Short communication

Bioproduction of indole acetic acid by *Rhizobium* strains isolated from root nodules of green manure crop, *Sesbania sesban* (L.) Merr.

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Abstract
Twenty six *Rhizobium* strains were isolated from root nodules of *Sesbania sesban* (L.) Merr. collected from different regions of Andhra Pradesh. All the 26 *Rhizobium* strains produced indole acetic acid (IAA), but maximum amount was produced by only five strains in yeast extract mannitol (YEM) medium supplemented with L-tryptophan. The strains were found to elaborate maximum IAA when fed with 2.5 mg/ml L-tryptophan. Cultural requirements were optimized for maximum growth and IAA production. The strains differ in their growth and production of IAA on different carbon and nitrogen sources. Addition of cell wall affecting agents increased the IAA production over controls. The compound was extracted, purified and structurally confirmed as IAA.
Keywords: IAA production; LC-MS; *Rhizobium* species; *Sesbania sesban.*

INTRODUCTION

Plant growth promoting rhizobacteria (PGPR) are involved with host plants in mutual interaction. They promote plant growth by the production of phytohormones, biocontrol of host plant diseases or improvement of plant nutritional status. The beneficial effect of rhizobia in terms of biological nitrogen fixation has been the main focus of study in the past. Besides biological nitrogen fixation, some strains of *Rhizobium* are also involved in PGPR activity (Deshwal *et al*., 2003). PGPR activity includes production of phytohormones like indole acetic acid (IAA) and gibberlins, but IAA production was studied in *Rhizobium* strains associated only with a few legume hosts (Basu and Ghosh, 2001; Ghosh and Basu, 2002; Roy and Basu, 2004). It is well known that the *Rhizobium* strains isolated from single host species vary in their cultural and biochemical characters. Hence the present work was taken up to study the IAA synthesizing capacity of different strains of *Rhizobium* isolated from root nodules of *Sesbania sesban* (L.) Merr. and to find out the cultural requirements for maximum IAA production.

Twenty six *Rhizobium* strains were isolated from root nodules of *Sesbania sesban* (L.) Merr., collected from different regions of Andhra Pradesh, using yeast extract mannitol agar (YEMA) medium. The identity of the cultures was established by plant infection test (Vincent, 1970). A representative isolate from *S. sesban* was identified as *Rhizobium radiobacter* MTCC 8917 (=*Agrobacterium radiobacter*). Since *Agrobacterium* and *Rhizobium* are still treated as separate genera in Bergey's Manual of Systematic Bacteriology (Kuykendall *et al*., 2005), we used the term *Rhizobium* sp. with strain numbers as 1 to 26. The strains were tested for their production of indole compounds in 1% tryptone broth followed by the method described by Dubey and Maheshwari (2002). The IAA in culture supernatant was quantified by growing the cultures in yeast extract mineral (YEM) broth supplemented with L-tryptophan (Bhattacharya and Basu, 1992) and estimated by using colorimetric assay suggested by Gordon and Weber (1951). Qualitative test showed that all the 26 *Rhizobium* strains produced indole compounds in 1% tryptone broth. The amount of IAA produced varied from strain to strain and relatively more amount was observed in *Rhizobium* strains

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Table 1. Effect of incubation period on growth and IAA production.

Incubation Period (hrs)	*Rhizobium* strain 6		*Rhizobium* strain 12		*Rhizobium* strain 13		*Rhizobium* strain 16		*Rhizobium* strain 18	
	OD_{540}	IAA ($\mu g\ ml^{-1}$)	OD_{540}	IAA ($\mu g\ ml^{-1}$)	OD_{540}	IAA ($\mu g\ ml^{-1}$)	OD_{540}	IAA ($\mu g\ ml^{-1}$)	OD_{540}	IAA ($\mu g\ ml^{-1}$)
24	0.08	2.0	0.06	1.5	0.19	5.0	0.03	0.02	0.13	3.5
48	0.40	12.0	0.12	3.5	0.50	14.5	0.12	3.00	0.49	14.2
72	0.37	12.3	0.15	4.0	0.78	28.0	0.20	12.0	0.55	16.2
96	0.46	11.2	0.10	3.2	0.62	19.2	0.10	3.20	0.46	13.2
120	0.42	9.2	0.08	2.0	0.46	11.4	0.07	2.00	0.29	6.0
144	0.32	8.6	0.06	—	0.22	5.6	0.04	—	0.09	—
168	0.18	4.2	0.04	—	0.05	—	0.02	—	0.06	—

