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Assessing phosphate solubilization ability of *sesbania grandiflora* rhizobia isolated from root nodules using diverse agroecological zones of Indian soils for biofertilizer production

Kuldeep Singh and Rajesh Gera

Abstract
A total of 20 rhizobia were isolated from *Sesbania grandiflora* root nodules by using trap plant method from diverse agroecological zones of Indian soils. These rhizobial isolates produced round, whitish, smooth surfaces on YEMA medium plates and were short rod in shape, motile in nature and gram negative in reaction. On Pikovskaya's medium plates around 80% of rhizobial isolates were found to be phosphate solubilizers. P-solubilisation index (P-SI) of these isolates ranged from 1.96 to 4.85. In the present investigation, isolates SGBh from Bhadra (Rajasthan) and SGKr(ii), SGKr(iv), SGKr(v) from CSSRI, Karnal (Haryana) were found to have excellent P-solubilization ability and can be exploited as biofertilizers for better yield of *Sesbania grandiflora.*

Keywords: *Sesbania grandiflora*, rhizobia, phosphate solubilization, biofertilizer

Introduction
It is undoubtedly clear that phosphorus (P) is one of the most essential macroelement required for growth and development of plants (including photosynthesis, energy and sugar production) and also promotes N_2 fixation in legumes (Saber *et al*. 2005; Xiao *et al*., 2011) [16, 26]. In soils, only 0.1% of the total P (0.5%) is available to plants, while rest of the total P is present in the insoluble form and therefore, cannot be taken up by plants (Scheffer and Schachtschabel, 1988) [17]. Rock phosphate (RP) is the only economic source of P but its availability is restricted and skewed. India has very limited sources of RP, which is mostly low to medium grade in quality, thus making it almost entirely dependent on import (Subba Rao *et al*., 2015) [23]. Even though most of the agricultural soils have sufficient amounts of phosphorus, low availability of native soil P remains a key limiting factor to improve crop productivity. It remains in a precipitated form in the soil as mono or orthophosphate or is adsorbed by Fe or Al oxides through ligand exchange, while the plants absorb it only in two soluble forms, the monobasic ($H_2PO_4^-$) and the dibasic (HPO_4^{2-}) ions (Bhattacharyya and Jha, 2012) [4]. Mobilization of unavailable P to plant available P is a prerequisite to sustain crop productivity. Usually, the phosphate solubilizing microorganisms (PSM) plays vital role in phosphorus nutrition by exchanging its availability to plants through lowering the soil pH (Baby *et al.*, 2016) [2]. Phosphate solubilizing bacteria (PSB) are useful bacteria capable of solubilizing inorganic phosphorus. P-solubilization ability of rhizospheric microorganisms is considered to be one of the most important characters associated with plant phosphate nutrition to improve the plant growth and yield. The mineralization of organic phosphorus also occurs through the synthesis of wide varieties of phosphatases like acid and alkaline phosphatase, catalyzing the hydrolysis of phosphoric esters (Zhang *et al.* 2014) [27]. The phosphate solubilizing bacteria and plant growth promoting rhizobacteria (PGPR) together could lower phosphorus fertilizer application by 50% without any significant reduction of crop yield (Jilani *et al.*, 2007) [11]. Bacterial genera like *Azotobacter*, *Bacillus*, *Beijerinckia*, *Burkholderia*, *Enterobacter*, *Erwinia*, *Flavobacterium*, *Microbacterium*, *Pseudomonas* and *Serratia* are reported as the most significant phosphate solubilizing bacteria (Bhattacharyya and Jha, 2012) [4]. The ability to P-solubilization is found even among *Rhizobiacae*, comprising of *Rhizobium*, *Bradyrhizobium*, *Mesorhizobium* and other non-specified legume-nodulating bacteria (LNB).

Sesbania grandiflora is a small, erect, fast-growing and sparsely branched perennial tree in the genus *Sesbania* belonging to the *Leguminosae* family. The other scientific names of *Sesbania grandiflora* are *Robinia grandiflora* Linn, *Aeshynomene grandiflora* Linn, *Sesban grandiflora* Poir and *Agathi grandiflora* (L.) Desv. In India it is distributed in West Bengal, Assam, Karnataka and North-Eastern parts (Reji and Alphonse, 2013) [15]. It can be grown on a wide range of soils including those that are poor in nutrients, tolerate saline, alkaline and acidic conditions of the soils down to water pH 4.5. It grows promptly and rapidly accumulates nitrogen (N_2) -rich biomass appropriate for soil fertility replenishment and also provides fuel wood, fodder and mulch. It occur naturally in wet or flooded soils and these have considerable potential as green manure in wetland rice production due to their ability to fix large quantities of N_2 (James *et al*., 2001) [10].

