonism was performed on PDA on petri dishes by dual culture method (Zivkovic *et al.,* 2010). The mycelial plugs (5 mm diameter) of pathogens and then on the same petri dish a loopful of bacteria was then streaked 3 cm away from the disc of the pathogens individually. Paired cultures were incubated at 25 °C for 7 days. The experiment was repeated twice with four replications of each treatment. The percent growth inhibition was calculated using formula:

PGI (%) = KR-R1/KR × 100

Where KR represents the distance (measured in mm) from point of inoculation to the colony margin on the control dishes, and R1 the distance of fungal growth from the point of inoculation to the colony margin on the treated dishes in the direction of the antagonist.

Stress Tolerance

The bacterial isolate was grown at different temperatures and different PEG concentrations and temperature ranges as per the local climatic conditions to observe its ability to grow in possible extreme conditions of the zone.

Plant growth promoting effect of *P. fluorescens* strain smppsap5

Brown sarson seeds (variety Gulchan) were collected from seed processing unit SKUAST Kashmir and surface sterilized with NaOCl (5%) and washed by autoclaved distilled water. The sterilized seeds were sown (after dipped in tryptic soy broth containing 3 days old *Pseudomonas fluorescens* strain smppsap5 culture with 10^8 cfu/mL but the control was dipped in TS broth without bacterial culture) in autoclaved plastic pots containing soil in a greenhouse at 23+2 °C. The isolated bacterial strain *P. fluorescens* strain smppsap5 was inoculated in the pots (with 10^8 cfu/mL) after 3 week interval from the date of sowing. The shoot length, root length, plant nutrient status and other yield attributes were measured at the time of harvesting of the crop. The germination percentage and chlorophyll content was determined as per the procedure adopted by Padder *et al.* (2015).

Effect on soil physio-chemical and biological properties

Various physical and chemical properties of the soil were determined by adopting standard procedures outlined in Jackson (1973), soil nutrient status was determined by adopting various procedures viz. available nitrogen by Subbiah and Asija (1956), available forms of phosphorus, potassium, calcium, magnesium, by Jackson (1973), available Sulphur by Che-Chesnien and Yien (1951), available micronutrients by Lindsay and Norwell (1978).

Soil enzymatic activities

Dehydrogenase activity (DHA) in soil

was determined, using the reduction of 2,3,5-triphenyltertrazolium chloride (3%) method (Klein *et al*., 1971), and the color intensity was measured at 485 nm. The method used for estimating urease activity (URE), involved incubating the soil with an aqueous urea solution (2%), and the residual urea was determined colorimetrically at 527 nm, described by Bremner and Douglas (1971). Phosphatase activity was determined following the method reported by Tabatabai and Bremner (1969), after soil incubation with modified universal buffer, and the produced color intensity was measured colorimetrically at 440 nm. Amidase activity of the inoculated soil was determined by using a modification of the method given by **Frankenberger and Tabatabai (1980**) in which the

Table 1. Morphological, physiological and biochemical characteristics *Pseudomonas fluorescens* strain smppsap5

1. Colony morphology	2. Pigment production	3. Gram reaction	4. Bacterial morphology
Brown, entire, circular, flat	Negative	Negative	Long rods
5. Bacterial arrangement	6. Endospore position	7. Indole production	8. Methyl red test
Singly	No endospore	Negative	Negative
9. Voges Proskauer reaction	10. Citrate utilization	11. Oxidase	12. Catalase
Negative	Positive	Positive	Positive
13. H2S production	14. Starch hydrolysis	15. Cellulose hydrolysis	16. Acid production
Negative	Negative	Negative	Positive
17. Glucose catabolism	18. Galactose catabolism	19. Maltose catabolism	20.Glycerol catabolism
Positive	Positive	Positive	Positive

