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Isolation and characterization of siderophore producing rhizobia from *Sesbania sesban* using different types of Indian soils

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Abstract
In present study fourteen rhizobia were isolated from root nodules of *Sesbania sesban* by using trap plant method from diverse agroecological zones of Indian soils. These isolates produced round, whitish, smooth surface colonies on YEMA medium plates, short rod in shape, motile in nature and gram negative in reaction. All the rhizobial isolates were studied for its ability to produce siderophore on Chrome-Azurol S agar medium plates after 7th days of incubation at 28±2 ºC. The results of these revealed that 43% of the rhizobial isolates were siderophore producers. Siderophore producing isolates were categorized as high, moderate and poor on the basis of yellow halo zones formation around the colonies, due to decolourization of the blue-coloured ferric dye complex spotted on the CAS agar medium plates. These results exposed the potential value of some isolates to be developed as bioinoculant for *Sesbania sesban* crop.

Keywords: *Sesbania sesban*, diverse, siderophore, bioinoculant

Introduction
Plant growth promoting rhizobacteria (PGPR) are known to improve nitrogen fixation in legumes by promoting nodulation through various mechanisms, regardless of their role in direct plant growth promotion, such as production of siderophore, phosphate solubilization (Patten and Glick, 2002; Brahmaprakash and Sahu, 2012; Sharma *et al.* 2014; Solanki *et al.* 2017) [18, 4, 22, 24], decrease of plant ethylene level by ACC utilization (Glick *et al.* 2007) [10]. PGPR may use one or more of these mechanisms in the rhizosphere which can be a significant component of management practices to achieve the attainable yield. Some bacterial strains directly regulate plant physiology by mimicking synthesis of plant hormones, whereas, others increase mineral and nitrogen accessibility in the soil as a way to supplement growth. *Rhizobium* with the plant growth promoting (PGP) attributes have potential for increased tolerance to high salt, water potential, pH and temperature stresses, therefore could enhance production of food and forage legumes in semi-arid and arid regions of the world (Singh *et al*. 2017) [23].

Iron is one of the most essential microelement for all living cells but its availability is limited, as the dominant form of iron in soil is present as ferric iron (Fe^{3+}), which has very low solubility. Rhizobia fix atmospheric nitrogen symbiotically in leguminous plants using the iron containing enzyme nitrogenase. Rhizosphere inhabiting bacteria usually live in micro colonies where the transient concentration of available iron can vary greatly from that of bulk soil solution. During evolution, microorganisms have developed sound strategies to acquire iron from both the environment and superior organisms, including direct uptake of iron ions from exogenous iron/heme sources and the synthesis of specialized Fe^{3+} chelators called siderophores (Schwyn and Neilands, 1987; Carvalho *et al*. 2011; Pahari and Mishra *et al*. 2017) [21, 5, 17]. Siderophores are commonly produced by aerobic, facultative anaerobic bacteria and fungi under iron limiting conditions (Neilands, 1995; Chaudhary *et al*. 2017) [15, 6]. As a chelating agent, they transport iron molecules inside the bacterial cell for various biochemical reactions. Siderophore and their derivative have large application in agriculture as to increase soil fertility and biocontrol for fungal pathogen (Ali and Vidhale, 2013) [1]. To date nearly 500 siderophores are reported from selected microorganisms. In general, siderophores are classified as hydroxamates, catecholates, salicylates, carboxylates and recently with new group polycarboxylates (Renshaw *et al*. 2002; Kannahi and Senbagam, 2014) [20, 13].

Sesbania Sesban is a short-lived shrub or small tree with a narrow crown (Nigussie and Alemayehu, 2013) [16] providing food, medicines, fibre, fuel etc. Apart from this, its capacity to control soil erosion and hence restore and maintain soil fertility makes it a useful component of traditional agroforestry (Degefu *et al*. 2011) [7]. It has outstanding ability to withstand waterlogging conditions and tolerate soil alkalinity to a considerable degree. In India, these crops have a long history of agricultural use, primarily as green manures and as sources of forage. But there is very little information about siderophore synthesizing capacity of *Rhizobium* strains isolated from this host. Hence, the present study was done to study the siderophore synthesizing capacity of 14 rhizobia from *Sesbania Sesban* using different types of Indian soils.

