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SIDEROPHORE QUANTIFICATION OF BACTERIA FROM SUNDARBAN AND ITS EFFECT ON GROWTH OF BRINJAL (*SOLANUM MELONGENA* L.)

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ABSTRACT
Bacteria produce low molecular weight siderophore for efficient iron transport mechanism. On account of that, an attempt was made in the present investigation to study the siderophore production by the bacteria isolated from rhizospheric region of Rice plant from Sundarban, India. A total of five siderophore producing bacteria were isolated from the soil sample and named as SBBA-1 to SBBA-5. Amongst them SBBA-5 was found the most efficient siderophore (77.84% SU) producer. These potential traits were further characterized for their plant growth promoting activities like production of IAA, ammonia, HCN, nitrate, phosphate solubization and N_2-fixation. All the isolates were positive for N_2 -fixation and only SBBA-2 was unable to produce ammonia. The potential isolates were tried with brinjal seeds and determine effect on germination, root length and shoot length. A significant increase in germination (71.25% and 81.25%), root length (6.16 cm and 5.06 cm) and shoot length (7.48 cm and 8.21 cm) observed by SBBA-1 and SBBA-5 respectively which was statistically significant ($p \leq 0.05$).

INTRODUCTION

In the era of sustainable agriculture production with emphasis on organic farming, the interactions between rhizosphere and soil microorganisms plays a pivotal role as microbes helps in transformation, mobilization and solubilization minerals from a limited nutrient pool in the soil and make available for plant (Mantelin and Touraine, 2004).Bacteria colonizing at the rhizospheric region of plant roots and enhancing plant growth are referred to as plant growth-promoting rhizobacteria (PGPR). They are known to improve plant growth by various direct and indirect mechanisms such as phosphate solubilization, nitrogen fixation, indole-3-acetic acid (IAA), repression of soil borne pathogens by production of hydrogen cyanide, siderophore, antibiotics etc. (Glick, 1995).

Iron is an essential trace element for living organisms, but the availability is limited due to very low solubility of the dominant ferric iron (Fe^{3+}) in soils (Bodek *et al*., 1988). The divalent state can be oxidized to the trivalent state, where it may form oxide or hydroxide precipitates and become unavailable to plants as a micronutrient (Thompson and Troeh, 1973).

Siderophores are low molecular weight bio-molecules secreted by specific micro-organisms in response to iron starvation for acquisition of iron from insoluble forms by mineralization and sequestration (Lankford, 1973). Although some siderophores are known to chelate other ions, their specificity and avidity for iron is the most consistent feature (Chincholkar *et al*., 2007a). Siderophores produced by rhizosphere inhabitants has been studied well and it has been reported that the ability to produce siderophores not only improve rhizosphere colonization of producer strain but also play an important role in iron nutrition of plant (Vansuyt *et al.,* 2007) and antagonism against phytopathogens (Chincholkar *et al*., 2007b). Siderophores which are produced by bacteria are divided in three categories basing on the chemical nature of their co-ordination sites. Hydroxamate siderophore possesses N-hydroxylated amide bonds as co-ordination sites, catecholates co-ordinate iron with catecholate hydroxyl group and carboxylates co-ordinate iron with carboxyl and hydroxyl groups (Bholay *et al*., 2012). In addition, PGPR can be very effective and a potential microbe for enriching the soil fertility and yield of the agricultural crops. In recent years, the role of siderophore-producing PGPR in biocontrol of soil-borne plant pathogens has created a great interest as it prevent growth of pathogens by chelating iron.

On account of that, the present investigation has been under taken to isolate the potential siderophore producing bacteria from rhizospheric soil of rice plant from Sundarban, West Bengal, India and the potential isolates were tried with brinjal seeds to evaluate the efficacy in increasing germination (%), root length and shoot length under *in-vitro* conditions and qualitative and quantitative analysis of siderophore production by the isolates was undertaken.