Seed inoculation of pulse crops with superior rhizobial strains is essentially required to improve nodulation, N_2-fixation and phosphate solubilisation, which in turn is translated into enhanced growth and grain yield. Free-living soil bacteria beneficial to plant growth, usually referred to as plant growth promoting rhizobacteria (PGPR), are capable of promoting plant growth by colonizing the plant root (Kumar *et al*., 2013) [12]. PGPR, as biocontrol agents, can act through various mechanisms, regardless of their role in direct plant growth promotion, such as by known production of auxin phytohormone like indole-3- acetic acid (IAA) and gibberellins, NH_3 excretion, phosphate solubilization and siderophore production (Arora *et al.*, 2001; Sharma *et al*., 2014) [1, 18]. Rhizobial strains having plant growth promoting (PGP) attributes have been found to improved nodulation and plant growth of legumes (Dhull *et al*., 2016) [5] with increased tolerance to high salt, water potential, pH and temperature stresses, therefore could enhance production of food and forage from legumes in semiarid and arid regions of the world (Singh *et al*., 2017) [20].

A fully functional symbiosis requires successful survival ability of bacteria even under adverse environmental conditions (Singh *et al*., 2018) [21]. The use of host-specific native rhizobial isolates is suggested because they adjust better to the local environmental and soil conditions. Additionally, native rhizobial isolates have improved survival rate which could raise the probability of successful nodulation and nitrogen fixation in the host plant. In order to achieve maximum legume productivity, screening of native rhizobial isolates for their nitrogen fixation efficiency along with P-solubilization activity is vital. The multiplicity of beneficial effects of microbial inoculants, particularly plant growth promoters, emphasizes the need for further strengthening the research and their use in modern agriculture (Gopalakrishnan *et al*., 2015) [7]. *Sesbania grandiflora* occupy a major position in the cropping system, but little is known of the rhizobia that associate with this agriculturally important species in terms of their P-solubilization ability. Hence, in the present study the rhizobial isolates from *Sesbania grandiflora* root nodules were characterized for phosphate solubilisation ability along with N_2 fixation from diverse agroecological zones of Indian soils for biofertilizer production.

Materials and Methods

Seeds

Seeds of *Sesbania grandiflora* were collected from Tamil Nadu Agricultural University, Coimbatore, Tamil Nadu.

Isolation of rhizobia nodulating *Sesbania grandiflora* using trap plant method

Seeds of *Sesbania grandiflora* were grown in cups, containing soil samples collected from different locations of India to trap the rhizobia. The healthy pink nodules were separated after 45 days of growth. The nodules were surface sterilized by using 0.1% $HgCl_2$ and 70% ethanol (Vincent, 1970) [24]. The nodules were crushed in a sterilized petri plate and a loopful of nodule sap was streaked on YEMA medium plates containing Congo red dye. The plates were incubated at 28±2ºC and growth was observed daily for 2-7 days. Single white gummy colony of rhizobial isolates were picked up and restreaked on YEMA medium plates for purification and maintained on YEMA medium slants at 4^0C in a refrigerator for further studies (Plate 1).

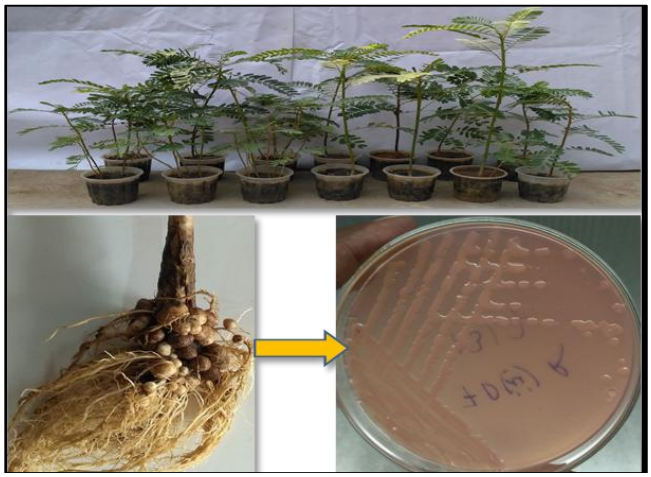

Plate 1: Isolation of rhizobia nodulating *Sesbania grandiflora* plant using trap plants method from different type of Indian soils

