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Bagmare RR
Department of Soil Science and Agricultural Chemistry, Vasantrao Naik Marathwada Agricultural University, Parbhani, Maharashtra, India

Syed Ismail
Head, Department of Soil, Science and Agricultural Chemistry, College of Agriculture, VNMKV, Parbhani, Maharashtra, India

Swami YP
Department of Soil Science and Agricultural Chemistry, Vasantrao Naik Marathwada Agricultural University, Parbhani, Maharashtra, India

Ghode MK
Department of Soil Science and Agricultural Chemistry, Vasantrao Naik Marathwada Agricultural University, Parbhani, Maharashtra, India

Correspondence
Bagmare RR
Department of Soil Science and Agricultural Chemistry, Vasantrao Naik Marathwada Agricultural University, Parbhani, Maharashtra, India

Effect of siderophore producing microorganisms on plant growth promotion in green gram

Bagmare RR, Syed Ismail, Swami YP and Ghode MK

Abstract
At present fertilizer has become essential to modern agriculture to feed the growing population. Though chemical fertilizers increase crop production; their overuses has hardened the soil, decreased fertility, strengthened pesticide, polluted air, water and release the green house gases, thereby bringing hazards to human health and environment as well. Due to the adverse effect of chemical fertilizers and the presence of plant pathogens, apart from using chemically based methods present study provides a biological platform to increase plant productivity. It has been reported that many siderophore producing bacterial and fungal strains have their potentials in plant growth promotions because although iron is abundant in the soil it is unavailable to plants because of its low solubility. The eight microbial isolates were screened for siderophore production by the universal Chrome Azurol S assay (CAS). *Pseudomonas fluorescens* produced maximum % siderophore. *Pseudomonas fluorescens* was further tested as a seed inoculants and found to be very effective in plant growth promotion in green gram.

Keywords: Chemical fertilizer, biological platform, siderophore, CAS, *Pseudomonas fluorescens*, plant growth promotion

Introduction
In modern cultivation process indiscriminate use of fertilizers, particularly the nitrogenous and phosphorus, has led to substantial pollution of soil, air and water. Excessive use of these chemicals exerts deleterious effects on soil microorganism, affects the fertility status of soil and also pollutes environment. The application of these fertilizers on a long term basis often leads to reduction in pH and exchangeable bases thus making them unavailable to crops and the productivity of crop declines (Alemu, 2013) [2]. Thus due to such adverse effect of chemical fertilizers at present there is an urgent need of biological agent which can be used in place of such chemical fertilizers.

Iron is a crucial element for proper plant development. Iron is the fourth most abundant element in the earth's crust and in most types of soil occurs in excess. This element can exist in aqueous solution in two states: $Fe^{2+}$and Fe^{3+}; however, Fe^{3+} forms are not readily utilizable by plants and microbes because they often form insoluble oxides or hydroxides which limit bioavailability (Desai *et al.* 2011) [6]. Even though it is required in a number of major physiological processes like N_2- fixation, photosynthesis, respiration, etc. (Dudeja *et al*. 1997) [7]. To meet their iron requirement, microorganisms and plants have evolved specific mechanisms to chelate insoluble iron through the release of siderophores and uptake of iron-siderophore complexes through specific outer membrane receptor proteins.

Siderophores are low molecular weight (500-1500 daltons) organic compounds, that are synthesized by many microbes to chelate Fe^{3+} from insoluble Fe compounds when growing under Fe deficient conditions (Miethke and Marahiel, 2007) [14]. There are over 500 different siderophores, of which the chemical structure of 270 siderophores have been identified (Hider and Kong, 2010) [11]. Siderophores scavenge iron from mineral phases by formation of soluble Fe^{3+} complexes that can be taken up by energy dependent membrane transport mechanism and thus bind it and transport it to plants or bacterial cells. Thus, siderophores act as solubilizing agents for iron from minerals or organic compounds under conditions of iron limitation. Different *Pseudomonas sp.* has been known for their siderophore production for many years (Decheng *et al.* 2005) [5]. There are mainly two types of siderophores. The functional groups responsible for the binding are most frequently hydroxymates and catechols. (Raymond *et al.* 1984) [19] Like iron other micronutrients such as Cu, Mn, Zn, Co, B, Al are also necessary for proper plant growth.

In addition to iron, siderophores also form stable complexes with other metals. Many studies demonstrated that microbial siderophores are used by plants (Crowley *et al*. 1992, Johnson *et al.* 2002) [4, 12]. So recently the importance of siderophore extends their applications in agriculture, biotechnology and medicine.

The present study has been undertaken for production, detection and estimation of siderophores produced by microbial isolates and their role in eco friendly practice to promote plant growth of 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) [21].

