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Evaluation of rhizobia isolated from nodules of *Sesbania* spp. for intrinsic antibiotic resistance pattern

Kuldeep Singh, Rajesh Gera, Jagdish Parshad, Dinesh Kumar and Sushil Kumar Singh

Abstract
A total of 50 *Sesbania* rhizobial isolates growing in different zones of India were used to investigate their intrinsic resistance to different concentrations of eight antibiotics *viz*., ampicillin, chloramphenicol, Gentamycin, Kanamycin, nalidixic acid, Tetracyclin streptomycin and neomycin. Most of the isolates were found to be resistant to five antibiotics *viz*., ampicillin, chloramphenicol, gentamycin, nalidixic acid and streptomycin up to a concentration of 100 µg ml^{-1} after 4 days of incubation. However, most of the isolates were sensitive to kanamycin, neomycin and tetracycline antibiotics even at a concentration 20 µg ml^{-1} indicating that different isolates have different antibiotic resistance pattern and can be used as tool to identify the inoculated rhizobia in soil under unsterilized conditions.

Keywords: *Rhizobium*, antibiotics, *Sesbania* spp., YEMA medium

Introduction
Contest for nodulation is commonly calculated by measuring the capability of introduced *Rhizobium* isolates to form large digit of nodules on the selected host. Inspite of this, the rhizosphere constitutes huge populations of diverse microorganisms. A number of these microorganisms typically generate antibiotics which are fatal to sensitive rhizobial populations in the soil (Naamala, *et al*., 2016) [1]. Resistance to antibiotics increases the *Rhizobium* probability of continued existence in the rhizosphere. Antibiotic resistance has been regularly applied in differentiating the applied inoculant strain from native rhizobia and observes their continuity and habitation of legume nodules (Bromfield, *et al*., 1985; Simon & Kalalova, (1996) [2, 3]. Rhizobial strains ought to resistant to concentrations of antibiotics that obstruct the development of other soil bacteria and they must be adept to keep their affectivity and symbiotic efficacy. The resistance may be grown up for single or manifold antibiotic groups (Anand, *et al*., 2012) [4]. Although bulks of rhizobial isolate in the soil are sensitive to antibiotics, remains have advanced resistance in reaction to commonly produced antibiotics (Beringer, 1974; Wiener, 1996; Xavier, *et al*., 1998) [5, 6, 7]. Hence, intrinsic resistance to antibiotics is an advantageous feature for the rhizobial population. It boosts the rhizobial probability of development, reproduction and determination in the soil. The logic for making of antibiotics by a few soil habiting microbes is not yet understandable. The antibiotics lyse living cells of sensitive bacterial group, thus given that food for the organism (Kumbhar & Watve, 2013) [8]. Different group of antibiotics show diverse modes of response on the bacteria like interference with cell wall synthesis, inhibition of protein, nucleic acid and metabolic pathway synthesis, disruption of bacterial membrane structure. Benefits of this process of detection enclosed its loyalty in that resistance to low levels of antibiotics came to be a durable assets of the *Rhizobium* strains investigated, as outcome were reproducible over lengthy stage of period. The resistance of rhizobial strains to antibiotics is being broadly applied in study of the stability in soil or other environment and their competitiveness as a marker for nodulation of the host plant and success of nitrogen fixation (Levin & Montgomery, 1974; Schwinghamer, 1979; Yosey, *et al*., 1979; Pugashetti & Wagner, 1980; El Hasan, *et al*., (1986) [9, 10, 11, 12, 13]. Intrinsic antibiotic resistance pattern of different rhizobial strains belonging to the same species is considered to be a significant phenotypic characteristic (Hartman and Amarger, 1991; Tas *et al*., 1996) [14, 15]. Different rhizobial strains show different degrees of susceptibility to various antibiotics, that is why this property is being used for the identification of inoculated strain (Obaton, 1971; Antoun *et al*., 1982; Kremer and Paterson, 1982) [16, 17, 18].

The genus *Sesbania* contains about 70 species widespread over tropical and subtropical regions, including annual, perennial, herbaceous shrubs and trees. Dhaincha belongs to the family Leguminosae (sub-family Papilionoideae), and is well known for its diversified use in India. It grows quickly and rapidly accumulates nitrogen (N_2) rich biomass suitable for soil fertility replenishment and also provides fuel wood, fodder and mulch. In deserts the growing of *Sesbania* spp. lead to the addition of organic matter in the soils. This examination shows the necessity for advance information of biological criterion, for example the dynamics of native rhizobial populations, continuity and competitiveness. For identification of strains high concentration of antibiotic resistance markers have been used (Schwinghamer & Dudman, 1973) [19]. It has advantage of ease of isolation and recognition of inoculum strains from nodules and also from the soil. Antibiotic resistance pattern has traditionally been used as a marker in competition studies because this method is simple and requires no specialized equipment (Ramirez, *et al*., 1998) [20]. Therefore, aim of this study was to characterize 50 strains of rhizobia isolated from root nodules of *Sesbania* spp. plant based on their intrinsic resistance to different concentrations of eight antibiotics.

