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Assessment of functional diversity of culturable phosphate solubilizing fluorescent pseudomonads

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ABSTRACT

The experiment was conducted during 2006-08 in SKUAST-K, Shalimar, Srinagar Rhizosphere soil samples were collected from different strawberry fields in Srinagar and Baramulla districts. Among phosphate solubilizing bacteria that colonize aggressively the plant roots fluorescent pseudomonads have been considered as an important group due to their biofertilizing and biocontrol properties. Fluorescent pseudomonad bacteria were isolated on Kings medium B (KB) and were identified under UV light (366nm). Phosphate solubilizing activity was performed by growing the cells in Pikovskaya's broth, soluble free phosphate in culture supernatant was estimated spectrophotometrically by recording absorbance value at 600nm, using the calibration curve with KH_2PO_4. All the isolates were screened for multifaceted plant growth promoting activities like production of IAA, sidrophore, HCN, ACC deaminase, ammonia, activities of enzymes like protease, chitinase, cellulase, pectinase, antagonistic potential and mineral solubilization (K, Zn and Fe) by using standard methods. All the isolates except PS3, PS7 and PS9 produced IAA, siderophore, HCN and ammonia. Screening of antagonistic activity of all the isolates confirmed that out of 20, 17 isolates exhibited antagonistic potential against *Fusarium sp.* with varying levels of inhibition of mycelial growth. However the maximum inhibition was recorded in PS2 isolate. Out of 20 isolates only PS1, PS7 and PS10 produced ACC deaminase. The isolates PS3 and PS7 were negative for proteinase and chitinase activity. Cellulase enzyme was almost abundantly produced by each isolate except PS3, PS7 and PS9 which showed poor cellulose activity. Moderate to poor pectinase activity was shown by all the isolates barring PS9 which did not produce any pectinase. All the isolates except PS7, PS11 and PS16 utilized with varying degrees several carbon sources as identified by Hi-Carbohydrate ™ kit test. The isolates PS3, PS8 and PS9 were unable to solubilize any mineral nutrient while as rest of the 17 strains solubilized insoluble Phosphate, zinc and iron compounds. Numerical taxonomy of isolates based on the basis of their carbon source utilization profile resulted in to two major phenons at 0.27 similarity coefficient level. On the basis of these results it was concluded that there existed some level of variability among the fluorescent pseudomonads found in the rhizosphere of strawberry fields.

INTRODUCTION

Phosphorus being an essential element for growth of plants is critically involved in, photosynthesis, respiration, cellular function, gene transfer, reproduction, root growth and energy production, transfer and storage. At present 5, 49.3, 48.8 and 1.19 % of the Indian soils have adequate, low, medium and high available phosphorus content respectively. A large portion of native as well as applied phosphorus in soils is converted to insoluble form and hence becomes unavailable to the plants.

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Microorganisms have the ability to solubilize the insoluble phosphate and maintain the soil health and quality (**Richardson, 2001**). Bacteria use several direct and indirect mechanisms of action to improve plant growth and health. Mechanisms such as phosphate solubilization, aminocyclopropane-1-carboxylate (ACC) deaminase, phytohormone production, chelate formation, nitrogen cycle, production of antifungal metabolites and HCN production are considered as popular mechanisms. Among phosphate solubilizing bacteria that colonize aggressively the plant roots fluorescent pseudomonads have been considered as an important group due to their biofertilizing and biocontrol properties.

In addition to phosphate solubilization, these bacteria have been found as potential producers of plant growth promoting hormones and enzymes.

MATERIALS AND METHODS

The experiment was conducted during 2006-08 in SKUAST-K, Shalimar, Srinagar Rhizosphere soil samples were collected from different strawberry fields in Srinagar and Baramulla districts. Samples were stored at 4 ^{0}C before being processed with in 24 hours of collection. Isolation of fluorescent pseudomonad bacteria was performed as described by **Kumar *et al.* (2005)**. Briefly soil suspension was obtained by shaking 10 g soil sample having roots with tightly adhering soil in 90 ml of 1M $MgSO_4$. $7H_2O$ buffer for 10 minutes at 180rmp on a rotary shaker, resulting suspensions were serially diluted and 0.1 ml aliquot of each dilution were spread on to Kings medium B (KB) agar in triplicate. After incubation at 28^{0}C for two days fluorescent pseudomonad colonies from replicate plates were identified under UV light (366nm). Purified single colonies were further streaked on to KB agar plates to obtain pure cultures. Stock cultures were made in Luria Bertani (LB) broth containing 50 % glycerol and stored at -86 ^{0}C.