Materials and Methods

Isolation of native rhizobia nodulating *Sesbania sesban*

Seeds of *Sesbania sesban* were collected from Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu. Seeds were grown in plastic cups, each containing soil samples collected from different locations of India. After 45 days proper nodules were formed and the healthy pink nodules from root were removed. The nodules were surface sterilized by using 0.1% $HgCl_2$ and ethanol (Vincent, 1970) [25]. The nodules were crushed in a sterilized petri plate and a loopful of nodule sap were streaked on YEMA medium plates containing congo red. The plates were incubated at 28±2 ^oC and growth was observed daily for 2-7 days. White gummy colony of rhizobial isolates were picked up and were restreaked for purification. Single rhizobial clones were picked up and maintained on YEMA medium slants. The slants were stored at 4 ^0C in a refrigerator for further studies.

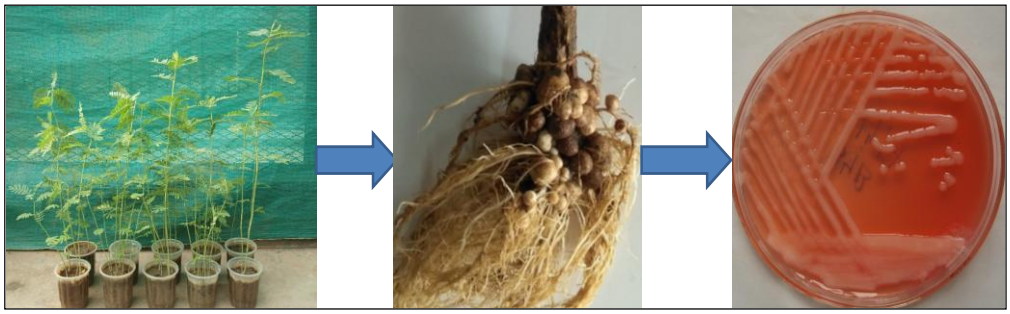

Plate 1: Isolation of rhizobia nodulating *Sesbania sesban* using trap plant from the different types of Indian soil

Authentication of rhizobial isolates

The pure cultures of rhizobia were subjected to Koch's postulates to check the authenticity of isolates.

A). Hofer's alkaline medium: In order to differentiate the isolates from the *Agrobacterium*, the isolates were grown in the Hofer's alkaline medium with the pH adjusted at 11 (Hofer, 1935) [11].

B). Ketolactase test: In this test, the isolates were streaked on the lactose agar medium for 2-5 d at 28±2^0C. Five milliliters of Benedict's reagent was poured on the plates and kept at room temperature for 1 h. Absence of yellowish zones of Cu_2O around the *Rhizobium* colonies indicated the purity of the isolates (Bernaerts and Deley, 1963) [3].

Siderophore production

Siderophore production was detected by CAS (Chrome Azurol S) assay (Modified method of Schwyn and Neilands, 1987) [21]

Solutions

Chrome azurol S (CAS) agar medium

(i) Dye solution (Solution A): Fifty ml of dye solution containing 60.5 mg CAS was prepared in distilled water and mixed with 10 ml of iron (III) solution (1 mM $FeCl_3.6H_2O$) in 10 mM HCl. The mixture was slowly added to a solution containing 72.9 mg HDTMA in 40 ml of distilled water. The resultant dark blue liquid was autoclaved at 15 lbs in^{-2} pressure for 10 min.

(ii) Solution B: A basal medium containing deferrated 1M sucrose (3 ml); deferrated 1M $CaCl_2$ (0.4ml): deferrated 1M $MgSO_4.7H_2O$ (0.8ml); 10% NaCl (2ml); 5% Na_2MoO_4 (0.1ml); Pipes buffer (30.24g) and agar (15g) in 800 ml of distilled water was prepared. pH of the medium was adjusted to 6.8 by 50% (w/w) NaOH and autoclaved at 121 ^0C under 15 lbs in^{-2} pressure for 20 min.

(iii) After cooling to 50 ^oC, 30 ml of mannitol solution (10%) was added as a carbon source and then dye solution (solution A) was added to it with gentle mixing to avoid the formation of air bubbles. The molten medium was distributed in sterilized petri plates.

Five µl of each log phase grown cells of rhizobia was spotted on the CAS agar medium plates and incubated at 28±2 ^oC for 7 days. The presence of iron chelator or siderophore producer is indicated by yellow halo zones around the colonies due to decolourization of the blue-coloured ferric dye complex.