numbered as 6, 12, 13, 16 and 18 after 72 h of incubation (Table 1). Among these five strains, maximum amount of IAA was produced by *Rhizobium* strain 13 (28.0 µg/ml) after 72 h. This could be due to better utilization of medium components for IAA production by this isolate compared to other isolates. IAA production started within 24 h of incubation and reached maximum at 72 h and then decreased. The decrease in IAA level might be due to the release of IAA degrading enzymes such as IAA oxidase and peroxidase as has been reported earlier in *Rhizobium* sp. from *Cajanus cajan* (Datta and Basu, 2000).

The effect of different concentrations of L-tryptophan revealed that the maximum growth and IAA production were observed in all the five strains at 2.5 mg ml^{-1} concentration after 72 h (Table 2). In earlier reports, the *Rhizobium* sp. isolated from root nodules of *Dalbergia lanceolaria* also produced high amount of IAA at 2.5 mg/ml L-tryptophan concentration (Ghosh and Basu, 2002), while the *Rhizobium* species from root nodules of *Roystonea regia* produced maximum amount of IAA at 3 mg/ml L-tryptophan concentration (Basu and Ghosh, 2001). The data were statistically analyzed using ANOVA (Two way classification technique) and it was found that F- calculated (Fc) value was greater than F-tabulated (Ft) value. If Fc >Ft then it is considered as significant.

Effect of carbon sources (1.0%) in the basal YEM medium by the replacement of 11 carbon sources revealed that the *Rhizobium* strains vary in their utilization and production of IAA on different carbon sources (Table 3). *Rhizobium* strain 13 produced highest amount in glucose (28.3 µg/ml) followed by fructose (28.0 µg/ml) and mannitol (28.0 µg/ml). The maximum growth and IAA production in glucose containing medium may have been due to the better utilization of glucose compared to other carbon sources. *Rhizobium* sp. from *Cajanus cajan* also produced a maximum amount of IAA in glucose containing medium as reported earlier by Datta and Basu (2000). The *Rhizobium* strains 12, 16 and 18 produced highest amount in sucrose containing medium (13.0, 13.3 and 24.0 µg/ml respectively), while the *Rhizobium* strain 6 produced highest amount in inositol (18.3 µg/ml). The production of IAA was minimum when rhamnose, sorbitol, dulcitol and maltose were used as carbon source and almost nil in ribose. But, ribose was reported to be the best promoter at 1.5% concentration by the *Rhizobium* sp. isolated from *Psophocarpus tetragonolobus* (Bhattacharya and Basu, 1992). IAA production on different carbon sources was also found to be statistically significant.

Table 2. Effect of different concentrations of L-tryptophan on growth and IAA production.

L-tryptophan concentration (mg ml^{-1})	*Rhizobium* strain 6		*Rhizobium* strain 12		*Rhizobium* strain 13		*Rhizobium* strain 16		*Rhizobium* strain 18	
	OD_{540}	IAA* ($\mu g\ ml^{-1}$)	OD_{540}	IAA* ($\mu g\ ml^{-1}$)	OD_{540}	IAA* ($\mu g\ ml^{-1}$)	OD_{540}	IAA* ($\mu g\ ml^{-1}$)	OD_{540}	IAA* ($\mu g\ ml^{-1}$)
0.5	0.09	6.0	0.06	2.0	0.28	2.0	0.09	—	0.06	—
1.5	0.16	12.0	0.08	2.6	0.40	13.3	0.16	5.3	0.10	3.3
2.5	0.37	12.3	0.15	4.0	0.78	28.0	0.20	12.0	0.55	16.2
3.0	0.09	3.2	0.10	4.0	0.11	11.6	0.24	11.8	0.02	0.06

* Significant at 5%. Between tryptophan concentration (Fc= 5.766, Ft= 3.490).