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 4ºC 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) [18].

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 growth, biometric and yield 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 no 1). It was obvious that all positive isolates produce siderophore on CAS assay. *Rhizobium phaseoli* do not shows growth on CAS agar plate. *Pseudomonas fluorescens, 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 fluorescens* 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: Sidero phoregenesis 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

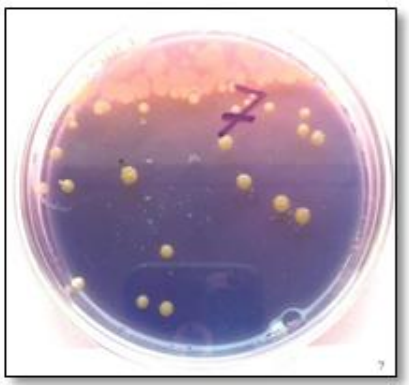

Fig 1: Formation of yellow-orange colonies on CAS agar plate

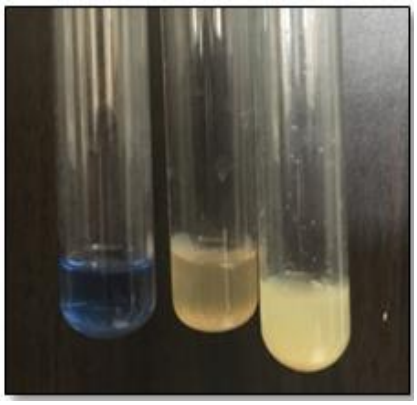

Fig 2: Colour chages of CAS reagent from blue to orange in Qualitative CAS assay.

Earlier, siderophore produced by both the *Pseudomonas spps.* 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) [3].

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) [20]. Further similar finding was also reported by Gupta and Gopal (2008) [10] on different bacteria.

Plant growth characters

The inoculation of siderophore producing microorganisms along with *Rhizobium phaseoli* had significant influence on different plant growth characters. Based on the efficiency of Fe solubilization, the eight selected microbial isolates were further examined for their performance to enhance Growth characters such as plant height and number of leaves, number of pods per plant over control**.**

The effect of siderophore producing microorganisms along with *Rhizobium phaseoli* on height of plant, number of branches per plant, number of leaves and number of pods per plant are presented in Table 2. The application of RDF + *Rhizobium phaseoli* + *Pseudomonas flurescens*(T_4) produced significantly more effect on height of plant, number of branches per plant, number of leaves per plant and number of pods per plant. The significant maximum plant height (55.10), number of branches (5.67), number of leaves (21.67) and number of pods per plant (12.00) were recorded with application of RDF + *Rhizobium phaseoli* + *Pseudomonas fluorescens*(T_4) followed by RDF + *Rhizobium phaseoli* + *Azospirillum lipoferum* (T_{10}) which was found superior over control.

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

Sr. No.	Treatments	Height of plant(cm)	No of Branches per plant	Number of leaves per plant	Number of pods per plant
T1	Absolute control	39.70	3.67	14.00	4.33
T2	Only RDF	43.23	4.33	16.67	6.67
T_3	RDF+*Rhizobium phaseoli*	48.17	5.00	17.33	8.33
T_4	T_3 + *Pseudomonas flurescens*	55.10	5.67	21.67	12.00
T_5	T_3+ *Pseudomonas striata*	40.83	4.67	17.33	10.33
T_6	T_3+ *Bacillus subtilis*	46.13	4.33	16.00	8.00
T_7	T_3+*Bacillus polymyxa*	47.67	4.67	19.00	9.67
T_8	T_3+*Bacillus megaterium*	50.93	4.67	17.00	8.00
T_9	T_3+ *Azotobacter chroococcum*	41.00	4.67	18.00	9.00
T_{10}	T_3+ *Azospirillum lipoferum*	52.10	4.67	17.67	11.00
	S.Em.±	3.13	0.30	1.08	0.50
	C.D. at 5 %	9.31	0.88	3.22	1.47
	C.V. %	11.68	11.07	10.73	9.83

The growth promotion in green gram might be due to greater availability of nutrient through organic and biological sources by enhancing the cambium activity of root hair, root proliferation and cell development in the root surface area resulting in better absorption of water and nutrients. Gamit and Tank (2014) [9] found that inoculation with siderophore producing organism *Pseudomonas pseudoalcaligenes* affect the germination of *Cajanus cajan* seeds significantly. There was a strong effect of inoculation with isolates on the height of plant, number of leaves root growth of germinated seedlings.

Nodule Attributes

Results narrated in Table 3 related to nodules attributes in green gram indicates significant effect of siderophore producing microorganisms along with *Rhizobium phaseoli* on nodulation in green gram. 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 fluorescens*(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 fluorescens* (T_4) treated plots Whereas, significantly lower fresh weight of nodules per plant was noted in absolute control.

