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Short Communication

Isolation and authentication of bacterial strains from root nodules of *Sesbania aculeata* in diverse agroecological zones of India

Kuldeep Singh and Rajesh Gera

Abstract
Root nodules were collected from young and healthy seedling of *Sesbania aculeata* using trap plant method from the soil samples collected from field at different locations of India. Ten isolates were isolated from the root nodule of *Sesbania aculeata* and characterized by regular tests. All strains were gram-negative and did not absorb red color when cultured in YEMA containing congo red. In the ketolactose test yellowish zone of Cu2O not found. Moreover isolates have either deprived or no growth on the Hofer's test which is representing nature of rhizobia. All isolates were fast grower which is confirmed by acid-alkaline production test.

Keywords: *Sesbania aculeata*, root nodules, rhizobia, ketolactose test

Introduction
Overcoming the lack of fertilization without soil pollution implies exploring alternative routes of nutrient use and input, possible through biological rhizosphere processes. Soil microbes are used as an ecofriendly approach for maintaining soil health and nutrient cycling. Rhizobia are differentiated from all nitrogen fixing bacteria as they formed a unique specialized structure called nodule as a consequence of symbiotic association with their legume host. The symbiosis among rhizobia and legume are a reasonable and generally more useful agronomic exercise for provide an enough source of N for legume based crop (Zahran, 1999) [13] and therefore can play a major function in enhancing the fertility and effiency of soils. Recognition and assortment of efficient rhizobial strains are vital for preserving them for potential study. The issue of symbiotic usefulness and competitiveness of rhizobia in Indian prospective assumes additional consequence and has attracted a lot of Indian workers (Appunu and Dhar, 2006; Appunu *et al*., 2008) [2, 1]. Selected rhizobial isolates which have multi-trait plant growth promoting ability could be further used as a biofertilizer for enhancing growth, yield and productivity for different leguminous crops. *Sesbania aculeata* (Dhaincha) is one of the potential nonwood plants. It is usually cultivated for its nutritive worth to soil, in monsoon seasons, approximately all over the India, as it grows properly in loamy, clay, black and sandy soils. It is an ultimate green manure crop as it is quick developing, succulent, simply decomposable, with low moisture supplies, producing utmost amounts of organic material and nitrogen in the soil. There is enormous quantity of text accessible, reporting rhizobia from diverse pulses (Riah *et al*. 2014; Wadhwa *et al*. 2011; Hou *et al*. 2009 and many others) [8, 12, 6] but incomplete studies are there about the biochemical description of rhizobia inhabiting *Sesbania aculeata*. Rhizobia are characterized on the source of biochemical tests. So, this study was aimed to isolate and identify rhizobia on the basis of biochemical tests from root nodules of *Sesbania aculeata* for improved agriculture growth.

Materials and Methods
Isolation of rhizobia nodulating *Sesbania aculeata* using trap plant method
Seeds of *Sesbania aculeata* 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) [11]. 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±2 ºC 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 ^{0}C in a refrigerator for further studies (Plate 1).

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

Authentication of rhizobial isolates retrieved from *Sesbania aculeata*

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) [5] (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) [10] (Plate 2c).