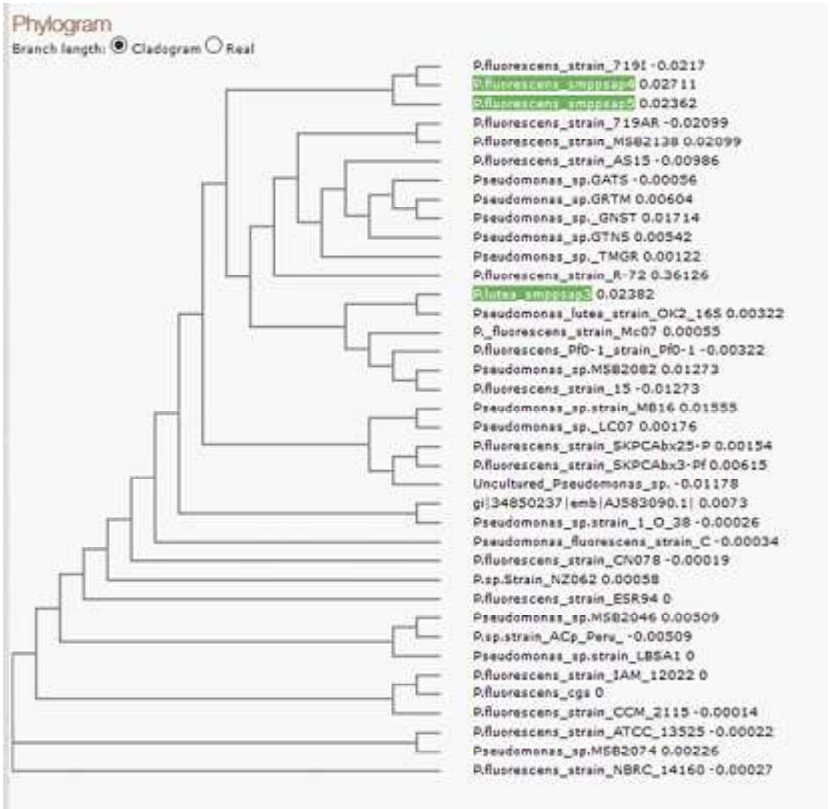

Fig. 1. Phylogenetic relationship of the endophytic *Pseudomonas fluorescens* strain smppsap5 isolated from brown sarson roots

inoculated soil was exposed to toluene. Ten milliliters of THAM (tris (hydroxymethyl) amino methane buffer (0.1M pH 8.5) was added to the assay mixture and incubated at 37 °C for 24 hours. Protease activity was determined by the modified method of Ladd and buttler (1972) in which the wet soil was incubated with 0.05M Tris-HCl buffer and 1% casein for a period of 2h. Cellulase activity was determined by the method of Hope and burns (1987), which measures glucose as the final product of cellulose metabolism, which was quantified by dinitrosalicylic acid method (Miller *et al.,* 1960). Xylanase activity was assayed using 1% oat spelt xylan as the substrate as described by Baily *et al.,* (1992) and glucose content was quantified in the same way as in case of cellulase activity determination.

Statistical Analysis

The experiment was arranged in randomized block design and analysis was performed using SPSS software. The mean values were compared at $p \leq 0.05$.

RESULTS AND DISCUSSION

Bacterial endophytes ubiquitously colonize the internal tissues of plants, being found in nearly every plant worldwide and promote the growth of plants (Santoyo *et al.,* 2016). *Pseudomonas fluorescens* has widely been observed to be an efficient phosphate solubilizer (Yadav *et al*., 2016). In the current study, the plant growth promoting endophytic bacterial isolate *P. fluorescens* strain smppsap5 was selected among 31 phosphate solubilizers and 81 bacterial endophytes of brown sarson roots. Among all the phosphate solubilizers the solubilization index was observed to be as low as 1.17 in SB39 and as high as 3.19 in strain smppsap5 and phosphate released in liquid assay varied from 37 in SB80 to 313.58 mg/L in strain smppsap5 (Fig. 1). While documenting the behavior of *P. fluorescens* towards phosphate solubilization Yadav *et al*. (2016) observed the solubilization index of *P. fluorescens* strain PSM1 to be equivalent to 3.24 but contrary to our findings