Authentication of rhizobial isolates retrieved from *Sesbania grandiflora*

Rhizobial isolates were grown in YEM liquid media having pH 7.0 and incubated under shaking conditions at 200 rpm. The log phase actively grown cultures were subjected to check their authenticity using following methods.

a). Hofer's alkaline test: In order to differentiate the rhizobial isolates from the *Agrobacterium*, log phase actively grown isolates were grown in the Hofer's alkaline medium (pH 11) at 28±2^{0}C for 3-7 days (Hofer, 1935) [8] (Plate 2a). Normally, *Rhizobium* cannot grow in Hofer's medium and help to detect the contamination of *Agrobacterium.*

b). Ketolactase test: The rhizobial isolates were streaked on the lactose agar medium for 2-5 days at 28±2^{0}C. Five millilitres of Benedict's reagent was poured on the plates and kept at room temperature for 1 hour. Absence of yellowish zones of Cu_2O around the colonies of *Rhizobium* indicated the purity of the isolates (Bernaerts and Deley, 1963) [3] (Plate 2b).

c). Acid alkaline production test: The production of acid and alkali was detected in this test by allowing the isolates to grow on YEM broth supplemented with bromothymol blue (BTB) at a concentration of 1.5 mL/100 mL. The change in color and pH of the YEM broth was recorded after incubation at 28±2^{0}C for 24-48 hours (Somasegaran and Hoben, 1994) [22] (Plate 2c).

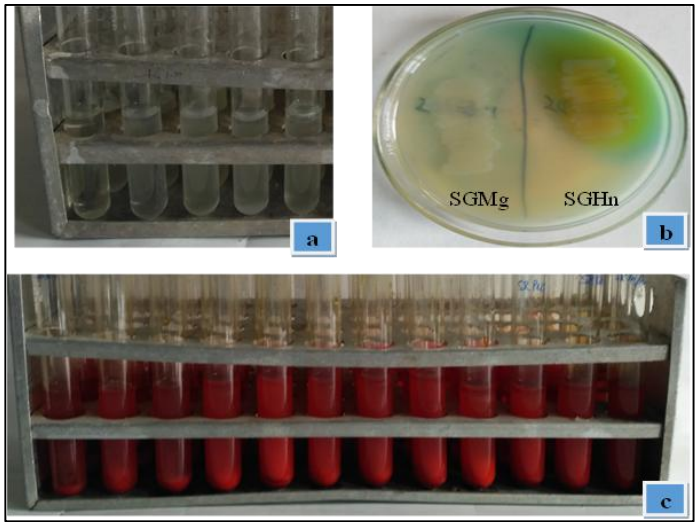

Plate 2: Hofer's alkaline test (a), Ketolactase test (b), Acid-alkaline production test (c) of *Sesbania grandiflora* rhizobial isolates

Phosphate solubilization

Phosphate solubilization ability of *Sesbania grandiflora* rhizobial isolates were detected by spotting separately on Pikovskaya's agar medium plates. Plates were then incubated at 28±2°C for 3-7 days, and observed for the clearing zone around the colonies (due to the solubilization of inorganic phosphate by bacteria) (Pikovskaya, 1948) [13]. Solubilization index (SI) of these isolates was determined by measuring diameter of solubilization zone using the following formula.

$$\text{P-Solubilization Index (P-SI)} = \frac{\text{Colony diameter (mm)} + \text{Zone diameter (mm)}}{\text{Colony diameter (mm)}}$$

Isolation of rhizobia nodulating *Sesbania grandiflora* using trap plant method

A total of 20 rhizobial isolates were isolated from root nodules of *Sesbania grandiflora* plants after 45 days of growth. These were named as SG with site of collection of soil like SGKe where Ke stand for Kerala and so on, and maintained separately on YEMA medium slants at 4°C for further study (Plate 1; Table 1). Similarly, Gera *et al*. (2014) [6] isolated 64 rhizobial isolates from root nodules of fababean grown in pots holding soils collected from arid and semiarid regions of Haryana, India. Similarly, Singh *et al*. (2018) [19] also isolated 14 rhizobial isolates from root nodules of *Sesbania sesban* plant using different types of Indian soils.

