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Siderophore production by microbial isolates and their effect on nodulation attributes in green gram

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Abstract
Many bacteria secrete ferric iron-specific ligands, generically termed siderophore, which aid in sequestering and transport of iron under iron limiting conditions. Eight microbial isolates were tested for their ability to produce siderophores using CAS-agar plate assay, a general test for siderophore detection, which is independent of siderophore structure. Among the isolates *Pseudomonas flurescens* was noted to 75 per cent siderophore production followed by *Azospirillum lipoferum* (67 per cent). The siderophore producing microbial isolates significantly enhanced nodulation attributes in green gram compare to absolute control. The ecological advantages in the synthesis of microbial siderophore encourage the use of such microbes as inoculants with root nodule bacteria.

Keywords: Ligands, siderophore, inoculants, CAS-agar assay

Introduction
The acquisition of iron by microorganisms in aerobic environment presents a difficult problem since the solubility product constant for ferric hydroxide is about 10^{-38} (Lindsay and Schwab, 1982) [6]. Thus at pH 7, the free available iron is at a concentration for no more than 10^{-17} M, which is far below that required for microbial isolates and plant growth. Iron is an aerated environment exist in the ferric form and so highly insoluble in neutral to alkaline soil (Shenkar *et al*., 1995). To solve this problem, microorganisms are generally observed to utilized a high-affinity iron transport system. The synthesis and secretion of a low molecule at weight ferric specific chelating agent to solubilize iron is termed as Siderophore (Neilands, 1981; Abd-Alla, 1998) [10].

Microbial siderophore may stimulate the plant growth directly by increasing the availability of iron in soil surrounding the roots or indirectly by competitively inhibiting the growth of plant pathogens with less efficient iron uptake system (Marek-Kozaczuk *et al*., 1996) [9]. In soil plant roots normally coexist with bacteria and fungi which may produce siderophore capable of sequestering the available soluble iron and so interfere with plant growth and function. Alternatively, plant roots might be capable of taking up ferric complexes of siderophore and using these as a source of iron (Neilands, 1982) [11].

Iron deficiency in crop is very common on alkaline soil, and affects such common agricultural crops as chick pea, green gram (Rai *et al*., 1982) [14]. There is, however, little evidence that iron deficiency in soil actually decreases the number of root nodules, implying either that these organisms have lesser demands for iron during normal growth and survival that the plant, or they have other mechanisms for acquiring iron under iron deficient conditions (Abd-alla, 1999).

The aim of the present study was to investigate the ability of different microbial isolates to produce siderophore and their effect on nodulation attributes in green gram.

Material and Method
Microbial strains and culture conditions
The laboratory stock cultures *Rhizobium phaseoli, Pseudomonas fluorescens, Pseudomonas striata, Bacillus subtilis, Bacillus polymyxa, Bacillus megaterium, Azotobacter chroococcum, Azospirillum lipoferum* and few others were procured from All India Network Project on Soil Biodiversity-Biofertilizers, VNMKV Parbhani and National Collection of Industrial Microorganisms **(**NCIM) Pune on the basis of their iron solubilizing ability in laboratory

condition. The solubilization potential was evaluated both qualitatively and quantitatively under in-vitro condition as outlined in the following paragraphs:

Detection of siderophore

Siderophore production by plant growth promoting microorganisms was tested qualitatively by Chrome Azural S (CAS) liquid as well as plate assay. The strains were spread over CAS agar plate and incubated for 48 hrs at 28ºC. After incubation a thin layer of CAS reagent in 0.7% agar was spread on the bacterial growth and plates were again incubated for 24 hrs at 28ºC, formation of yellow orange colour zone around the colonies in plate assay and colour changes from blue to orange in liquid assay, indicated the siderophore production **(**Schwyn and Neilands, 1987) [16].

Estimation of siderophore

The quantitative estimation of siderophore produced by different plant growth promoting microorganisms was done by CAS-shuttle assay, in which both the strains were grown on CAS agar medium and incubated for 24-30 hrs at 28ºC with constant shaking at 120 rpm on shaking incubator separately. During incubation, every 20 min 5 ml broths were centrifuged at 10,000 rpm at 4 ºC in cooling centrifuge for 10 minute and cell free supernatant was mixed with 0.5 ml CAS solution. The colour obtained was measured using the spectrophotometer at 630 nm with reference containing 0.5ml uninoculated succinate medium and 0.5 ml CAS solution. The percentage of siderophore unit was estimated as the proportion of CAS colour shifted using the formula: % Siderophore units = [(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) [13]

Field experiment

Field experiment was conducted at experimental farm, Department of Soil Science and Agril. Chemistry, Vasantrao Naik Marathwada Krishi Vidyapeeth, Parbhani during *kharif*, 2018. The experiment was laid out in randomized block design with three replications and ten treatments. The eight strains of microbial cultures used in the present study were procured from All India Network Project on Soil Biodiversity-Biofertilizers, VNMKV, Parbhani and National Collection of Industrial Microorganisms **(**NCIM) Pune. Influence of siderophore producing microbial isolates on nodule attributes in green gram was checked by field experiment.