Fig. 1. Phosphate solubilization (mg/l) activity of isolated cultures

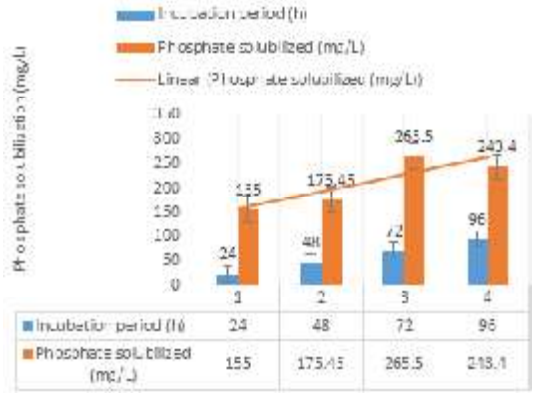

Fig. 2. Phosphate solubilization by *P. fluorescens* strain smppsap5 at different incubations

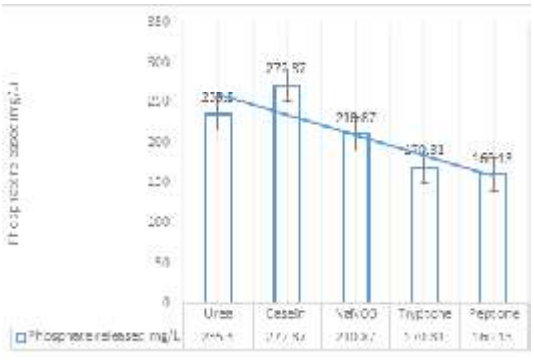

Fig.3. Effect of various nitrogen sources onphosphate solubilization from tri-calcium phosphate

peak phosphate solubilization in liquid assay was observed to be very less (8 mg /L). But endophytic bacterial strain BC-52 produced 400 mg/L phosphate in liquid assay as reported by Lins *et al.* (2014) which is almost similar to our findings. Release of P from mineral phosphate is related to the levels of organic acids produced mainly the gluconc acid (Lugtenberg and Kamilova 2009). In fluorescent pseudomonads, gluconic acid production is catalyzed by periplasmic oxidation of glucose by membrane-bound glucose dehydrogenase and its cofactor, pyrroloquinoline quinone (Meyer *et al.,* 2011). Among all the isolates *P. fluorescens* strain smppsap5 with highest solubilization index of and ability to release phosphate in liquid assay was selected its sequence matched accurately with *P. fluorescens*. The sequence of smppsap5 was submitted to NCBI GenBank and was assigned Accession No. KU883600. The result of phylogenetic analysis revealed that bacterial isolate smppsap5 was identified as *P. fluorescens* on the basis of sequence similarity (Fig.a).

Morphological, biochemical and physiological characteristics of *P. fluorescens* strain smppsap5

The isolate strain smppsap5 was characterized morphologically, biochemically and physiologically based on various tests (Table 1).

Table 2. Effect of yeast extract and incubation period on ammonia production

Incubation period (hr.)	48	96	144	Mean						
Yeast extract concentration (%)	0.5	1.0	1.5	0.5	1.0	1.5	0.5	1.0	1.5	
Isolates										
Strain smppsap5	31.23	35.13	42.10	45.40	66.36	39.29	47.61	48.65	35.26	43.44

C.D ($p \leq 0.05$)Incubation period (A): 0.034; Concentration (B): 0.034; Isolates (C) : 0.075 ;
A X B: 0.058; A X C: 0.130; B X C: 0.130; A X B X C: 0.225

Table 3. Antifungal activity of *Pseudomonas fluorescens* strain smppsap5 against Fusarium solani using dual culture technique

Bacterial isolate	Antifungal activity (% growth inhibition) *Fusarium solani*	
Pseudomonas	Inhibition zone (mm)	Percentage inhibition
*fluorescens*strain smppsap5	43.25 ± 0.47	37.31 ± 0.69