Results and Discussion

Table 1: Details of rhizobia isolated from root nodules of *Sesbania grandiflora* plant using soil samples collected from diverse agroecological zones of India

State	Agroecological zones	Rhizobial isolates name	No. of rhizobial isolates obtained
Kerala	Western Ghats	SGKe(i), SGKe(ii), SGKe(iii)	3
Rajasthan	Northern plane	SGBh, SGUd	2
Tamil Nadu	Eastern Ghats	SGTn	1
Maharastra	Deccan platue	SGPr	1
Haryana	Northern plane	SGKr(i), SGKr(ii), SGKr(iii), SGKr(iv), SGKr(v), SGKr(vi), SGSn, SGHs, SGHn, SGGh, SGMg, SGBn	12
Uttar Pradesh	Northern plane	SGMa	1
		Total	20

SG= *Sesbania grandiflora*, Ke= Kerala, Bh= Bhadra, Tn= Tamil Nadu, Pr= Parbhani, Kr= Karnal, Sn= Sonipat, Hs= Hisar, Hn= Hansi, Ma= Mau, Gh= Gohana, Mg= Mahendergarh, Bn= Bhiwani and Ud= Udaipur

Authentication of rhizobial isolates

All 20 *Sesbania grandiflora* rhizobia thus obtained were characterized for colony morphology and cell shape through Gram staining. It was observed that all the rhizobial isolates formed white gummy colony on YEMA medium supplemented with Congo red dye. The colonies did not absorb the Congo red color and this differentiates *Rhizobium* from other contaminants. Colonies of *Rhizobium* were found to be circular, semi-translucent, single and mucilaginous in nature. All the isolates were Gram –ve having small rods in appearance. In the present study out of twenty rhizobial isolates, only SGKr(i) showed mild growth in Hofer's medium doubting its authenticity to be *Rhizobium* (Table 2 and Plate 2a). Out of 20 rhizobial isolates, only four showed yellow zone (YZ) formation on Ketolactose agar medium plates, indicating that these isolates belongs to *Agrobacterium* species (Table 2 and Plate 2b). Inoculation of these isolates in YEM broth supplemented with bromothymol blue changed the color of broth to yellow after five days of growth showing the production of acid which is the characteristic of *Rhizobium*. The pH of the culture broth was also decreased to 4.1-5.1 from an initial pH of 7.0 (Table 2 and Plate 2c). The authentication of these isolates by Koch's postulation showed that 15 out of 20 isolates belong to *Rhizobium* species, while rests of the isolates were tentatively characterized as *Agrobacterium* species. Similarly Rai and Sen (2015) [14] used these methods to differentiate 18 *Rhizobium* strains of French bean (*Phaseolus vulgaris* L.) from *Agrobacterium* and found that 17 isolates to be *Rhizobium*. Moreover, according to new classification of rhizobial taxonomy *Agrobacterium* also forms nodules and fix nitrogen (Willems, 2006) [25] and recently named as *Rhizobium radiobactor*. Therefore, all the 20 rhizobial isolates were used for further studies.

Table 2: Authentication of rhizobia isolated from root nodules of *Sesbania grandiflora* plant

Sr No.	Rhizobial isolates	Hofer's alkaline test	Ketolactose test	Acid-alkaline production test
1	SGKe (i)	0.086	NYZ	5.1
2	SGKe (ii)	0.131	NYZ	4.7
3	SGKe (iii)	0.037	NYZ	4.5
4	SGBh	0.065	YZ	4.1
1.	SGTn	0.077	NYZ	4.6
2.	SGPr	0.041	YZ	4.7
3.	SGKr(i)	1.132	NYZ	4.8
4.	SGKr(ii)	0.022	NYZ	4.8
5.	SGKr(iii)	0.130	NYZ	4.8
6.	SGKr(iv)	0.127	NYZ	4.5
7.	SGKr(v)	0.074	NYZ	4.3
8.	SGKr(vi)	0.038	NYZ	4.6
9.	SGSn	0.050	NYZ	4.6
10.	SGHs	0.083	NYZ	5.0
11.	SGHn	0.038	YZ	4.9
12.	SGMa	0.043	NYZ	4.7
13.	SGGh	0.027	YZ	4.6
14.	SGMg	0.079	NYZ	4.1
15.	SGBn	0.064	NYZ	4.8
16.	SGUd	0.131	NYZ	4.2