Table 3: Effect of siderophore producing microorganisms on nodulation in green gram

Sr. No.	Treatments	No. of nodules plant^{-1}	Nodule fresh wt plant^{-1} (mg)	Nodule Dry wt plant^{-1} (mg)
T1	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
T4	T_3 + *Pseudomonas flurescens*	39.00	74.67	39.33
T5	T_3+ *Pseudomonas striata*	30.00	40.67	22.67
T_6	T_3+ *Bacillus subtilis*	25.33	40.33	19.33
T7	T_3+*Bacillus polymyxa*	25.67	50.67	31.00
T8	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

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) [13]. These results are in agreement with the findings of Sindhu *et al*. (2002) [22]. Gamit and Tank (2014) [9] who observed significantly higher number of nodules produced in *Cajanus cajan* inoculated plants with inoculation of siderophore producing microorganisms *Pseudomonas pseudoalcaligenes*.

Yield Attributes

Data given in Table 4 reveal significant increase in seed and straw yield due to application of siderophore producing microorganisms along with *Rhizobium phaseoli.* Siderophore producing microorganisms along with *Rhizobium phaseoli* influenced the seed yield which ranged between 662.17 to 848.80 kg ha^{-1}and straw yield 1230.67 to 1503.87 kg ha^{-1} showing significantly higher seed and straw yield in RDF + *Rhizobium phaseoli* + *Pseudomonas fluorescens*(T_4) at par with treatment RDF + *Rhizobium phaseoli* + *Azospirillum lipoferum* (T_{10}) treated plots and RDF + *Rhizobium phaseoli* + *Azotobacter chroococcum* (T_9) treated plots. Whereas, significantly lower seed and straw yield per plot was noted in absolute control. Significant percent increase in seed and straw yield due to application of RDF + *Rhizobium phaseoli* + *Pseudomonas fluorescens* (T_4) was noted 28% and 22% respectively over absolute control.

Table 4: Seed and straw yield of green gram as influenced by siderophore producing microorganisms along with *Rhizobium phaseoli*

Sr. No.	Treatments	Seed yield	Straw yield
T_1	Absolute control	661.67	1230.67
T_2	Only RDF	669.33	1258.43
T_3	RDF+*Rhizobium phaseoli*	675.00	1310.70
T_4	T_3 + *Pseudomonas flurescens*	848.80	1503.87
T_5	T_3+ *Pseudomonas striata*	696.93	1400.10
T_6	T_3+ *Bacillus subtilis*	737.50	1434.10
T_7	T_3+*Bacillus polymyxa*	775.00	1444.17
T_8	T_3+*Bacillus megaterium*	777.53	1448.80
T_9	T_3+ *Azotobacter chroococcum*	794.10	1455.53
T10	T_3+ *Azospirillum lipoferum*	824.17	1484.00
	S.Em.±	14.44	22.99
	C.D. at 5 %	42.91	68.30
	C.V. %	3.35	4.85

Omidvari *et al.* (2010) [15] observed that inoculation of *Pseudomonas fluorescent* significantly increased the bean growth factor and yield of bean crop. Similarly, Ahmad *et al*. (2012) [1] found that combined application of *Rhizobium* and *Pseudomonas strains* improve the productivity of mung bean. Further Gajera *et al*. (2014) [8] studied the enhancement of green gram due to coinoculation of *Rhizobium* over liquid PSB inoculation.

Gamit and Tank (2014) [9] observed that effect of siderophore producing microorganisms on *Cajanus cajan.* They found that inoculation with siderophore producing organism *Pseudomonas pseudoalcaligenes* affect the germination of *Cajanus cajan* seeds significantly. There was a strong effect of inoculation with isolates on the root growth of germinated seedlings. Increase in seed yield and straw yield was observed in case of treated plant as compared to uninoculated control. These results are in agreement with the findings of Parmar and Chakraborty (2016) [17] who observed that effect of siderophore producing microorganism (*Pseudomonas fluorescens)* on *Lens Culinaris* (masoor dal) and *Phaseolus lunatus* (large white Lima Beans) were grown under iron limiting conditions with siderophore supplements. Siderophore which was proved to be useful for plant growth promotion due to increase in root length, shoot length and number of leaves, seed and straw yield of masoor dal and large white lima bean.

Conclusion

Eight plant growth promoting microorganisms isolates were tested for siderophore production. *Pseudomonas fluorescens*

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 useful for plant growth promotion due to increase in height of plant, number of branches, number of pods and number of leaves of green gram when grown under iron limiting conditions with siderophore supplements. Thus siderophore can be used in combination with other biofertilizers to increase crop productivity.

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