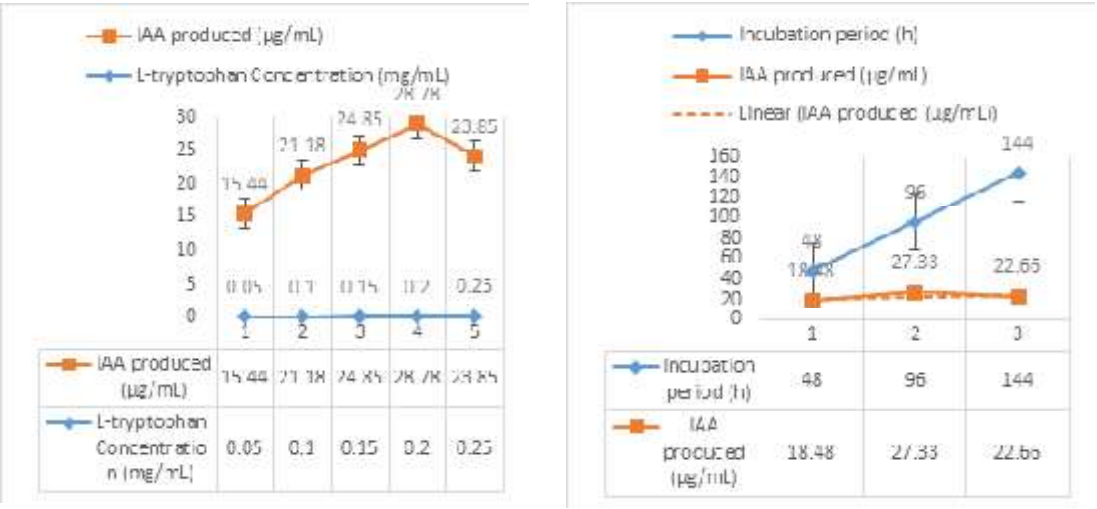

Fig. 4. Effect of incubation period (a) and L-tryptophan concentration (b) on IAA production

Phosphate solubilization at different incubations and various nitrogen sources on phosphate solubilization from tri-calcium phosphate

The endophytic bacterial isolate *P. fluorescens* strain smppsap5 containing 1× 10^8 CFU/mL (» 625 nm) were inoculated into Pikovskaya's broth with different nitrogen sources (Tryptone, peptone, $NaNO_3$, casein, urea and incubated for 24, 48, 72 and 96 h at 28+2 °C. It was observed that *P. fluorescens* strain smppsap5 caused maximum solubilization at 72 h incubation (Fig. 2) and with casein as nitrogen source (Fig. 3), beyond this incubation period the solubility decreased. Contrary to our findings Yadav *et al*. (2016) found maximum phosphate solubilization by *P. fluorescens* at 144 h incubation the possible reason may be that they carried the experiment at different set of pH. But similar to our findings Song *et al.* (2008) observed the maximum phosphate solubilization at 72 h incubation in *Burkholderia cepacia* DA23. Effect of nitrogen sources and incubation period on phosphate solubilization has been studied widely. Sagervanshi *et al*. (2012) observed that among various nitrogen sources the broth with casein as a nitrogen source has the maximum soluble phosphate.

Indole-3-acetic acid (IAA) production

To investigate the effect of incubation period and L-tryptophan concentration on IAA production, the samples were drawn at an interval

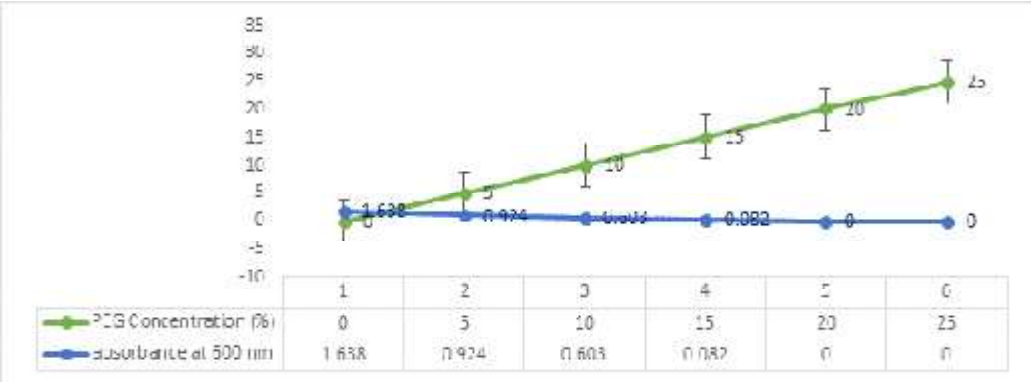

Fig. 5. Growth of strain smppsap5 in terms of absorbance at 600 nm

Table 4. Effect of Temperature on root endophytic bacterial strain smppsap5 population dynamics