MATERIALS AND METHODS

Sample collection and bacterial isolation

Soil sample was collected from the rhizosphere region of Rice plant from Sundarban, west Bengal and intact root system was dug out and the rhizospheric soil sample was carefully collected in plastic bags and stored at 4ºC. A total of forty eight bacterial isolates were isolated from the rhizospheric soil sample by spread plate technique.

Screening for Siderophore production

All rhizobacterial isolates obtained were screened for siderophore production by Chrome Azurol S (CAS) method (Schwyn B. and Neilands J, 1986). All glass wares were soaked overnight in 6N HCl and rinsed with distilled water for several times to remove traces of iron. Freshly grown bacterial cultures were inoculated as spot on CAS agar plates and incubated at 28ºC for 24-48 hours. After incubation, siderophore production was confirmed by the presence of orange colour zone around the colony on CAS agar plates. Total five positive colonies were isolated and named as SBBA-1 to SBBA-5.

Quantification of Siderophore

Siderophore production by the isolates was tested qualitatively by CAS- shuttle assay, in which the isolates were grown on succinate medium (Meyer and Abdallah, 1978) and incubated for 24-48 hrs at 28ºC with constant shaking at 120 rpm on shaking incubator separately. Following incubation, fermented broth was centrifuged (10,000 rpm for 15 min) and 0.5 mL of cell free supernatant was mixed with 0.5mL of CAS reagent, and the absorbance was measured at 630 nm against a reference consisting of 0.5 Ml of uninoculated broth and 0.5 mL of CAS reagent. Siderophore content in aliquot was calculated by using following formula: [(Ar - As)/Ar] x 100, where Ar is the absorbance at 630nm of reference (CAS assay solution + uninoculated media) and As is the absorbance at 630nm of the sample (CAS assay solution + supernatant) (Payne, 1994).

Identification, biochemical characterization and enzymatic activities of bacterial isolates

The isolates were further characterized on the basis of their staining characteristics and were further investigated for their biochemical properties like Indole, catalase, urease, citrate, ammonia, nitrate producing abilities and enzymatic activities like amylase, cellulase, gelatinase, caesinase and fermentation of various sugars and this helped in the bacterial identification up to the genus level (Gupta *et al.*, 2000) by Bergey's manual of Determinative bacteriology (Holt *et al.*,1994) and ABIS online software.

In vitro screening of isolates for different plant growth promoting activities

The bacterial isolates were screened for Plant growth promoting activities. IAA equivalents production by the rhizobacterial isolates were assayed using the qualitative method developed by Bric *et al.* (1991). Bacterial cultures were inoculated in the nutrient broth with tryptophan (1mg/mL) incubated at 35 ± 2ºC for 7 days. Cultures were centrifuged at 3000 rpm for 30 min. 2mL of thesupernatant was mixed with 2 drops of orthophosphoric acidand 4 ml of Salkowski's reagent (50mL, 35% perchloric acid; 1mL 0.5 $FeCl_3$). Development of a pink colour indicated 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 ineach tube and incubated for 48 h at 35 ± 2ºC. Nessler's reagent (0.5 ml) was added in each tube. Development of brown to yellow colour was a positive test for ammonia production (Cappuccino and Sherman., 1992).The bacteria were grown in NB containing 0.4 % glycerine containing a filter paper (Whatman no. 1) strip (10 90.5 cm) saturated with alkaline picrate solution (1 gm of picrate and 5 g of Na_2CO_3 dissolved in 200 ml of dis-tilled water) placed inside the conical flasks in a hanging position without touching the media and grown at30 ± 2ºC for 48 h on a rotary shaker. The reddish colour of the filter paper developed due to hydrocyanic acidevolution was eluted in 10 ml distilled water and the absorbance was measured at 625 nm (Reddy *et al.*, 2008). The ability of the microorganisms to reduce nitrate to nitrite is detected through the test. Bacteria were inoculated into nitrate broth, incubation at 30 ±0.16ºC for 96 h. After inoculation sulphanillic acid and á-naphthyl amine mixture (1:1) was added. Appearance of deep pink colour indicated positive result. Phosphate solubilization activity of the isolates were checked by spot inoculation (Pikovaskya *et al.*,1948) on the Pikovskaya agar medium and incubated at 28ºC for 72 h. Clear zone around the colonies were considered as positive for Phosphate solubilization. N_2-fixation ability of the isolates were checked by the using N-freeagar basedJensen (1951) media and incubated for 72 h at30 ± 1ºC.