Table 3. Effect of carbon sources on growth and IAA production.

Carbon Source (1%)	*Rhizobium* strain 6		*Rhizobium* strain 12		*Rhizobium* strain 13		*Rhizobium* strain 16		*Rhizobium* strain 18	
	OD_{540}	IAA* ($\mu g\ ml^{-1}$)	OD_{540}	IAA* ($\mu g\ ml^{-1}$)	OD_{540}	IAA* ($\mu g\ ml^{-1}$)	OD_{540}	IAA* ($\mu g\ ml^{-1}$)	OD_{540}	IAA* ($\mu g\ ml^{-1}$)
Control	0.16	2.0	0.09	3.0	0.07	5.0	0.15	5.0	0.40	6.0
Mannitol	0.37	12.3	0.15	4.0	0.78	28.0	0.20	12.0	0.55	16.2
Glucose	0.30	3.0	0.40	11.6	0.80	28.3	0.35	13.0	0.32	3.6
Maltose	0.16	1.3	0.04	—	0.06	1.0	0.03	—	0.05	1.6
Galactose	0.20	6.0	0.30	2.30	0.02	0.6	0.08	2.3	0.17	4.0
Fructose	0.25	12.3	0.35	4.00	0.78	28.0	0.15	5.3	0.29	16.6
Inositol	0.36	18.3	0.30	3.30	0.20	6.6	0.49	12.6	0.03	1.6
Sucrose	0.50	14.0	0.30	13.0	0.03	0.3	0.50	13.3	0.69	24.0
Sorbitol	0.24	6.3	0.35	7.3	0.02	0.3	0.02	0.3	0.09	2.3
Lactose	0.33	3.3	0.53	12.6	0.29	7.3	0.29	7.0	0.25	8.6
Dulcitol	0.20	2.3	0.17	2.0	0.02	0.6	0.25	6.6	0.02	0.3
Rhamnose	0.10	1.0	0.04	—	0.04	—	0.28	3.0	0.06	2.0
Ribose	0.08	—	0.09	1.0	0.08	0.6	0.04	1.0	0.04	—

* Significant at 5%. Between carbon sources (Fc= 3.908, Ft= 1.960).

Effect of different nitrogen sources (0.1%) was studied by replacing yeast extract in the original YEM medium supplemented with L-tryptophan**.** It revealed that the *Rhizobium* strains 6, 12 and 18 utilized inorganic nitrogen sources like KNO_3, $NaNO_2$ and $NaNO_3$ and produced maximum amount of IAA, while amino acid glycine as additional nitrogen source reduced growth and IAA production. Some amino acids were shown earlier to inhibit IAA production by *Rhizobium meliloti* (Garcia-Rodrigaez *et al*., 1981) due to inhibition of conversion of tryptophan to IAA. The *Rhizobium* strain 13 showed maximum growth and IAA production on organic nitrogen sources (casamino acid, cystine, tyrosine), while the *Rhizobium* srrain 16 utilized and produced IAA on both organic and inorganic nitrogen sources (Table 4). Among the five *Rhizobium* strains, the maximum growth and IAA production were observed in *Rhizobium* strain 16 in the medium amended with L-glutamic acid as nitrogen source. When L-glutamic acid used as nitrogen source , a *Rhizobium* sp. from root nodules of *Cajanus cajan* was also reported to produce maximum IAA (Datta and Basu, 2000). The effect of different nitrogen sources on IAA production was found to be statistical-

Table 4. Effect of nitrogen sources on growth and IAA production.