Microbial population : Log_{10}(cfu/mL)			
Pseudomonas	Temperature		
fluorescens strain smppsap5	15°C	25°C	35°C
	7.38 ± 1.4	7.49 ± 1.29	7.09 ± 0.57

Table 5. Plant growth promoting effect of *P. fluorescens* strain smppsap5

Treatment	Germ-nation percentage	No. of primary branches	No. of secondary branches	No. of siliqua per plant	No. of seeds per siliqua	Siliqua length (cm)	Oil content (%)
Uninoculated control	83.12	2.25 ± 0.05	5.26 ± 0.25	201.5 ± 0.28	10.5 ± 0.28	4.25 ± 0.29	21.04 ± 0.01
Inoculation with strain smppsap5	88.32	4.25 ± 0.25	7.25 ± 0.25	237.5 ± 0.28	15.5 ± 0.28	5.37 ± 0.25	27.12 ± 0.00
C.D (pd"0.05	2.54	0.71	0.71	0.91	0.71	0.07	0.05
C.V	3.55	14.36	7.20	0.28	3.50	0.96	0.15

of 48 h up to 144 h. The data obtained suggests that there was maximum IAA production (27.33 µg/mL) at 96 h incubation and 28.78 µg/mL at 0.2 mg/mL L-tryptophan (Fig. 4). Our results are in agreement with the findings of Bharucha *et al.* (2013) who worked on impact of L-tryptophan concentration and incubation period on IAA production by *Pseudomonas putida* UB1 and observed that L-tryptophan stimulated the auxin biosynthesis. As per our studies, L-tryptophan concentrations used (ranging from 0.05 to 0.25 mg/mL) which resulted in increase in IAA production and 0.2 mg/mL L-tryptophan concentration resulted in maximum IAA production.

Production of ammonia and hydrocyanic acid

Ammonia production by *P. fluorescens* strain smppsap5 was observed to be equivalent to 66.16 µg/mL of ammonia in peptone water containing 1% peptone similar to our findings Nimnoi *et al* (2010) also reported the production of

Table 6. Effect on plant and soil nutrient status and soil physio-chemical and biological properties

Treatment	Plant nutrient content (%)					
	N	P	K	Ca	Mg	S
Uninoculated control	1.34±0.06	0.13±0.01	1.06±0.00	1.22±0.05	0.12±0.01	0.21±0.01
Inoculation with strain smppsap5	1.95±0.14	0.24±0.02	1.33±0.03	1.34±0.16	0.19±0.01	0.32±0.01
C.D (p≤0.05	0.043	0.004	0.037	0.036	0.020	0.040
C.V	1.861	1.702	2.017	1.920	7.946	1.923
Treatment	Plant nutrient content (ppm)					
	Zn	Cu	Fe	Mn		
Uninoculated control	22.38±0.30	7.78±0.08	81.59±0.22	69.50±0.16		
Inoculation with strain smppsap5	41.71±0.18	14.96±0.13	149.64±0.23	105.28±0.02		
C.D (p≤0.05	0.427	0.266	0.462	0.521		
C.V	0.801	1.369	0.240	0.380		
Treatment	Available soil nutrient content (ppm)					
	N	P	K	Ca	Mg	S
Uninoculated control	176.52±0.10	6.75±0.03	83.10±0.07	12.54±0.12	1,931.20±0.49	458.07±0.45
Inoculation with strain smppsap5	197.40±0.05	12.25±0.03	102.12±0.02	18.23±0.02	2,066.1±0.0	489.35±0.13
C.D (p≤0.05	0.164	0.100	0.145	0.186	1.889	1.137
C.V	0.060	0.723	0.106	0.818	0.065	0.165

Treatment	Available soil nutrient content (ppm)			
	Zn	Cu	Fe	Mn
Uninoculated control	1.27 ± 0.01	1.46 ± 0.01	54.34 ± 0.15	47.38 ±0.25
Inoculation with strain smppsap5	1.71 ± 0.03	1.85 ± 0.03	97.10 ± 0.24	84.20 ± 0.26
C.D (p≤0.05	0.054	0.059	0.064	0.216
C.V	2.341	2.365	0.056	0.228