Materials and Methods

Isolation of native rhizobia nodulating *Sesbania* species using trap plant method

Seeds of *Sesbania* species *viz., S. sesban*, *S. grandiflora*, *S. aculeata* and *S. rostrata* were grown in pots, each containing soil samples collected from different locations of India to trap the rhizobia nodulating *Sesbania* species. In case of *S. sesban*, *S. grandiflora*, *S. aculeata* and *S. rostrata*, the healthy pink nodules were removed after 45 days of growth, while in case of *S. rostrata*, the green stem nodules were also separated after 75 days of growth. The nodules were surface sterilized by using 0.1% $HgCl_2$ and ethanol (Vincent, 1970) [21]. 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 3-7 days. White gummy colony of rhizobial isolates were picked up and restreaked on same medium 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.

Evaluation of rhizobia for intrinsic antibiotic resistance pattern

Intrinsic antibiotic resistance of different rhizobia was evaluated according to Eaglesham's technique as described by Hashem *et al*. (1998) [22] on YEMA medium plates supplemented with different concentrations of antibiotics. Fifty *Sesbania* spp. rhizobial isolates were grown in TY broth. Stock solutions of various antibiotics (ampicillin, chloramphenicol, Gentamycin, Kanamycin, Tetracycline, nalidixic acid, streptomycin and neomycin) were prepared. TY media plates supplemented with different concentrations (10, 25, 50 and 100 µg ml^{-1}) of filter sterilized antibiotics were prepared. The TY medium without antibiotics was used as control. Two µl of log phase grown cells of each isolate was spotted on TY medium plates without antibiotics and incubated at 28±2ºC for 2-3 days till appearance of growth. All the 50 *Sesbania* rhizobial isolates were then spotted on TY medium plates containing different concentration of all the above antibiotics through replica plating method. The plates were incubated at 28±2ºC for 4-7 days until the appearance of growth.

Results and Discussion

A total of 50 *Sesbania* rhizobial isolates were obtained from different *Sesbania* spp. out of which 8 belonged to *Sesbania sesban*, 12 from *Sesbania grandiflora*, 9 from *Sesbania aculeata*, 9 from *Sesbania rostrata* (root nodulating) and 12 from *Sesbania rostrata* (stem nodulating) as described in table 1. Similarly Singh *et al*. (2017) [23] also isolated forty-nine isolates from root nodules of Pigeon pea [*Cajanus cajan* (L.) Millspaugh] growing in Haryana. In present study, the development of rhizobial strains tested to various antibiotic concentrations was visibly decided depend on improvement on colony diameter. All the 50 *Sesbania* rhizobial isolates were tested for resistance to various antibiotics at different levels of concentration. Most of these isolates were found to be resistant to five antibiotics namely; ampicillin, chloramphenicol, gentamycin, nalidixic acid and streptomycin up to a concentration of 100 µg ml^{-1} after 4 days of incubation and sensitive to kanamycin, neomycin and tetracycline antibiotics even at a concentration 20 µg ml^{-1} indicating that different isolates have different antibiotic resistance pattern (Tables 1 and 2). The antibiotic resistance pattern of these isolates will be quite useful in studying their survival and nodulation efficiency under field conditions. Young and Chao (1989) [24] described that both the quickly and slow developing isolates of rhizobia display extensive fluctuation in resistance to antibiotics. Milicic *et al*. (2006) [25] investigated thirty six strains of *Bradyrhizobium japonicum* and three strains of *Rhizobium galegae* to study their intrinsic resistance to different concentrations of several antibiotics. The results clearly indicate diversity among the strains tested regarding their intrinsic resistance to different concentrations of antibiotics. Kaur and Sharma (2013) [26] tested ten rhizobacteria of *Pseudomonas* spp. isolated from rhizosphere of chickpea for their reactivity to antibiotics along with reference strain LK884. All the isolates showed resistance against ampicillin, whereas LK884 was sensitive to this antibiotic. Ansari *et al*. (2014) [27] reported that slow growing and fast growing Bradyrhizobia, also differed with respect to intrinsic antibiotic resistance pattern as mostly slow growers were resistant to trimethoprim, nalidixic acid, polymyxin B, vancomycin and novobiocin, while fast growing rhizobia, were resistant to trimethoprim, nalidixic acid and novobiocin. Boora (2016) [28] observed the similar type of results with pigeon pea rhizobia. Zohra *et al*. (2016) [29] reported that the intrinsic antibiotic resistance level in all strains was tested against nine antibiotics; they revealed a variability of resistance against spectinomycin (10 µg , erythromycin (15 µg ml^{-1} , rifampicin (30 µg ml^{-1}), streptomycin (500 µg ml^{-1}), kanamycin (30 µg ml^{-1}), naldixic acid (30 µg ml^{-1}), penicillin (6 µg ml^{-1}) and tetracycline (30 µg ml^{-1}) except for gentamycin (500 µg ml^{-1}). Dhull *et al*. (2018)[30] reported that all the 14 strains of *Rhizobium*/*Bradyrhizobium* from clusterbean showed good growth on nalidixic acid, chloramphenicol and ampicillin up to the concentrations of 100 µg ml^{-1}, while sensitive to neomycin and kanamycin upto a concentrations of 10-20 µg ml^{-1}. Mir *et al*. (2020) [31] reported that out of 19 isolates, six isolates showed resistance to Chloramphenicol. Rhizobial strains showed resistance to