Screening of phosphate solubilizers and estimation of phosphate solubilization

For detecting phosphate solubilizing bacteria, isolates were streaked on to Pikovskaya's agar medium. After 3 days of incubation at 28^{0}C isolates that induced clear zones around the colonies were considered as positive. Phosphate solubilizing activity was performed by growing the cells in liquid medium (pH 7. 0) at 28^{0}C up to 10 days and on 2,4,6,8 and 10 days an aliquot of 5 ml was collected and cells were removed by centrifugation at 9000rpm for 20 minutes. Soluble free phosphate in culture supernatant was estimated spectrophotometrically by recording absorbance value at 600nm, using the calibration curve with KH_2PO_4. The variation in pH of Pikovskaya's medium on 2nd and 10th day of incubation was also observed.

Functional diversity of phosphate solubilizing bacteria

All the isolates were screened qualitatively for multifaceted plant growth promoting activities. These include productions of IAA (**Gorden and Paleg, 1957**), sidrophore (**Schwyn and Neilands 1987**), HCN (**Bakker and Schipper, 1987**), ACC deaminase (**Ramamoorthy *et al.*, 2001**), ammonia (**Dye, 1962**), activities of protease (Smibert and Krieg, 1994), chitinase (**Renwick *et al.*, 1991**), Cellulase (**Cattelan *et al.*, 1999**), pectinase (**Cattelan *et al.*, 1999**). The antagonistic potential of the isolates were determined against *Fusarium sp.* by dual culture method (**Rabindran and Vidyasekaran, 1996**). Solubilization of insoluble mineral nutrients like phosphate (**Katznelson and Bose, 1959**), potassium (**Hu *et al.*, 2006**), zinc (**Bunt and Rovira, 1955**) and iron (**Takayuki and yuzaburo, 1989**) were determined by using specific media.

Phenotypic characterization of phosphate solubilizing bacteria

In order to determine the phenotypic diversity of 20 phosphate solubilizing bacteria,characterization was done on the basis of fluorescence on Kings B medium,levan formation,gelatin liquification and growth at 4^{0}C and 42^{0}C. Gram's reaction was determined by KOH technique (**Ryu, 1940**). Carbon utilization profiles were tested using Hicarbohydates^{TM} kit from Himedia, laboratories Mumbai, India. Here the cells were grown in nutrient broth to reach density of 0. 5 OD at 600nm. An aliquot of 50µl of this suspension was inoculated to each well of Himedia carbohydrate^{TM} kit incubated at 30^{0}C for 24 hours and the results were registered according to the instructions of the manufacturer. The experiment was based on three replications.

Clustering analysis of isolates

On the basis of data derived from the carbon source utilization profile, a matrix with binary code composing positive (+) and negative (-) values was made. UPGMA algorithm was used for hierarchical cluster analysis. Pair wise comparison were calculated using Jaccard's coefficient (**Jaccard, 1912**) and dandrogram was built using the UPGMA method (**Nei and Li, 1979**) using NTSYS-PC2 package(Numerical taxonomy analysis programme package, External soft ware USA).

RESULTS AND DISCUSSION

Plant growth promoting activities

All the identified isolates were screened for multiple number of plant growth promoting activities. It was observed that all the isolates except PS3, PS7 and PS9 produced IAA, siderophore, HCN and ammonia Table 1. The production of these substances reflect the plant growth promoting properties of the fluorescent pseudomonads. Several studies reported that siderophores produced by beneficial pseudomonads reduce iron availability to competing harmful microflora by sequestering it (**Prasana, 2010**), while as HCN metabolite produced by fluorescent pseudomonads act as a biocontrol factor against plant pathogenic fungi (**Voisard *et al.*, 1981**).

Table 1. Production of plant growth promoting substances by *fluorescent pseudomonads*

Isolates	IAA(µ mol ml$^{-1)}$	Siderophore	HCN	Ammonia
PS1	34.12	++	++	++
PS2	28.16	++	++	++
PS3	ND	ND	ND	ND
PS4	14.3	+	+	++
PS5	13.7	+	+	+
PS6	26.9	++	+	+
PS7	ND	ND	ND	ND
PS8	36.08	++	+	+
PS9	ND	ND	ND	ND
PS10	12.4	+	+	+
PS11	11.8	+	+	+
PS12	10.7	+	+	+
PS13	12.9	+	+	+
PS14	11.6	+	+	+
PS15	12.2	+	+	+
PS16	10.1	+	+	+
PS17	9.6	+	+	+
PS18	11.14	+	+	+
PS19	12.4	+	+	+
PS20	14.2	+	+	+

ND = Not detected

Production of enzymes and antifungal metabolites

Out of 20 isolates only PS1, PS7 and PS10 produced ACC deaminase Table 2.

It is reported that ACC deaminase producing bacteria increase root elongation and seed germination by lowering natural plant ethylene levels (**Glick *et al.*, 1995**).