Results and Discussion

Isolation of rhizobia nodulating *Sesbania sesban*

The seeds of *Sesbania sesban* were sown in cups containing different soil samples. Nodule formation was observed after 45 days of growth in *S. sesban*. The healthy pink nodules from root were removed separately and surface sterilized using 0.1% $HgCl_2$ and ethanol as described in material and methods section. The nodules were crushed and streaked on YEMA medium plates containing congo red. Total fourteen rhizobia were isolated from *Sesbania sesban* on YEMA medium after incubation of 2-7 days at 28±2 ^oC (Plate 1). List of *Sesbania sesban* rhizobial isolates is given in Table 1. Similarly, Kuldeep *et al*. (2016) [14] isolated 49 pigeon pea rhizobial isolates from different soils collected from arid and semi-arid region of Haryana using trap plant method. Dhull and Gera (2017) [9] also isolated 158 cluster bean rhizobial isolates using trap plant method from sixty seven soil samples collected from Bhiwani, Hisar and Mahendergarh districts of Haryana.

Table 1: Rhizobial isolates of *Sesbania sesban* obtained from different soil samples using trap plant method

Species	Rhizobial isolates Number	No. of rhizobial isolates
Sesbania sesban	SSUd, SSTn, SSKe (i), SSKe (ii), SSGh, SSBh, SSKr (i), SSKr (ii), SSKr (iii), SSKr (iv), SSHn, SSSn, SSHs, SSPr	14

Authentication of rhizobial isolates obtained from *Sesbania sesban*

All fourteen rhizobial isolates were characterized using colony morphology and Gram staining. All these isolates were observed white gummy and Gram-ve with small rods shape in appearance. Colonies of *Rhizobium* were found to be circular, semi-translucent, single and mucilaginous in nature. The colonies did not absorb the congo red color and such nature differentiates *Rhizobium* from *Agrobacterium*. Out of total rhizobial isolates only one [SSKr (ii)] showed mild growth in Hofer's medium. The isolates inoculated in YEM with bromothymol blue changed to yellow color showing the production of acid which is the characteristic of *Rhizobium*. The pH of the culture broth which was initially 7 decreased 4.1 to 5.1 (Table 2). In the present work, only two rhizobial isolates namely SSBh and SSKr (i) showed yellow zone formation on Ketolactose medium which show contamination of *Agrobacterium*. The authentication of the isolates by Koch's postulation (Plate 2) showed 3 out of 14 isolates to be *Agrobacterium* but according to latest classification of rhizobial taxonomy *Agrobacterium* also cause nodulation and fix nitrogen. Rai and Sen (2015) [19] reported authentification of French bean associated rhizobia found in North Bengal and Sikkim using similar methods. Therefore, all 14 rhizobial isolates were used for further study.

Table 2: Details of authentication of rhizobial isolates obtained from *Sesbania sesban*

Sr. No.	Rhizobial isolates	Hofer's test	Acid alkaline production test	Ketolactose test
1	SSUd	0.047	4.5	NYZ
2	SSTn	0.093	4.9	NYZ
3	SSKe (i)	0.062	5.1	NYZ
4	SSKe (ii)	0.115	4.7	NYZ
5	SSGh	0.073	4.5	NYZ
6	SSBh	0.150	4.1	YZ
7	SSKr (i)	0.016	4.7	YZ
8	SSKr (ii)	1.232	4.8	NYZ
9	SSKr (iii)	0.150	4.6	NYZ
10	SSKr (iv)	0.177	4.6	NYZ
11	SSHn	0.083	5.0	NYZ
12	SSSn	0.022	4.8	NYZ
13	SSHs	0.027	4.5	NYZ
14	SSPr	0.093	4.9	NYZ

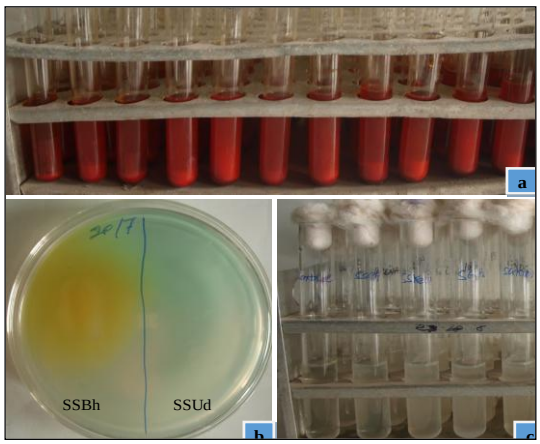

Plate 2: Acid-Alkaline production test (A), Ketolactose agar test (B), Hofer's alkaline test (C) of *Sesbania sesban* rhizobial isolates