the antibiotics such as erythromycin, fusidic acid, methicilin and pencicillin. All the isolated bacterial strains were susceptible to novobiocin and tetracycline

Table 1: Intrinsic antibiotic resistance pattern of *Sesbania* rhizobial isolates

Sr. No.	Rhizobial isolate	Concentration (ug/ml)																			
		Ampicillin			Chloramphenicol			Gentamycin	Kanamycin			Neomycin		Nalidixic acid			Tetracycline	Streptomycin		
		20	50	100	20	50	100	20	50	20	50	20	50	20	50	100	20	50	20	50	100
***Sesbania sesban*(root nodulating)**																					
1	SSUd	+++	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	+++	+++	+++	-	-	+++	+++	-
2	SSTn	+++	+++	+++	+++	+++	+++	++	+	+++	-	+++	++	+++	+++	+++	++	+	+++	+++	+++
3	SSKe(ii)	+++	+++	+++	+++	+++	+++	++	+	-	-	-	-	+++	+++	+++	-	-	+++	+++	+++
4	SSGh	+++	+++	++	+++	+++	+++	+++	++	-	-	-	-	+++	+++	-	+	-	+++	+++	+++
5	SSBh	+++	+++	+++	+++	+++	+++	++	++	-	-	-	-	+++	+++	+++	-	-	+++	+++	+++
6	SSKr(ii)	+++	+++	+++	+++	+++	+++	++	+	-	-	-	-	+++	+++	+++	-	-	+++	+++	-
7	SSHs	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	+++	+++	+++	-	-	+++	+++	+++
8	SSPr	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	+++	+++	+++	++	-	+++	+++	+++
***Sesbania grandiflora* (root nodulating)**																					
9	SGKe(i)	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	+++	+++	+++	+	-	+++	+++	+++
10	SGKe(ii)	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	+++	+++	+++	++	-	+++	+++	+++
11	SGBh	+++	+++	+++	+++	+++	-	+++	-	-	-	-	-	+++	+++	+++	-	-	+++	+++	-
12	SGTn	+++	+++	+++	+++	+++	+++	+++	+	-	-	-	-	+++	+++	+++	-	-	+++	+++	+++
13	SGPr	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	+++	+++	+++	-	-	+++	+++	-
14	SGKr(vi)	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	+++	+++	+++	+	-	+++	+++	+++
15	SGSn	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	+++	+++	+++	-	-	+++	+++	+++
16	SGHs	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	+++	+++	+++	-	-	+++	+++	+++
17	SGMa	+++	+++	+++	+++	+++	-	+++	-	-	-	-	-	+++	+++	+++	-	-	+++	+++	+++
18	SGMg	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	+++	+++	+++	-	-	+++	+++	+++
19	SGBn	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	+++	+++	+++	-	-	+++	+++	+++
20	SGUd	+++	+++	+++	+++	+++	+++	+++	+	-	-	-	-	+++	+++	+++	+	-	+++	+++	+++
***Sesbania aculeata* (root nodulating)**																					
21	SAKe(i)	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	+++	+++	+++	+	-	+++	+++	+++
22	SAKe(ii)	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	+++	+++	+++	++	-	+++	+++	+++
23	SATn	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	+++	+++	+++	+	-	+++	+++	+++
24	SAMa	+++	+++	+++	+++	+++	-	+++	-	-	-	-	-	+++	+++	+++	-	-	+++	+++	+++
25	SAUd	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	+++	+++	+++	++	-	+++	+++	+++
26	SAPr	+++	+++	+++	+++	+++	-	+++	-	-	-	-	-	+++	+++	+++	-	-	+++	+++	-
27	SAKr(ii)	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	+++	+++	+++	-	-	+++	+++	-
28	SAHn	+++	+++	+++	+++	+++	+++	+++	+	-	-	-	-	+++	+++	+++	-	-	+++	+++	+++