Statistical analysis

The data obtained from the field experiment was done by completely randomized design as per the methods described in "Statistical Methods for Agricultural Workers" by Panse and Sukhatme (1985). Appropriate standard error (S.E.) and critical differences (C.D.) at 5% level were worked out as and when necessary and used for data interpretation.

Results and Discussion

Screening for siderophore producing ability

Siderophore production by different microbial isolates was confirmed by colour changes of CAS agar reagent from blue to orange. The colour change from blue to orange resulted by siderophoretic removal of Fe from dye.

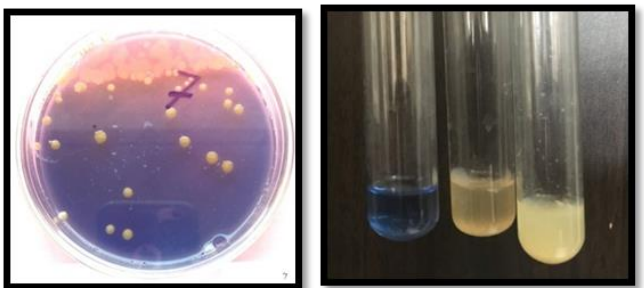
Eight plant growth promoting microorganisms isolates tested, for their ability to produce siderophore under iron limiting condition, seven isolates were positive (Table 1). It was obvious that all positive isolates produce siderophore on CAS assay. *Rhizobium phaseoli* do not shows growth on CAS agar plate. *Pseudomonas flurescens, Azotobacter chroococcum, Pseudomonas striata, Bacillus subtilis, Bacillus polymyxa, Bacillus megaterium, Azospirillum lipoferum* were positive on CAS agar test.

Quantitative CAS assay

In quantitative CAS assay, percent siderophore units were estimated as the proportion of CAS color shifted. *Pseudomonas flurescens* produced maximum amount of siderophore (75%), followed by *Azospirillum lipoferum (67%), Bacillus subtilis (62%), Pseudomonas striata (58%)* and *Bacillus megaterium (44%).* Same trend was observed in both qualitative and quantitative detection of siderophores produced by different plant growth promoting microorganisms.

Table 1: Siderophoregenesis by plant growth promoting organisms

Sr. No	Microbial inoculants	CAS Agar test	% Siderophore
1	*Rhizobium phaseoli*	-	0
2	*Azotobacter chroococcum*	+	35
3	*Pseudomonas striata*	+	58
4	*Bacillus subtilis*	+	62
5	*Bacillus polymyxa*	+	10
6	*Bacillus megaterium*	+	44
7	*Pseudomonas flurescens*	+	75
8	*Azospirillum lipoferum*	+	67



A B

Fig A: Formation of yellow-orange colonies on CAS agar plate
Fig B: Colour changes of CAS reagent from blue to orange in qualitative CAS assay.

Amount of siderophore produced by both the *Pseudomonas sps.* were estimated as percentage of siderophore units as the proportion of CAS color shifted. By liquid CAS assay, *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* have shown the highest yields of siderophore i.e. 88% and 83% siderophore units respectively (Bholay *et al.* 2012).

The two *fluorescent pseudomonads, Pseudomonas fluorescens* NCIM 5096 and *P. putida* NCIM 2847 produced maximum yield of hydroxamate type of siderophore (87% and 83% units, respectively). (Sayyed *et al.* 2005). *Pseudomonas fluorescens* NCIM 5096 shows the high yellow zone formation on the CAS agar plate. Similar finding was also reported by Gupta and Gopal (2008) studied on different bacteria includes *Bacillus coagulans, Bacillus* sp., *Bacillus polymyxa, Brevibacillus brevis, Enterobacter* sp., *Pseudomonas* sp., *Pseudomonas fluorescens, Pseudomonas striata, Azospirillum brasilens, Enterobacter* sp. Among that Pseudomonas fluorescens (76%) produced maximum amount of siderophores followed by *Enterobacter* sp., Pseudomonas sp., Enterobacter sp., Azospirillum brasilense and Brevibacillus brevis. Same trend was observed in both

qualitative and quantitative detection of siderophores produced by various PGPR isolate.