Screening of rhizobial isolates for siderophore production

The ability to synthesis siderophores were restricted to very few isolates as out of 14 *Sesbania sesban* rhizobial isolates tested only 6 were able to produce siderophore. From these, 7% high siderophore producer (HSP), 15% moderate siderophore producer (MSP), and 21% low siderophore producer (LSP) respectively while, 57% of the isolates did not produce siderophore (Table 3 and Plate 3). Rhizobial isolates SSKe (ii) was highly efficient for siderophore production which can be used for plant growth in *Sesbania sesban* but field level extended study is recommended. These results are in agreement with Arora *et al*. (2001) [2] who reported that the ability to synthesize siderophore by rhizobia is restricted to a limited number of strains rather than wide distribution. Similarly, Jenifer *et al.* (2013) [12] reported that out of the 11 isolated cultures from rhizospheric soil, 4 cultures namely C2, C3, C8 and C11 were found to produce siderophore. Singh *et al*. (2017) [23] reported that 35% of pigeon pea rhizobial isolates isolated from four districts of Haryana, India were able to produce siderophore. Dhull and Gera (2018) [8] reported that out of 13 clusterbean rhizobia only 6 rhizobia showed siderophore production on Chrome-Azurol S agar medium after 7 days of incubation. Out of 13 clusterbean rhizobia, 23% of the rhizobial isolates were found to be significant siderophore producers, 7% intermediate and 23% low producers, whereas 53% did not produce siderophore.

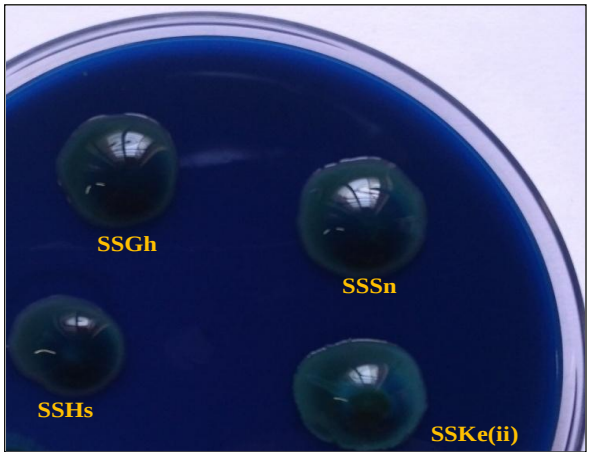

Plate 3: Siderophore production by *Sesbania sesban* rhizobial isolates

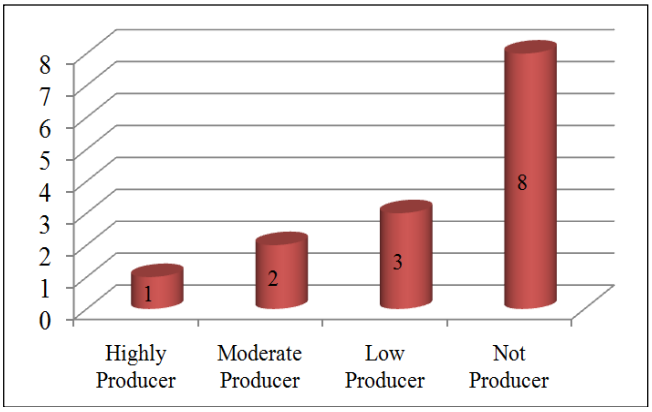

Fig 1: Siderophore production ability by different *Sesbania sesban* rhizobial isolates

Table 3: Characterization of *Sesbania sesban* isolates for siderophore production

Sr. No.	Rhizobial isolate	Siderophore production
1.	SSUd	-
2.	SSTn	-
3.	SSKe (i)	-
4.	SSKe (ii)	+++
5.	SSGh	+
6.	SSBh	-
7.	SSKr (i)	+
8.	SSKr (ii)	++
9.	SSKr (iii)	-
10	SSKr (iv)	-
11	SSHn	-
12	SSSn	++
13	SSHs	+
14	SSPr	-