Phosphate solubilization by *Sesbania grandiflora* rhizobia

Sesbania grandiflora rhizobial isolates were tested for P-solubilization on solid Pikovskaya's medium plates and it was observed that around 80% of the rhizobial isolate were found to be P-solubilizer. Out of which, only 20% of the isolates were found to be excellent P-solubilizer having P-solubilization index (P-SI) 3.0-5.0. However, 45 and 15% were moderate and poor P-solubilizer showing P-SI between 1.0 to 3.0, and ≤ 1.0 respectively. Only, 20% of the rhizobial isolates were unable to solubilise phosphate (Table 3, Plate 3 and Fig. 1). Similarly, Singh *et al.* (2017) [20] reported that out of forty nine pigeon pea rhizobial isolates from arid and semi arid zones of Haryana, 96% of the isolates were able to form significant zone of P-solubilization on Pikovskaya's medium plates and their P-solubilization index (P-SI) varied from 1.2 to 3.7. While Jadhav (2013) [9] reported contrary results that out of 10 rhizobial isolates from soybean crop, only 3 isolates showed P-solubilization activity.

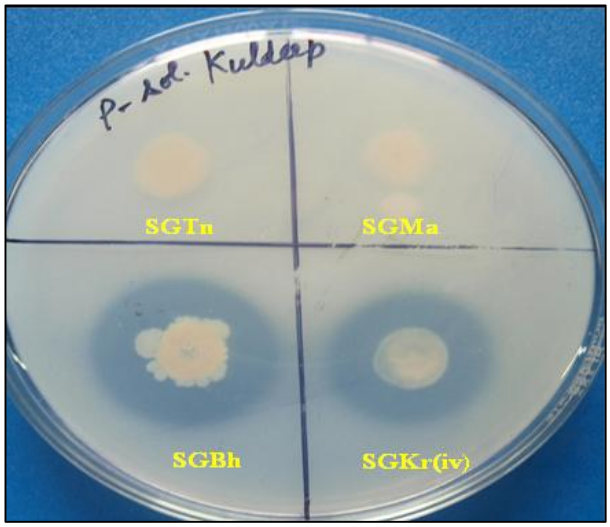

Plate 3: *Sesbania grandiflora* rhizobial isolates showing zone of P-solubilization on Pikovskaya's agar medium plate

Table 3: Phosphate solubilisation by *Sesbania grandiflora* rhizobia on Pikovskaya's agar medium plates

Sr. No.	Rhizobial isolates	P-Solublization Index (P-SI)
1.	SGKe(i)	1.96
2.	SGKe(ii)	2.17
3.	SGKe(iii)	2.05
4.	SGBh	4.85
5.	SGTn	-
6.	SGPr	1.98
7.	SGKr(i)	-
8.	SGKr(ii)	3.50
9.	SGKr(iii)	2.67
10.	SGKr(iv)	3.57
11.	SGKr(v)	4.24
12.	SGKr(vi)	2.83
13.	SGSn	2.30
14.	SGHs	2.17
15.	SGHn	-
16.	SGMa	-
17.	SGGh	1.98
18.	SGMg	2.55
19.	SGBn	2.70
20.	SGUd	2.92

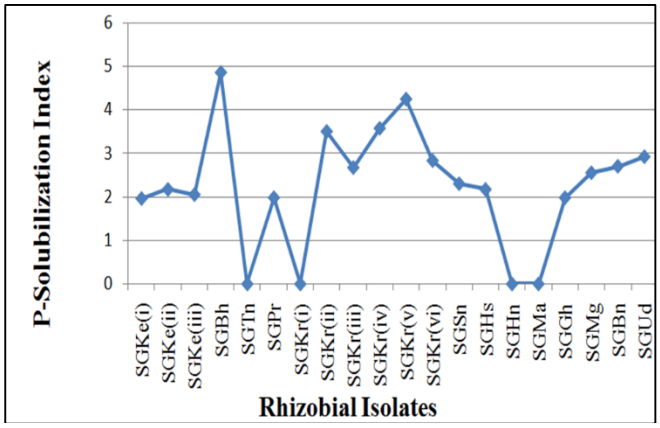

Fig 1: Categorization of *Sesbania grandiflora* rhizobia for P-solubilization ability

Therefore, from the present study, it was concluded that most of the *Sesbania grandiflora* rhizobial isolates showed good phosphate solubilization activity. Four rhizobial isolates *viz.*, SGBh, SGKr (ii), SGKr (iv) and SGKr (v) performed excellent in terms of P-solubilization as compared to remaining ones. These isolates have tremendous potential in near future to be used as biofertilizers, which will not only improve P-solubilization rather leading to enhancement in plant growth of *Sesbania grandiflora*.

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