Trial with seed germination

All the isolates weretested for seed germination under lab conditions. Brinjal seeds (*Solanum melongena* L.) and were surface sterilized with 0.1% $HgCl_2$ for 2 min and rinsed with sterile distilled water for ten times. Bacterial isolates were grown in respective broth on shaking incubator (180 rpm) at 28 ± 2ºC for 24h. Cell densities in the suspension were adjusted to a final density of approximately 10^8CFU seed^{-1}. The surface sterilized seeds of brinjal were inoculated in broth culture for 30 minutes (ISTA, 1993). Germination tests were carried out by the paper towel method and treated seeds and control were seeded onto paper towels.

Statistical analysis

Observations on germination, growth, shoot and root length were recorded and statistically analyzed.Germination percentage was measured with following formula: Germination percentage = Number of germinated seeds / Number of seeds in sample × 100. Data was analyzed statistically by one way ANOVA. The statistical significance of difference in germination, root and shoot length ofbrinjal were assessed using SPSS 16.0 software. All tests were conducted in triplicate and the significance of differences between mean values with ($p \leq 0.05$) level of significance was evaluated by DMRT (Duncan's Multiple Range Test).

RESULTS AND DISCUSSION

Isolation and screening of Siderophore producing bacteria

Table 1: PGP activities by the Siderophore producing bacterial isolates from Sundarban

Test	Isolate No. SBBA-1	SBBA-2	SBBA- 3	SBBA-4	SBBA- 5
Siderephore production	+	+	+	+	+
HCN production	-	-	-	-	-
NH_3 production	+	-	+	+	+
IAA production	+	-	-	-	-
N_2 fixation	+	+	+	+	+
Phosphate solubilization	-	-	-	-	-

Table 2: Physiological and biochemical properties of the producing bacterial isolates from Sundarban

TEST	ISOLATE NO. SBBA-1	SBBA-2	SBBA- 3	SBBA-4	SBBA- 5
Gram staining	+	+	+	+	+
Endospore staining	+	+	+	+	+
Catalase	+	+	+	-	-
H_2S production	-	-	-	-	-
Indole	-	-	-	-	-
Methyl red test	+	-	-	+	+
VP	-	-	-	-	-
Nitrate reduction	+	+	+	+	+
Urease production	+	+	+	+	+
Citrate utilization	+	+	-	-	-
Oxidase	+	-	+	-	+
Mannitol	+	-	-	-	-
Aesculin hydrolysis	+	+	+	+	+
Anaerobic growth	+	+	+	+	+

Table 3: Extracellular enzymatic activities of the Siderophore producing bacterial isolates from Sundarban

TEST	ISOLATE NO. SBBA-1	SBBA-2	SBBA- 3	SBBA-4	SBBA- 5
Gelatinase	+	+	+	+	-
Casein hydrolysis	+	+	-	+	+
Tributyrin	+	+	+	+	+
Amylase	-	+	+	-	-
Cellulase	+	-	+	+	+
Chitin hydrolysis	-	-	-	-	-
Pectin hydrolysis	+	+	+	+	+
DNase	-	-	-	-	-
Lecithinase	-	-	-	-	-

Table 4: Sugar utilization by the Siderophore producing bacterial isolates from Sundarban

Isolate No.	Tre	De	Du	Sa	Ga	Ino	Me	So	Ma	Su	La	Rh	Mn	Ce	Glu
SBBA-1	+	+	+	+	-	+	+	+	+	-	+	+	-	+	+
SBBA-2	-	+	+	+	-	+	+	+	+	+	+	+	-	+	+
SBBA-3	+	+	+	+	-	+	+	+	+	-	+	+	-	+	+
SBBA-4	+	-	+	+	-	+	+	+	+	-	+	+	-	+	+
SBBA-5	+	+	+	-	-	-	+	+	+	-	+	+	-	+	+

