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Characterization of Siderophore Producing Rhizobacteria and its Effect on Growth of Pea

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A B S T R A C T

Bacteria can produce low molecular weight iron chelating compound called siderophore. Siderophore are low molecular weight iron-binding ligands which can bind to ferric ion and make it available to the producer microorganism. On account of that, an attempt was made in the present investigation to isolate potential siderophore producing bacteria from different places of Kashmir and study their effect on pea crop. A total of ten siderophore producing bacteria was isolated from rhizospheric soil sample and amongst them PGP26 was found the most efficient siderophore (18.00). The potential isolates were further characterized for their different plant growth promoting activities like Indole acetic acid production (IAA), ammonia production, phosphate solubilization, and HCN production. The potential isolates were further tried with pea to study the germination percentage, root length and shoot length by Roll towel method.

Introduction

Numerous species of soil bacteria which flourish in the rhizosphere of plants, but which may grow in, on, or around plant tissues, stimulate plant growth by a plethora of mechanisms. These bacteria are collectively known as PGPR (plant growth promoting rhizobacteria) (Akhtar *et al.*, 2012). In the view of increasing demand for food with deteriorating environmental quality due to application of agrochemicals, plant growth promoting rhizobacteria is steadily increasing in agriculture as, it supplements fertilizers and prevent growth of phytopathogens by a wide range of mechanisms. PGPR can promote the plant growth by various direct and indirect mechanism such as phosphate solubilization, nitrogen fixation,indole-3-acetic acid (IAA) production, siderophore production and repression of soil borne pathogens by production of hydrogen cyanide and antibiotics (Glick,1995). Fe is one of the most predominant elements in the earth`s crust its availability to plants

and microorganism is insufficient due to very low solubility of the dominant ferric iron (fe^3) in soil and become unavailable to plants as a micronutrient Siderophore producing plant growth promoting rhizobacteria (PGPR) is required iron in plants by causing solubilization and chelation of organic and inorganic complexes in soil. It helps to protect phytopathogens.

Siderophore producing PGPR for the solubilization and transport of iron from phases to soluble fe^3 complexes that can be taken up by active transport mechanism. The siderophores are non-ribosomal peptides bond. It is multidentate, organic, oxygen donar ligands and classified to hydroxamate, catecholate, caboxyylate and to facilitate solubilization and chelation of transport of iron to cells. Siderophore produced by rhizospheric bacteria improve rhizosphere colonization and play an important role in iron mineralization and supplement to plant (Vannsuyt *et al.,* 2007). In recent years the role of siderophore -producing PGPR in biocontrol of soil borne plant pathogens has created a great interest as it prevents growth of pathogens by chelating iron.

On account of that, the present investigation has been undertaken to isolate the potential siderophore producing bacteria from rhizosphere soil of two pea growing districts of Kashmir valley, viz Srinagar and Baramulla and the potential isolates were tried with pea to evaluate the efficacy in increasing germination (%), root length and shoot length under in viro conditions.

Materials and Methods

Sample collection and bacterial isolation

Soil sample was collected from the rhizosphere region of pea plant from different locations of Srinagar and Baramulla districts. The rhizospheric soil sample was carefully collected in plastic bags under aseptic conditions. The soil sample was air dried and subjected to the isolation of bacteria by spread plate technique. A total of 40 bacteria were isolated from the rhizospheric soil sample and they are further characterized for siderophore production.

Screening for siderophore production

The siderophore production was detected by chrome-azurol-S (CAS) plate assay method (Schwyn and Neilands, 1987). Sterilized blue agar was prepared by mixing CAS (60.5 mg/50ml distilled water) with 5ml iron solution (1mMFeCl3.6H2O and 5ml 10mM HCl). This solution was slowly added to hexadecyltrimethyl ammonium bromide (HDTMA) (72.9 mg/40ml distilled water). Thus, 50 ml CAS dye was prepared and poured into 500 ml nutrient agar and the plates were prepared.

Twenty-four hours old culture of test bacteria was spotted on pre-poured blue coloured CAS agar plates. Plates were incubated for 72 hours at 37^oC. After proper incubation period, siderophore production was confirmed by the presence of orange colour zone around the colony on CAS agar plates and total ten positive colonies were isolated.