Table 7. Effect of inoculation on soil enzymatic activity

Treatments	Dehydrogenase (µg of TPF g^{-1} soil h^{-1}).	Protease (µg tyrosine g^{-1} soil h^{-1}).	Phosphatase (µg p-NP g^{-1} soil h^{-1}).	Cellulase (µg GE g^{-1} soil $24h^{-1}$).	Xylanase (µg GE g^{-1} soil $24h^{-1}$).	Urease (NH^{4+}-N g^{-1} soil h^{-1}).	Amidase (NH^{4+}-N g^{-1} soil h^{-1}).
Uninoculated control	148.75	17.00	83.25	173.5	146.25	0.08	0.47
Inoculation with strain smppsap5	208.00	37.00	162.75	194.00	196.00	0.19	0.95
C.D. (pd"0.05)	1.892	1.701	1.882	1.295	1.713	0.005	0.028
C.V.	0.739	4.293	1.164	0.461	0.688	2.223	2.845

20 to 60 mg/mL ammonia by root endophytic bacteria and when peptone was replaced by yeast extract the ammonia produced varied from 41.41, 50.04, and 38.88 µg/mL ammonia at 0.5, 1, 1.5 % yeast extract concentrations respectively indicating that yeast extract although at very low concentrations compared to peptone is metabolized by the bacteria to produce ammonia but beyond 1 % concentration the yield of the ammonia reduces. When the experiment with yeast extract was conducted at different incubations the isolate produced 36.23, 50.35 and 43.84 µg/mL ammonia at 48, 96 and 144 h incubations respectively (Table 2). Thus with yeast extract as nitrogen source the incubation of 96 h and concentration of 1% produced the highest ammonia (µg/mL). Mishra *et al* (2010) also observed *P. fluorescens* to be the efficient ammonia producer. Our findings are also supported by Sharma and Saharan (2015) who also reported highest ammonia production at 96 h incubation, but the optimization with different nitrogen sources is not reported yet.

Hydrocyanic acid (HCN), a volatile metabolite is well known for its role against various pathogens thus plays a vital role in biological control of various plant pathogens and *P. fluorescens* has widely been observed to produce hydrocyanic acid (Schippers, 1988) and hence exposing plants to the volatile metabolites of antagonism causes a prominent increase in peroxidase activity, which may contribute induction of disease resistance. In quantitative estimation, *P. fluorescens* strain smppsap5 recorded the absorbance of 0.109. Similar to our findings Lukkani and Reddy (2014) also reported the production of hydrocyanic acid by *P. fluorescens* in the same absorbance range.

Siderophore production

Siderophores are an important tool of various bacterial isolates by which they bring about the iron starvation of pathogens and as a result is an important tool in the biological control of plant diseases. The root endophyte *P. fluorescens* strain smppsap5 was recorded to produce a zone of 13.50 mm and percent siderophore produced was recorded to be 10.22 %. Shobha and Kumudhini (2012) also reported that *Pseudomonas* sp. is an efficient siderophore producer and they observed that *Bacillus* isolate JUMB7 produces 10 % siderophores, which is similar to our findings. Pal *et al.* (2010) also reported that *Klebsiella* sp. produced 3.22 % and 11.99 %, which falls under the range of our observations. Kaushal and Kaushal (2013) also reported that isolate MK7 produced the zone of 13.33 mm which is same to our findings.

Antifungal activity

Bacterial plant growth promotion is a well-established and complex phenomenon and is often achieved by various plant growth promoting traits exhibited by the associated bacterium, such as antagonism against phytopathogenic fungi (Haas and Defago 2005). The isolate *Pseudomonas fluorescens* strain smppsap5 characterized for antifungal activity against various pathogens viz. *Fusarium oxysporum, Fusarium solani*, *Dematophora necatrix* and *Pythium amphanidermatum*. It was observed that the strain smppsap5 showed antifungal activity against *Fusarium solani* only, the inhibition zone was observed to be 43.25 ± 0.47 and percent growth inhibition of 37.31 ± 0.69 (Table 3). Jenifer *et al*. (2013) also observed that *Pseudomonas fluorescens* causes an inhibition of *Fusarium* sp. by 38.1% but not against *Aspergillus niger* which is similar to our findings. Sagahon *et al*., (2011) also reported that *Pseudomonas fluorescens* showed antifungal activity against phytopathogens. Microbial production of extracellular metabolites like HCN has been demonstrated to contribute to biocontrol of root pathogens (Haas and Defago 2005). It has newly been reported that inorganic phosphate solubilization potential of pseudomonads is often combined with the production of other metabolites taking part in the biological control of soil-borne phytopathogens (Jha *et al*.2009). Our results indicate that the inhibition of the phytopathogens by *Pseudomonas fluorescens* strain smppsap5 could be as a result of the ammonia toxicity brought about in the fugal pathogen niches by the bacterial isolate as it produces 66.16 µg/mL ammonia under *in vitro* conditions and even the isolate has considerable siderophore and HCN activity which could also aid it to bring about the iron starvation for pathogenic fungi or their toxicity respectively.