Mansoureh Sadat *et al*. (2012) [8] reported that Pseudomonas fluorescens forms a major constituent of Rhizobacteria that encourage the plant growth through their diverse mechanisms. In this investigation, 20 strains of Pseudomonads isolated from the rhizosphere soils of paddy areas in Malaysia and were screened for their plant growth promoting activity. All the 20 tested isolates of Pseudomonads were positive for the production of siderophores and HCN, while of the 20 antagonist bacteria strains, 15 strains (75%) showed positive for the production of plant growth-promoting hormone, IAA.

Nodule attributes

Results narrated in Table 2 related to nodules attributes in green gram indicates significant effect of siderophore producing microorganisms along with *Rhizobium phaseoli* on nodulation in green gram.

Table 2: Effect of siderophore producing microorganisms on nodulation attributes in green gram

Sr. No.	Treatments	No. of nodules plant^{-1}	Nodule fresh wt plant^{-1} (mg)	Nodule Dry wt plant^{-1} (mg)
T_1	Absolute control	14.67	20.00	8.67
T_2	Only RDF	21.67	35.00	13.00
T_3	RDF+*Rhizobium phaseoli*	25.00	47.33	22.00
T_4	T_3 + *Pseudomonas flurescens*	39.00	74.67	39.33
T_5	T_3+ *Pseudomonas striata*	30.00	40.67	22.67
T_6	T_3+ *Bacillus subtilis*	25.33	40.33	19.33
T_7	T_3+*Bacillus polymyxa*	25.67	50.67	31.00
T_8	T_3+*Bacillus megaterium*	25.67	40.00	20.00
T_9	T_3+ *Azotobacter chroococcum*	30.00	46.67	31.67
T_{10}	T_3+ *Azospirillum lipoferum*	28.33	48.00	27.00
	S.Em.±	1.62	2.64	1.75
	C.D. at 5 %	4.81	7.84	5.19
	C.V. %	10.58	10.31	12.90

Microbial inoculants influenced the number of nodules which ranges from 14.67 to 39.00 per plant showing significantly higher number of nodules in RDF + *Rhizobium phaseoli* + *Pseudomonas flurescens* (T_4) treated plots followed by RDF + *Rhizobium phaseoli* + *Pseudomonas striata* (T_5) and RDF + *Rhizobium phaseoli* + *Azotobacter chroococcum* (T_3). Whereas, significantly lower number of nodules per plot were noted in absolute control. Microbial inoculants influence the nodule fresh weight and nodule dry weight which sowing significantly higher fresh weight of nodules and nodule dry weight in RDF + *Rhizobium phaseoli* + *Pseudomonas flurescens* (T_4) treated plots Whereas, significantly lower fresh weight of nodules per plant was noted in absolute control.

The increase in number of nodules, fresh weight and dry weight of nodules per plant with siderophore producing microorganisms along with *Rhizobium phaseoli* might be a result of more iron availability in nodulating period of green gram which might have enhanced nodulation process. Earlier report shows that the treatment of *Bradyrhizobium* (mung bean) USDA 3447 + *P. chrysogenium* exhibited an increase in nodule number and nodules activity in mung bean as reported by Mahmoud and Abd-alla (2001) [7]. These results are in agreement with the findings of Sindhu *et al*. (2002) [18]. Gamit and Tank (2014) [4] who observed significantly higher number of nodules produced in *Cajanus cajan* inoculated plants with inoculation of siderophore producing microorganisms *Pseudomonas pseudoalcaligenes*.

Conclusion

Among eight plant growth promoting microbial isolates tested for siderophore production. Only seven were found to produce more siderophore. *Rhizobium phaseoli* do not shows growth on CAS agar plate. *Pseudomonas flurescens* produced maximum amount of siderophore (75%) followed by *Azospirillum lipoferum* (67%). Thus in the present study *Pseudomonas fluorescens* was able to overcome the major problem related to the adverse effects of chemical fertilizers on plant growth and productivity. Thus a biological platform was built to combat this problem. *Pseudomonas fluorescens* produce extracellular water soluble Siderophore which was proved to be enhancement of nodulation, nodule fresh and dry weight in green gram.

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