Hi-Carbohydrate™ kit test. The utilization of wide variety of carbon sources by the isolates reflects their ability to accommodate under different soil and plant environments Table 3.

Table 2. Production of enzymes and antifungal metabolites by *fluorescent pseudomonads*

Isolates	ACC deaminase	Protease(µ mol ml$^{-1)}$	Chitinase(µ mol ml$^{-1)}$	Cellulase	Pectinase	Antagonistic activity against *Fusarium sp.*
PS1	+	516.12	6.8	++	++	+
PS2	-	422.16	4.5	++	++	++
PS3	-	-	-	+	++	-
PS4	-	411	4.2	++	++	+
PS5	-	354	5.3	++	++	+
PS6	-	297	3.2	++	++	+
PS7	+	-	-	+	++	+
PS8	-	443	5.1	++	++	+
PS9	-	298.15	2.10	+	-	-
PS10	+	342.9	4.1	++	++	+
PS11	-	426.24	4.7	++	+	+
PS12	-	356.3	6.1	++	+	+
PS13	-	295.9	3.6	++	++	+
PS14	-	387.43	3.8	++	+	+
PS15	-	301.11	5.4	++	+	+
PS16	-	263.8	4.2	++	++	+
PS17	-	461.2	5.3	++	++	-
PS18	-	304.6	4.0	++	++	+
PS19	-	281	2.9	++	+	+
PS20	-	347.2	3.6	++	++	+

Table 3. Carbon source utilization by *fluorescent pseudomonads*

Isolates	Carbon sources										
	Dextrose	Galactose	Mannose	Citrate	Lactose	Xylose	Fructose	Melibiose	L-arabinose	Glycerol	Mannitol
PS1	+	+	+	+	+	+	+	+	+	+	+
PS2	+	+	+	+	+	+	+	+	+	+	+
PS3	+	+	+	+	+	+	+	+	+	+	+
PS4	+	+	+	+	+	+	+	+	+	+	+
PS5	+	+	+	+	+	+	+	+	+	+	+
PS6	+	+	+	+	+	+	+	+	+	+	+
PS7	-	-	-	-	-	-	-	-	-	-	-
PS8	+	+	+	+	+	+	+	+	+	+	+
PS9	+	+	+	+	+	+	+	+	+	+	+
PS10	+	+	+	+	+	+	+	+	+	+	+
PS11	-	-	-	-	-	-	-	-	-	-	-
PS12	+	+	+	+	+	+	+	+	+	+	+
PS13	+	+	+	+	+	+	+	+	+	+	+
PS14	+	+	+	+	+	+	+	+	+	+	+
PS15	+	+	+	+	+	+	+	+	+	+	+
PS16	-	-	-	-	-	-	-	-	-	-	-
PS17	+	+	+	+	+	+	+	+	+	+	+
PS18	+	+	+	+	+	+	+	+	+	+	+
PS19	+	+	+	+	+	+	+	+	+	+	+
PS20	+	+	+	+	+	+	+	+	+	+	+

The isolates PS3 and PS7 were negative for proteinase and chitinase activity. Cellulase enzyme was almost abundantly produced by each isolate except PS3, PS7 and PS9 which showed poor cellulase activity. Moderate to poor pectinase activity was exhibited by all the isolates barring PS9 which did not produce any pectinase. Screening of antagonistic activity of all the isolates confirmed that out of 20, 17 isolates exhibited antagonistic potential against *Fusarium sp*. with varying levels of inhibition of mycelial growth. However the maximum inhibition was recorded in PS2 isolate. This was supported by the findings of **Gnanamanickam *et al.* (2001)** who reported the ability of *Pseudomonas fluorescence* strains to suppress various diseases in rice.

Carbon utilization

All the isolates except PS7, PS11 and PS16 utilized with varying degrees several carbon sources as identified by These findings are supported by the results of **Naik *et al*., (2008)** who reported that fluorescent pseudomonad strains utilized several carbon sources which revealed their adaptability to variety of crop and soil types.