Stress tolerance

As J&K is a geographical area with extreme climatic conditions, both temperature and

water are the challenges during harsh climatic conditions, therefore the bio inoculant isolated need to be tolerant to such conditions. With this intention the drought tolerance of *Pseudomonas fluorescens* strain smppsap5 was observed by recording the absorbance (at 600 nm) at 0, 5, 10, 15, 20 and 25% Polyethylene glycol (PEG 6000) and temperature tolerance was observed by recording the CFU/mL at 15, 25, 35 ^{o}C as during the Brown sarson growing season the temperature of the area falls in three ranges. It was observed that the strain smppsap5 tolerated the concentration of PEG up to 15% (Fig. 5) and hence can be classified to be moderately resistant to drought conditions. In the same way the strain smppsap5 grew at all the temperature ranges but showed maximum CFU/mL at 25 ^{o}C equaling Log_{10} 7.49 ± 1.29 with little less in other temperature ranges (Table 4), hence it can be concluded that the strain smppsap5 has better adaptability to a set of temperatures with least impact on CFU/mL.

Similar to our findings Marulanda *et al*. (2009) observed that various *Pseudomonas* sp. were resistant to drought stress induced by polyethylene glycol (PEG) although some of the strains showed reduced drought stress tolerance. Michiels *et al.* (1995) reported an increased synthesis of six heat shock proteins in heat-tolerant bacterial isolates at 45 ^{0}C, in context to our findings which may be one of the reason in maintaining the good CFU/mL even at high temperatures of 35 ^{0}C by an isolate *Pseudomonas fluorescens* strain smppsap5 isolated from a temperate climate where temperature usually does not go beyond 30 ^{0}C.

Plant growth promoting effect of *P. fluorescens* strain smppsap5

The brown sarson plants were grown in a greenhouse for the evaluation of inoculation effects of *P. fluorescens* strain smppsap5 on their growth. The uninoculated plants were recorded to have low growth rate compared to the plants inoculated with strain smppsap5 in terms of germination percentage, chlorophyll content, plant nutrient uptake and yield attributes like number of primary/ secondary branches, no. and length of siliqua, no of seeds per siliqua and oil content of the seeds (Table 5).

Ardebili *et al*. (2011) reported that the beneficial effect of *P. fluorescens* CHAO on plant growth enhancement in tomato and observed that bacterial inoculation caused significant growth compared to control. Endophytic microorganisms are able to enhance plant growth through various mechanisms, such as production of plant hormones and antimicrobial metabolites, as well as to provide the soil with nutrients (Lins *et al.,* 2014). Hoon *et al.* (2007) reported that the beneficial effect of *Pseudomonas* sp. on plant growth of pepper and observed an enhanced nutrient uptake and overall yield. Overbeek and Elsas (2008) reported that endophytic bacteria are more often capable of triggering physiological changes that promote the growth and development of the plants. Shi *et al*. (2009) found significant increase in various plant growth parameters compared with control after inoculating selected endophytic bacterial isolates obtained from beet roots. Supporting our findings, Muthukumar *et al*. (2010) found increased germination percentage and other yield parameters of chilli when inoculated with bacterial root endophyte *P. fluorescens*. The bacterial strains of *P. fluorescens* have been reported to increase the growth, yield and germination percentage in maize (Noumavo *et al.,* 2013) and hence supporting our findings. Similar to our investigation, Padder *et al.* (2015) reported that *Pseudomonas* sp. isolates cause increased chlorophyll, carotenoid and other growth parameters. In the same way Jog *et al.* (2014) also reported the increased no. of yield attributes with respect to uninoculated control upon bacterial inoculation which also testifies our findings. This may be as a result of the secretion of some beneficial metabolites by inoculated bacteria or their impact on changed indigenous microbial population in the soil. In general, plant inoculation with *P. fluorescens* strain showed positive effects on brown sarson growth and yield. Differences obtained with the control can be attributed to nutrients being available from the insoluble sources. Thus inoculation with phosphate solubilizing *P. fluorescens* strain smppsap5 made more soluble phosphates available to the growing plants. This may be the reason for improved growth and yield of the host plants. Many bacteria (Rodriguez and Fraga, 1999) are able to promote plant growth by solubilizing sparingly soluble inorganic phosphates in the soil. Moreover *P. fluorescens* strains are considered to be good plant growth promoters through the production of