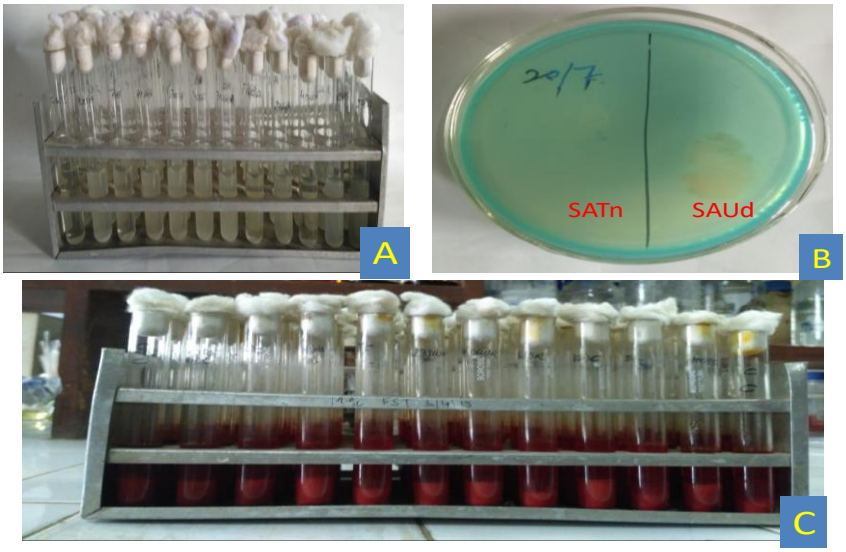

Plate 2: Hofer's alkaline test (a), Ketolactase test (b), Acid-alkaline production test (c) of *Sesbania aculeata* rhizobial isolates

Results and Discussion

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

A total of 10 rhizobial isolates were isolated from root nodules of *Sesbania aculeata* plants after 45 days of growth. These were named as SA with site of collection of soil like SAKe 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, Singh *et al*. (2018) [9] isolated 14 rhizobial isolates from root nodules of *Sesbania sesban* grown in pots holding soils collected from diverse regions of India. Similarly, Dhull *et al*. (2018) [4] also isolated fourteen rhizobia from healthy pink root nodules of clusterbean plants grown in farmer's field belongs to Hisar, Bhiwani and Mahendergarh districts of Haryana.

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

***Sesbania* species**	**Name of rhizobial isolates**	**No. of rhizobial isolates**
Sesbania aculeata	SAKe(i), SAKe(ii), SATn, SAMa, SAUd, SAPr, SAKr(i), SAKr(ii), SAHn, SAMg	10

SA= *Sesbania aculeata*, Ke= Kerala, Tn= Tamil Nadu, Pr= Parbhani, Kr= Karnal, Hn= Hansi, Ma= Mau, Mg= Mahendergarh, and Ud= Udaipur

Authentication of rhizobial isolates

All 10 *Sesbania aculeata* 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 ten rhizobial isolates, only SAKe (ii) showed mild growth in Hofer's medium doubting its authenticity to be *Rhizobium* (Table 2 and Plate 2a). Out of 10 rhizobial isolates, none showed yellow zone (YZ) formation on Ketolactose agar medium plates, indicating that these isolates belong to *Rhizobium* 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 4.1-4.8 from an initial pH of 7.0 (Table 2 and Plate 2c). The authentication of these isolates by Koch's postulation showed that 90% isolates belong to *Rhizobium* species, while 10% of the isolates were tentatively characterized as *Agrobacterium* species. Similarly Rai and Sen (2015) [7] used these methods to differentiate 18 *Rhizobium* strains of French bean (*Phaseolus vulgaris* L.) from *Agrobacterium* and found that 17 isolates to be *Rhizobium*.

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

Sr. No.	Rhizobial isolates	Hofer's alkaline test	Ketolactose test	Acid-alkaline production test
1	SAKe(i)	0.044	4.8	NYZ
2	SAKe(ii)	1.030	4.1	NYZ
3	SATn	0.133	4.7	NYZ
4	SAMa	0.135	4.8	NYZ
5	SAUd	0.104	4.6	NYZ
6	SAPr	0.150	4.2	NYZ
8	SAKr(i)	0.055	4.2	NYZ
9	SAKr(ii)	0.057	4.2	NYZ
10	SAHn	0.046	4.7	NYZ
11	SAMg	0.137	4.4	NYZ

Therefore, from the present study, it was concluded that, all 10 *Rhizobium* strains from *Sesbania aculeata* did not take up red color while cultured in YEMA containing congo red medium. Pseudo-nodule forming bacteria *Agrobacterium* appropriate congo red however *Rhizobium* strains didn't consume congo red. This test is important to distinguish *Rhizobium* and *Agrobacterium*. Further biochemical tests confirmed that isolated strains were *Rhizobium.*

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