29	SAMg	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	+++	+++	+++	-	-	+++	+++	+++
***Sesbania rostrata* (root nodulating)**																					
30	SRKe(i)/r	+++	+++	+++	+++	+++	-	+++	-	-	-	-	-	+++	+++	+++	-	-	+++	+++	+++
31	SRKe(ii)/r	+++	+++	+++	+++	+++	-	+++	-	-	-	-	-	+++	+++	+++	-	-	+++	+++	+++
32	SRTn/r	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	+++	+++	+++	+	-	+++	+++	+++
33	SRMa/r	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	+++	+++	+++	+	-	+++	+++	+++
34	SRUd/r	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	+++	+++	+++	-	-	+++	+++	+++
35	SRPr/r	+++	+++	+++	+++	+++	+++	+++	+	-	-	-	-	+++	+++	+++	+	-	+++	+++	+++
36	SRKr(i)/r	+++	+++	+++	+++	+++	-	++	-	-	-	-	-	+++	+++	+++	-	-	+++	+++	+++
37	SRKr(ii)/r	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	+++	+++	+++	-	-	+++	+++	+++
38	SRSn/r	+++	+++	+++	+++	+++	-	+++	-	-	-	-	-	+++	+++	+++	-	-	+++	+++	+++
***Sesbania rostrata* (stem nodulating)**																					
39	SRKe(i)/s	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	+++	+++	+++	+	-	+++	+++	+++
40	SRKe(iii)/s	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	+++	+++	+++	++	-	+++	+++	+++
41	SRTn/s	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	+++	+++	+++	+	-	+++	+++	+++
42	SRMa/s	+++	+++	+++	+++	+++	-	+++	-	-	-	-	-	+++	+++	+++	-	-	+++	+++	+++
43	SRUd/s	+++	+++	+++	+++	+++	-	+++	-	-	-	-	-	+++	+++	+++	-	-	+++	+++	-
44	SRPr/s	+++	+++	+++	+++	+++	+++	+++	+	-	-	+	-	+++	+++	+++	-	-	+++	+++	+++
45	SRKr(i)/s	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	+++	+++	+++	-	-	+++	+++	-
46	SRKr(iii)/s	+++	+++	+++	+++	+++	+++	+++	+	-	-	-	-	+++	+++	+++	+	-	+++	+++	+++
47	SRHn/s	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	+++	+++	+++	+	-	+++	+++	+++
48	SRMg/s	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	+++	+++	+++	-	-	+++	+++	+++
49	SRSn/s	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	+++	+++	+++	-	-	+++	+++	+++
50	SRHg/s	+++	+++	+++	+++	+++	+++	+++	-	-	-	-	-	+++	+++	+++	-	-	+++	+++	-

(+++= good growth, ++= moderate growth, += poor growth and - = no growth)

Table 2: Summary of antibiotic resistance pattern of *Sesbania* rhizobia

Concentration (ug ml^{-1})	No. of rhizobial isolates showing growth on different antibiotics							
	Amp	**Chl**	**Gen**	**Kan**	**Neo**	**Nal**	**Tet**	**Str**
20	50	50	50	1	2	50	19	50
50	50	50	12	0	0	50	1	50
100	50	40	0	0	0	49	0	9

Amp- Ampicillin, Chl- Chloramphenicol, Gen- Gentamycin, Kan- Kanamycin, Neo- Neomycin, Nal- Nalidixic acid, Tet- Tetracycline, Str- Streptomycin

Conclusion

It was concluded that different rhizobial strains have different antibiotic resistance pattern. Most of the rhizobial isolates were also found to be resistant to the antibiotics; ampicillin, chloramphenicol, gentamycin, nalidixic acid and streptomycin up to a concentration of 100 ug ml^{-1} and sensitive to kanamycin, neomycin and tetracycline antibiotics even at a concentration 20 µg ml^{-1} indicating that different isolates have different antibiotic resistance pattern.

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