growth-stimulating hormones (Schorth and Hancock, 1982) and this could be one of the reason to have affected growth, nutrient uptake and finally the yield. Enhancement of yield in numerous crops due to involvement of indirect biocontrol activities of *P. fluorescens* (ammonia, HCN, siderophore etc.) is well documented (Levenfors *et al*. 2008; Kumar *et al*. 2009). Several fluorescent and non-fluorescent strains have been found to increase yield in crops such as potato (Frommel *et al*. 1991), spring wheat (Kropp *et al.* 1996) and cereals (Validov et al. 2009). The both micro as well as macro nutrient content of plants increased upon inoculation when compared to uninoculated control. Similarly in comparison to control the available nutrient was high in the soil inoculated with strain smppsap5 (Table 6).

Measurements of soil enzymatic activities has been used as an indicator of the effect of soil manipulations (Naseby and Lynch, 1998) and may be very much important for gaining a better understanding of the nature of the perturbations caused to ecosystem function after microbial inoculations. Soil enzyme assessment has also been used as an indicator carbon leakage from the host plant roots (Naseby *et al*., 1999). In the present study the soil enzymatic activity increased upon inoculation (Table 7). So, the changes observed in the present study suggest a direct effect of bacterial isolate inoculated, as well as indirect effect through changes in microbial composition in the rhizospheric soil. Rana *et al.* (2015) reported that endophytic bacterial inoculation increased N, P uptake by plants besides microbial biomass carbon and soil biological properties like dehydrogenase activity, alkaline phosphatase activity etc. over uninoculated control. There may be many reasons behind it but the measure reason seems to be the increase in bacterial population inside the soil as a result of inoculation and hence all the dead organic matter gets metabolized to form cell constituents besides release of minerals from their respective sources. Similarly Dutta and Neog (2015) observed increased phosphatase, dehydrogenase and urease activity besides soil carbon content upon bacterial inoculation. Hassan and Bano (2015) reported that inoculation of *Pseudomonas* sp. in wheat resulted in increased grain yield, N, P, Ca, and K content availability in soil and total content in plant respectively. The bacterial inoculants of *Pseudomonas* fluorescent sp. are among the most important plant growth promotors through a number of mechanisms (Glick *et al*., 2007). Some species are known to provide plants with required nutrients like nitrogen, phosphorus, iron etc. (Sexana and Tilak, 1989). El-Ghany *et al*. (2010) reported that bacterial inoculations improve the soil physical properties like EC, bulk density, pH etc. are improved by organic matter degradation products, Microbial gums produced (EPS) and root growth promoting substances enhance soil aggregation process, subsequently soil penetrability resistance decreases. The net result is less cohesion relation to adhesion forces between soil particles, which is in complete synchronization with our findings.

CONCLUSIONS

The summarized findings of the present study on P-mobilizing potential and plant growth promoting traits of *Pseudomonas fluorescens* strain smppsap5 suggests that there are efficient phosphate solubilizers already adapted to agricultural soils and agro-climatic conditions with excellent potential to be used as bio-inoculants. The production of hydrogen cyanide, ammonia and siderophores etc. by the strain prompts us to consider these strains as putative biocontrol agents. These abilities of *Pseudomonas fluorescens* strain smppsap5 need to be subjected to further studies on the behavior with various carriers and concentration of the formulations to be used under field conditions.

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