Tre : Trehalose, De: Dextrose, Du: Dulcitol, Sa: Salicin, Ga: Galactose, Ino: Inositol, Me: Melibiose, So: Sorbitol, Ma: Maltose, Su: Sucrose, La: Lactos, Rh: Rahmmose, Mn: Mannose, Ce: Cellobiose, Glu: Glucose

Siderophore production by the isolates were confirmed by the colour change of CAS reagent from blue to orange in CAS agar plate. Among all the isolates it was found that five gram positive bacterial isolates were positive for the siderophore production. Due to colour change from blue to orange resulted by siderophore removal of Fe from the dye (Wilhelmina *et al*., 2004) an. Moreover chelation of soluble iron by microbial siderophores leads to growth inhibition of phytopathogens indirectly (Bano and Musarrat, 2003).

Quantitative estimation of Siderophore

In quantitative CAS assay, percent of siderophore units were estimated as the proportion of CAS colour shifted. In the present study it was found that SBBA-5 produced 77.84% (Fig.1) siderophore units after 48h.Similar observations were also found by the scientists that maximum siderophore production by the *Bacillus* sp. observed after 48h (Shobha and Kumudini, 2012).

Table 5: Effect of siderophore producing bacteria on germination percentage, root length and shoot length of Brinjal seeds in germination paper

Isolate No.	Germination %	Root length (cm)	Shoot length (cm)
Control	44.08 ± 1.45[a]	4.08± 0.12[a]	5.90±0.11[a]
SBBA-1	71.25 ± 5.20[bcd]	6.16± 0.13[c]	7.48±0.06[c]
SBBA-2	65 ± 1.91[bc]	6.09±0.11[c]	6.57±0.17[b]
SBBA-3	63.75 ± 2.89[b]	7.62±0.13[d]	8.23±0.08[d]
SBBA-4	75 ± 4.51[cd]	5.37±0.17[b]	8.33±0.07[d]
SBBA-5	81.25 ± 2.50[d]	5.06±0.14[b]	8.21±0.06[d]

*Values are the mean ± SEM and differ significantly as per DMRT by LSD (pd≤0.05). Mean values in each column with same superscript (s) do not differ significantly as per DMRT. Mean values in each column with different superscript (ab) differ significantly as per Duncan Multiple range Test.

Table 6: Identification of bacterial isolates by ABIS online software

Isolate No.	Identification	Matching %
SBBA-1	*Bacillus licheniformis*	80%
SBBA-2	*Bacillus circulans*	78%
SBBA-3	*Bacillus coagulans*	80%
SBBA-4	*Bacillus circulans*	72%
SBBA-5	*Bacillus coagulans*	82%

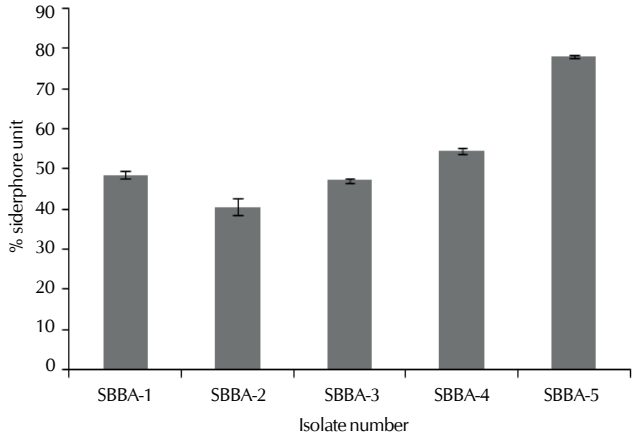

Figure 1: Quantification of Siderophore produced by bacteria isolated from Sundarban

Plant growth promoting activities of the bacterial isolates

Growth promotion may be attributed to other mechanisms such as production of plant growth promoting hormones in the rhizosphere. The bacterial isolate SBBA-1 only produced plant growth promoting hormone *i*.e. IAA. IAA in the rhizosphere depends onthe availabilityof precursors and uptake of microbial IAA by plant (Arshadand Frankenberger, 1991; Pradhan and Mishra, 2015). All the isolates also exhibited strong production of ammonia from peptone water except SBBA-2 and capable to produce more nitrate (Table 1) . None of the isolates were positive for HCN production.