In vitro screening of isolates for different plant growth promoting characters

All rhizobacterial isolates obtained were screened for different plant growth promoting traits. Each culture was placed on modified Pikovskaya agar (Pikovaskya *et al.,* 1948) with insoluble tricalcium phosphate (TCP) and incubated at 30±0.1ºC for 5 days to check the phosphate solubilization. IAA production was assayed using qualitative method developed by Bric *et al.,* (1991). Bacterial cultures were inoculated in nutrient broth with tryptophan (1mg/ml) incubated at 35±2ºC for 7 days. Cultures were centrifuged at 3000 rpm for 30 min. 2 mL of supernatant was mixed with 2 drops of orthophosphoric acid and 4 ml of Salkowski's reagent (50 ml, 35% perchloric acid; 1 ml 0.5 FeCl3). The development of a pink colour indicated Indole Acetic Acid (IAA) production (Loper and Schroth, 1986). Bacterial isolates were tested for the production of ammonia in peptone water. Freshly

grown cultures were inoculated in 10 ml peptone water in each tube and incubated for 48 h at 35±2°C. Nessler's reagent (0.5 ml) was added in each tube. Development of brown to yellow colour observed was a positive test for ammonia production (Cappuccino and Sherman, 1992). Isolates were further screened for HCN production. Bacterial cultures were streaked on nutrient agar medium containing 4.4 g/L of glycine. A Whatman filter paper No. 1 soaked in 0.5% picric acid solution (in 2% sodium carbonate) was placed inside the lid of a plate. Plates were sealed with parafilm and incubated at 35 ± 2ºC for 4 days Baker and Schippers (1987).

Biochemical characterization of bacterial isolates

The potential isolates were further characterized on the basis of their staining characteristics and further investigated in terms of biochemical properties like indole, catalase, urease, citrate, oxidase, nitrate producing abilities and fermentation of various sugars, which helped in identifying the bacteria up to genus level (Gupta *et al.,* 2000) by Bergey's manual of Determinative bacteriology (Holt *et al.,* 1994).

Trial with seed germination

Bacterial isolates, PGP1, PGP5, PGP8, PGP10, PGP13, PGP22, PGP26, PGP32, PGP34, PGP38 were tried with pea for seed germination under lab condition. seeds were collected from Dept. of Vegetable science, SKUAST Shalimar and were surface sterilized with 0.1% HgCl2 for 2 min and rinsed with sterile distilled water for 10 times. Bacterial isolates were grown in respective broth on shaking incubator (180 rpm) at 28±2°C for 24 h.

The surface sterilized seeds were inoculated in broth culture for 30 min (ISTA, 1993). Germination tests were carried out using the paper towel method.

Treated seeds and control were seeded onto paper towels. Germination percentage was measured with the following formula: Germination percentage = Number of germinated seeds / Number of seeds in sample × 100. Root length and shoot length of individual was then measured.

Results and Discussion

Screening of siderophore positive isolates

The siderophore production by selected bacterial isolates on chomeazurol-S (CAS) solid medium was compared on the basis of their zone size. Perusal of the data presented in(Table 1)indicates that amongst the 40 rhizobacterial isolates, only 10 isolates produced siderophores. The results further revealed that the isolate PGP26 exhibited highest siderophore production (18.00) and was statistically superior to all other isolates. This was followed by isolates PGP22 (17.50). While least zone size was observed in isolate PGP10 (2.30).

Plant growth promoting activities of the bacterial isolates

A total of ten siderophore positive bacterial isolates were further characterized for their different plant growth promoting activities. It was observed that out of ten bacterial isolates PGP1, PGP8, PGP13, PGP22, PGP26 and PGP38 were positive for IAA production. On Pikovskaya medium, PGP1, PGP5, PGP8, PGP13, PGP22, PGP26, PGP32 and PGP38 showed a development of sharp halo zones (Table 2).

Similar observations has been reported by Ngomle *et al.,* (2014), who state that microorganisms capable of producing a clear zone due to P solubilization in the surrounding medium were selected as potential phosphate solubilizers and where clear zones around the colonies indicated the capacity of phosphate solubilization on Pikovskaya medium. Furthermore, all of the bacterial isolates also exhibited strong production of ammonia from peptone water (Table 2), which is another important trait of PGPR and taken up by plants as a source of nitrogen for their growth (Ahmad *et al.,* 2008). None of the isolates were positive for HCN production.