Mineral solubilization

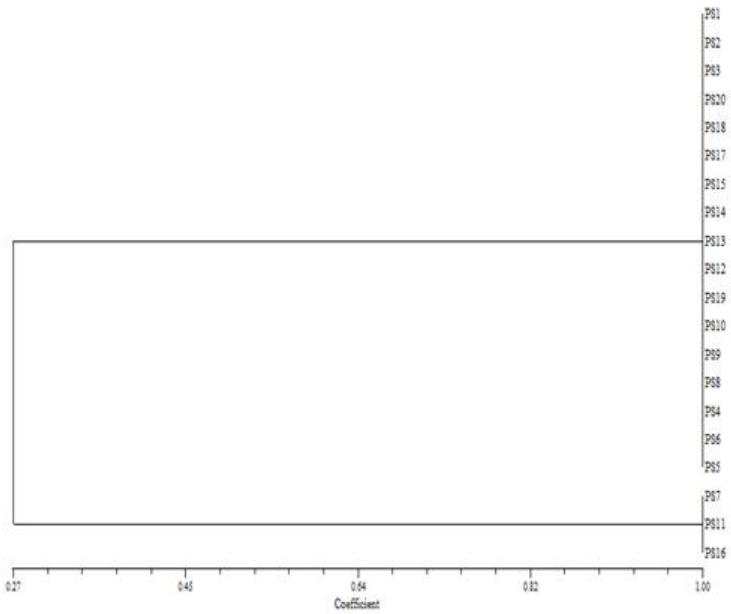
Mineral solubilization is an important mechanism by which the fluorescent pseudomonads enhance plant growth. The mineral (P, K, Zn and Fe) solubilization was recorded after 2nd and 10th day of incubation. From this study it is reported that the isolates PS3, PS7, PS9 and PS16 did not show any phosphate solubilization while as rest of the isolates solubilized the insoluble phosphate with varying degrees.

Table 4. Mineral solubilization (qualitative) by *fluorescent pseudomonads*

Isolates	Mineral solubilization			
	P	Zn	Fe	K
PS1	+++	++	++	++
PS2	+++	++	++	++
PS3	-	-	-	-
PS4	+	+	+	+
PS5	+	+	+	+
PS6	++	++	++	++
PS7	-	+	+	+
PS8	+	-	-	-
PS9	-	-	-	-
PS10	++	++	++	++
PS11	++	++	++	++
PS12	+	++	++	++
PS13	++	++	++	++
PS14	++	++	++	++
PS15	+	+	+	+
PS16	-	+	+	+
PS17	+	+	+	+
PS18	+	+	+	+
PS19	+	+	+	+
PS20	+	++	+	+

Table 5.Tricalcium Phosphate solubilization by *fluorescent pseudomonads*

Isolates	After 2 days of inoculation		After 10 days of inoculation	
	P-solubilized ($\mu g ml^{-1)}$	pH	P-solubilized $\mu g ml^{-1}$	pH
PS1	6.82	6.2	58.6	5.9
PS2	6.56	6.2	55.41	6.10
PS3	0.00	6.9	0.00	6.7
PS4	6.12	6.3	49.2	6.20
PS5	7.21	6.0	60.10	5.8
PS6	7.42	6.0	60.35	5.3
PS7	0.00	6.8	0.00	6.6
PS8	4.28	6.6	45.04	6.4
PS9	0.00	6.7	0.00	6.5
PS10	5.20	6.4	46.10	6.3
PS11	4.96	6.5	46.00	6.3
PS12	5.60	6.4	47.80	6.25
PS13	4.38	6.6	45.3	6.40
PS14	3.94	6.6	44.12	6.40
PS15	6.18	6.3	51.14	6.15
PS16	0.00	7.0	0.00	6.4
PS17	5.4	6.4	47.00	6.25
PS18	3.81	6.7	43.00	6.50
PS19	6.52	6.2	53.81	6.15
PS20	5.96	6.3	47.85	6.25



Jaccard's coefficient

Fig.1. Dendrogram showing two major clusters

The isolate PS6 was found to solubilize maximum phosphate to the tune of 7.42µg ml^{-1} and 60.35µg ml^{-1} on 2nd and 10th day of incubation respectively. Simultaneously all the isolates except PS3, PS8 and PS9 showed K, Zn and Fe solubilization qualitatively. The pH value of the growing medium also decreased with the increase in incubation period in case of all the mineral solubilizing isolates Table 4 and 5. These results revealed the potential of these isolates to secrete the mineral solubilizing enzymes. These observations are supported by the findings of **Priyanthi, (2007), Luis and Renato, (1971) and Venkatakrishnan *et al.* (2003).**

Phenotypic characterization of phosphate solubilizing bacteria

Out of 20 fluorescent pseudomonad isolates, 17 showed mineral solubilization potential. They were Gram negative, motile rod shaped and tested positive for cytochrome oxidase, arginine dihydrolase. The isolates showed variability for features such as gelatin hydrolysis, levan production and growth at 4^0C and 42^0C.

Clustering analysis of isolates

Numerical taxonomy of isolates based on their carbon source utilization profile resulted in to two major phenons at 0.27 similarity coefficient level. On the basis of these results it could be inferred that there existed some degree of variability among the fluorescent pseudomonads in the rhizosphere of strawberry fields. These findings are in conformity with the results of **Naik *et al.* (2008)** who showed that cluster analysis of 80 fluorescent pseudomonad strains on the basis of carbon source utilization resulted in to three distinct genomic clusters.

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