Biochemical characterization and Identification

The biochemical tests such as oxidase test, nitrate reduction, catalase, carbohydrate utilization, citrate utilization etc. were carried out for phenotypic identification of isolates. The isolates were examined for catalase, oxidase and for urease test. Biochemical characterization of all isolatesand enzymatic activities of the isolates were tabulated in the Table 2 and 3. Briefly, all rod shaped isolates were positive for oxidase, catalase, lactose, maltose, fructose, dextrose, galactoseand salicin utilization (Table 4). The bacterial isolate were characterized by biochemical attributes and were identified as SBBA-1 (*Bacillus licheniformis*), SBBA-2 (*Bacillus circulans*), SBBA-3 (*Bacillus coagulans*), SBBA-4 (*Bacillus circulans*), SBBA-5 (*Bacillus coagulans*) on the basis of ABIS online software (Table 5).

Seed germination test

In this study, an increase in the plant growth by seed bacterization has been demonstrated. It is well established fact that plant growth promoting rhizobacteria increases the synthesis of gibberellins, which would have triggered the activity of specific enzymes the promoted the early germination, such as amylase, which have brought an increase in availability of starch assimilation (Bharathi *et al.,* 2004). In the present study it was found that siderophore producing PGPR significantly increased the germination percentage, root and shoot length of brinjal seedlings, over control (Debbarma *et al.*, 2015) (Table 6).

From the present study it is concluded that five siderophore producing Gram positive bacterial isolates were obtained from the rhizosphere region of the genus *Bcillus*, also showed some other plant growth promoting characters like IAA production, Ammonia production, Nitrate reduction and N_2-fixation. These potential isolates also increased the germination percentage, root and shoot length of the brinjal seeds. So the use of this siderophore producing isolates as biofertilizers is a novel approach to replace the chemical fertilizers and pesticides for sustainable agriculture in India.

REFERENCES

Arshad, M. and Frankenberger, W. T. 1991. Microbial production of plant hormones. *Plant Soil.* **1(133):** 1-8.

Bano, N. and Musarrat, J. 2003. Charectarization of a new *Psudomonus aeruginosa* strain NJ-15 as a potential biocontrol agent. *Current Microbiology*. **46:** 324-328.

Bharati, R., Vivekananthan, R., Harish, S., Ramanathan, A and Samiyappan. R. 2004. Rhizobacteria-based bio-formulations for the management of fruit rot infection in chillies. *Crop Protec.* **23:** 835-843.

Bholay, A. D., Jadhav Priyanka, U., Borkhataria, B. V. and Mayuri, V. Dhalkari. 2012. Fluorescent Pseudomonads as Plant Growth Promoting Rhizobacteria and Their Siderophoregenesis. *IOSR J. Pharmacy and Biological Sciences (IOSRJPBS).* **3:** 27-32.

Bodek, I., Lyman, W. J., Reehl, W. F. and Rosenblatt, D. H. 1988. Environmental Inorganic Chemistry: Properties, Processes, and Estimation Methods. SETAC Special Publication Series, B.T. Walton

and R.A. Conway, editors. *Pergamon Press*. New York.

Bric, J. M., Bostock, R. M. and Silverstone, S. E. 1991. Rapid in situ assay for indole acetic acid production by bacteria immobilized on a nitrocellulose membrane. *Appl. Environ. Microbiol.* **57:** 535-538.

Cappuccino, J. C. and Sherman, N. 1992. Microbiology, (in A Laboratory Manual). 3rd edition new york, Benjamin/cummings Pub. Co. pp. 125-179.

Chincholkar, S. B., Chaudhari, B. L. and Rane, M. R. 2007a. Microbial Siderophores: State of art. In: Microbial Siderophores. Chincholkar, S. B. and Varma, A. (eds.) Springer Verlag, Germany, pp. 233-242.