Table.1 Screening of plant growth promoting rhizobacteria for siderophore production

Isolate No.	Qualitative siderophore estimation (zone size, mm)
PGP1	8.50
PGP5	14.50
PGP8	12.00
PGP10	2.30
PGP13	16.80
PGP22	17.50
PGP26	18.00
PGP32	10.50
PGP34	11.00
PGP38	8.50

Table.2 Plant growth promoting functions of the isolates

Test	PGP1	PGP5	PGP8	PGP10	PGP13	PGP22	PGP26	PGP32	PGP34	PGP38
HCN production	-	-	-	-	-	-	-	-	-	-
NH3 Production	+	+	+	+	+	+	+	+	+	+
IAA production	+	-	+	-	+	+	+	-	-	+
Phosphate production	+	+	+	-	+	+	+	+	-	+

Table.3 Biochemical properties of the siderophore producing bacteria

Test	PGP1	PGP5	PGP8	PGP10	PGP13	PGP22	PGP26	PGP32	PGP34	PGP38
Catalase	+	+	-	+	+	-	-	+	+	+
Methyl red test	-	+	-	-	-	+	-	-	+	+
Indole	+	+	+	_	+	+	-	-	+	-
VP	-	-	-	-	-	-	-	-	-	-
Citrate utilization	-	-	-	-	-	-	-	-	-	-
Urease Production	+	+	+	+	+	+	+	+	_	_
Oxidase	+	+	+	+	+	+	+	+	+	+
H_2S production	-	-	-	-	-	-	-	-	-	-

Table.4 Sugar utilization by siderophore producing bacteria

Isolate No.	De	GLu	Ino	Ce	Mn	Ma	Ga	La
PGP1	+	+	+	-	-	+	-	+
PGP5	+	+	-	+	-	+	-	+
PGP8	+	+	+	+	+	-	-	+
PGP10	+	+	+	+	+	-	-	+
PGP13	+	+	+	+	-	+	-	-
PGP22	+	+	-	+	-	+	-	+
PGP26	+	+	+	+	+	_	-	+
PGP32	-	+	+	+	+	+	-	+
PGP34	+	+	+	+	+	+	-	+
PGP38	+	+	-	-	+	-	-	+

De:Dextrose, Glu:Glucose, Ino:Inositol, Ce:Cellobiose, Mn:Mannose, Ma:Maltose, Ga:Galactose, La:Lactose

Table.5 Effect of siderophore producing plant growth promoting rhizobacteria on germination percentage, root length and shoot length of pea in germination paper

Isolate no.	Pea		
	Root Length(cm)	Shoot Length(cm)	Germination %
Control	5.13	6.23	45.23
PGP1	6.41	8.34	72.13
PGP5	7.13	12.16	77.12
PGP8	8.12	10.16	66.18
PGP10	9.11	13.12	82.16
PGP13	5.18	10.41	85.24
PGP22	10.14	11.51	89.51
PGP26	12.14	13.18	92.10
PGP32	8.10	9.14	90.31
PGP34	7.51	8.16	78.41
PGP38	6.42	7.10	84.61

Biochemical characterization and Identification

The biochemical tests such as oxidase test, H_2S production, catalase, urease production, citrate utilization, Indole were carried out for phenotypic identification of isolates (Holt *et al.*, 1994). All of the siderophore producing isolates were positive for maximum biochemical activities (Tables 3). All of the isolates were positive for maximum sugar utilization (Table 4).

Seed germination test

In this study, an increase in plant growth by seed bacterization has been demonstrated. Plant growth promoting rhizobacteria increased the synthesis of gibberellins, which would have triggered the activity of specific enzymes including amylase to promote early germination, which have brought an increase in availability of starch assimilation (Bharathi *et al.*, 2004).

It is a well-established fact that overall plant growth and root development influenced by improved phosphorous nutrition (Jones *et al.*, 1994). A large number of evidence suggests that PGPR enhance the growth, seed emergence and crop yield (Herman *et*

al., 2008). In the present study, it was found that all of the isolates significantly increased the germination percentage, root and shoot length of pea, over control (Table 5). Highest root Length (12.14cm), shoot elongation (13.18 cm) and germination (92%) was recorded when arkal seeds were pre-treated with PGP26.

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