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References

1. Ali SS, Vidhale NN. Bacterial Siderophore and their Application: A review. International Journal of Current Microbiology and Applied Sciences. 2013; 2(12):303-312.
2. Arora NK, Kang SC, Maheshwari DK. Isolation of siderophore producing strains of *Rhizobium meliloti* and their biocontrol potential against *Macrophomina phaseolina* that causes charcoal rot of groundnut. Currrent Science. 2001; 81:673-677.
3. Bernaerts MJ, Deley J. Biochemical test for crown gall bacteria. Nature. 1963; 8:406-407.
4. Brahmaprakash GP, Sahu PK. Biofertilizers for Sustainability. Journal of the Indian Institute of Science. 2012; 92(1):37-61.
5. Carvalho CRD, Marques MPC, Fernandes P. Recent achievements on siderophore production and application. Recent Patents on Biotechnology. 2011; 5(3):183-198.
6. Chaudhary DY, Gosavi P, Durve-Gupta A. Isolation and application of siderophore producing bacteria. International Journal of Applied Research. 2017; 3(4):246-250.
7. Degefu T, Wolde-meskel E, Frostegard A. Multilocus sequence analyses reveal several unnamed *Mesorhizobium* genospecies nodulating Acacia species and *Sesbania sesban* trees in Southern regions of Ethiopia. Systematic and Applied Microbiology. 2011; 34:216-226.
8. Dhull S, Gera R. Siderophore production by rhizobia isolated from Cluster bean [*Cyamopsis tetragonoloba* (L.) Taub.] Growing in semi-arid regions of Haryana, India. International Journal of Current Microbiology and Applied Sciences. 2018; 7(3):3187-3191.
9. Dhull S, Gera R. Assessing stress tolerant rhizobial isolates of clusterbean (*Cyamopsis tetragonoloba* (L.) Taub.) Retrieved from semi-arid regions of Haryana, India. International Journal of Current Microbiology and Applied Science. 2017; 6:744-753.
10. Glick BR, Cheng Z, Czarny J, Duan J. Promotion of plant growth by ACC deaminase-producingsoil bacteria. Europian Journal of Plant Pathology. 2007; 119:329-339.
11. Hofer AW. Methods for distinguishing between legume bacteria and their most common contaminant. Journal of American Society of Agronomy. 1935; 27:228-230.
12. Jenifer MRA, Reena A, Aysha OS, Valli S, Nirmala P, Vinothkumar P. Isolation of siderophore producing bacteria from rhizosphere soil and their antagonistic activity against selected fungal plant pathogens. International Journal of Current Microbiology and Applied Science. 2013; 2(1):59-65.
13. Kannahi M, Senbagam N. Studies on siderophore production by microbial isolates obtained from rhizosphere soil and its antibacterial activity. Journal of Chemical Pharmaceutical Research. 2014; 6(4):1142-1145.
14. Kuldeep Gera R, Padder SA. Evaluation of rhizobial strains for abiotic stress tolerance in pigeon pea from arid and semi-arid zones of Haryana, India. The Ecoscan. 2016; 9:401-407.
15. Neilands JB. Siderophores: Structure and Function of Microbial Iron Transport Compounds. The Journal of Biological Chemistry. 1995; 270(45):26723-26726.
16. Nigussie Z, lemayehu G. *Sesbania sesban* (L.) Merrill: Potential uses of an underutilized multipurpose tree in Ethiopia. African Journal of Plant Science. 2013; 7(10):468-475.
17. Pahari A, Mishra BB. Characterization of siderophore producing rhizobacteria and its effect on growth performance of different vegetables. International Journal of Current Microbiology and Applied Science. 2017; 6(5):1398-1405.
18. Patten CL, Glick BR. Role of *Pseudomonas putidaindole* acetic acid in development of the host plant root system. Applied Environment Microbiology. 2002; 68:3795-3801.
19. Rai R, Sen A. Biochemical characterization of french bean associated rhizobia found in North Bengal and Sikkim. Journal of Acadamic and Industrial Research. 2015; 4(1):10-18.
20. Renshaw JC, Robson GD, Trinci APJ, Wiebe MG, Livens FR, Collison D *et al*. Fungal siderophores: structures, functions and applications. Mycology Research. 2002; 106:1123-1142.
21. Schwyn B, Neilands JB. Universal chemical assay for the detection and determination of siderophores. Annals of Biochemistry. 1987; 160:47-56.
22. Sharma S, Rana S, Kaur M. Isolation and characterization of bacterial isolates for phosphate solubilization and other plant growth promoting activities from apple soil of Himachal Pradesh. The Bioscan. 2014; 9(1): 443-448.
23. Singh K, Rani A, Padder SA, Gera R. Plant growth promoting (pgp) attributes of stress tolerant rhizobial isolates from root nodules of pigeon pea [*Cajanus Cajan* (L.) Millspaugh] growing in Haryana, India. International Journal of Current Microbiology and Applied Science. 2017; 6(12):461-473.
24. Solanki MK, Wang Z, Wang FY, Li CN, Lan TJ, Singh RK. Intercropping in sugarcane cultivation influenced the soil properties and enhanced the diversity of vital diazotrophic bacteria. Sugar Technology. 2017; 19:136-147.
25. Vincent JM. A Manual for the practical study of root nodule bacteria IBP handbook No. 15, Blackwell, Edinburgh, U.K, 1970, 73-97.