Chincholkar, S. B., Chaudhari, B. L., Rane, M. R. and Sarode, P. D. 2007b. Fungal phytopathogen suppression using siderophoregenic bio-inoculants. In: Biological Control of Plant Diseases: Current Concepts. Chincholkar, S. B. and Mukerji, K.G. (Eds). Haworth Press, USA., pp. 401-417.

Debbarma, S., Rai, P. K. and Meghawal, D. R. 2015. Response of linseed (*Linum usitatissimum* L.) genotypes towards plant growth promoting rhizobacteria (PGPR) and pH stress. *The Ecoscan*. **V:** 143-146**.**

Glick, B. R. 1995. The enhancement of plant growth by free-living bacteria. *Can. J. Microbiol.* **41:** 109-117.

Gupta, A., Gopal, M. and Tilak, K. V. 2000. Mechanism of plant growth promotion by rhizobacteria. *Indian. J. Exp Biol*. **38:** 856-862.

Holt, J. G., Krieg, N. R., Sneath, P. H. A., Staley, J. T. and Williams, S. T. 1994. Bergey's Manual of Determinative Bacteriology,(in Williamsons and Wilkins). *9th edition Balitomore*.

ISTA. 1993. Proceedings of the international Seed Testing Association, International Rules for Seed Testing. *Seed Science Technology*. **21:** 25-30.

Jensen, H. L.1951. Notes on the biology of Azotobacter. In: Proceedings of the Society for Applied Bacteriology 14, No. 1; Blackwell Publishing Ltd.

Lankford, C. E.1973. Bacterial assimilation of iron. *Crit Rev Microbiol.* **2:** 273-331.

Loper, J. E. and Schroth, M. N. 1986. Influence of bacterial source of indole-3-acetic acid of root elongation of sugar beet. *Phyto pathol*. **76:** 386-389.

Mantelin, S. and Touraine, B. 2014. Plant growth promoting bacteria and nitrate aviability: impacts on root development and nitrate uptake, *J. Exp. Bot.* **394:** 27-34.

Meyer, J. M. and Abdallah, M. A. 1978. The florescent pigment of *Psudomonus fluorescens* : biosynthesis, purification and physico-chemical properties.*J. Gen. Microbol.* **107:** 319.

Payne, S. M. 1994. Detect6ion, isolation and characterization of siderophore, *Methods in Enzymology*. **235:** 329-344.

Pikovaskya, R. I. 1948. Mobilization of phosphorus in soil in connection with the vital activity of some microbial species. *Mikrobiologiya.* **17:** 362-370.

Pradhan, A. and Mishra, B. B. 2015. Effect of Plant Growth Promoting Rhizobacteria on germination and Growth of Rice (*Oryza Sativa* L.), *The Ecoscan*. **9(1&2):** 213-216.

Reddy, B. P., Reddy, K. R. N., Subba, R. M., Rao, K. S. 2008. Efficacy of antimicrobial metabolites of *Pseudomonas fluorescens* against rice fungal pathogens. *Curr. Trend. Biotechnol Pharm.* **2:** 178-182.

Schwyn, B. and Neilands, J. B. 1986. Universal Chemical Assay for the detection and determination of siderophores. *Anal. Biochem.* **140:** 47-56.

Shobha, G. and Kumudini, B. S. 2012. Antagonistic effect of the newly PGPR *Bacillus* spp. On *Fusarium oxysporum. Int. J. Applied Sci. and Engineering.* **1:** 463-474.

Thompson, L. M. and F. R. Troeh. 1973. Soils and Soil Fertility, third ed. McGraw-Hill Book Company.

Vansuyt, G., Robin, A., Briat, J. F., Curie, C. and Lemanceau, P. 2007. Iron acquisition from Fe-pyoverdine by Arabidopsis thaliana. *Molecular Plant-Microbe Interactions*. **20:** 441-447.

Wilhelmina, M. H., Potter, A. J., Jennings, M. P., Jordi, R., Hauser, A. R. and McEwan, A. G. 2004. *J. Clinical Microbiology*. **42:** 2806-2809.