Nitrogen Source (0.1%)	*Rhizobium* strain 6		*Rhizobium* strain 12		*Rhizobium* strain 13		*Rhizobium* strain 16		*Rhizobium* strain 18	
	OD_{540}	IAA* ($\mu g\ ml^{-1}$)	OD_{540}	IAA* ($\mu g\ ml^{-1}$)	OD_{540}	IAA* ($\mu g\ ml^{-1}$)	OD_{540}	IAA* ($\mu g\ ml^{-1}$)	OD_{540}	IAA* ($\mu g\ ml^{-1}$)
Control	0.12	1.80	0.10	0.9	0.19	0.7	0.27	8.5	0.05	2.5
KNO_3	0.80	26.6	0.86	28.6	0.55	18.3	0.85	28.8	0.84	29.6
$(NH_4)_2SO_4$	0.40	13.3	0.39	13.0	0.51	17.0	0.89	29.6	0.74	11.6
$NaNO_3$	0.77	25.6	0.84	28.0	0.25	8.3	0.98	39.3	0.83	27.6
$NaNO_2$	0.28	23.0	0.41	10.0	0.58	1.0	0.03	—	0.83	11.6
L-asparagine	0.21	7.0	0.16	5.3	0.17	5.6	0.80	26.6	0.08	26.6
L-glycine	0.02	0.6	0.04	1.3	0.03	1.3	0.08	2.3	0.08	2.6
Casamino acid	0.30	10.0	0.57	19.0	0.80	26.6	0.96	37.3	0.37	12.3
L-glutamic acid	0.35	11.6	0.08	0.3	0.58	19.3	0.98	44.3	0.26	8.6
Cystine	0.42	10.3	0.56	13.3	0.90	25.0	0.73	14.3	0.39	13.6
Tyrosine	0.16	3.0	0.18	4.3	0.88	25.0	0.25	8.3	0.09	2.3

* Significant at 5%. Between nitrogen sources (Fc= 3.570, Ft= 2.152).

Table 5. Effect of cell wall affecting agents on growth and IAA production.

Cell wall affecting agents	Concentration	*Rhizobium* strain 6		*Rhizobium* strain 12		*Rhizobium* strain 13		*Rhizobium* strain 16		*Rhizobium* strain 18	
		OD_{540}	IAA* (μg ml^{-1})	OD_{540}	IAA* (μg ml^{-1})	OD_{540}	IAA* (μg ml^{-1})	OD_{540}	IAA* (μg ml^{-1})	OD_{540}	IAA* (μg ml^{-1})
Control	—	0.37	12.3	0.15	0.40	0.78	28.0	0.20	12.0	0.55	16.2
EDTA	0.1 (μg ml^{-1})	0.70	21.6	0.90	26.0	0.62	29.6	0.84	44.2	0.79	21.2
	0.2 (μg ml^{-1})	0.78	24.5	0.93	29.3	0.83	30.1	0.89	55.0	0.85	22.0
	0.3 (μg ml^{-1})	0.60	20.2	0.75	22.0	0.60	26.0	0.52	46.2	0.62	19.2
SDS	0.1 (μg ml^{-1})	0.60	20.3	0.63	11.3	0.55	15.6	0.60	15.3	0.65	16.6
	0.2 (μg ml^{-1})	0.52	15.6	0.75	20.9	0.69	30.6	0.65	30.6	0.60	16.3
	0.3 (μg ml^{-1})	0.55	16.6	0.52	14.6	0.40	18.0	0.55	20.0	0.50	15.6
Penicillin	25 IU	0.90	19.0	0.70	8.3	0.72	13.0	0.50	7.3	0.90	18.0
	50 IU	0.88	15.3	0.62	17.0	0.63	11.3	0.65	12.0	0.62	13.6
	100 IU	0.60	10.6	0.69	19.3	0.42	6.3	6.30	0.30	0.40	5.3
Lysozyme	25 IU	0.89	25.0	0.40	32.6	0.90	32.0	0.82	49.3	0.92	32.0
	50 IU	0.93	33.0	0.80	26.0	0.95	27.3	0.78	80.6	0.81	22.6
	100 IU	0.88	25.0	0.69	19.0	0.60	24.0	24.0	0.6	0.72	19.6

* Significant at 5%. Between surfactants (Fc= 6.493, Ft = 1.960), Between *Rhizobium* strains (Fc = 5.120, Ft = 2.565).

ly significant.

Addition of cell wall affecting agents or surfactants revealed that the *Rhizobium* strains produced maximum amount of IAA in the medium amended with different concentrations of EDTA, SDS, penicillin and lysozyme. IAA production was maximum at 0.2 μg/ml of EDTA, 0.1 μg/ml of SDS in *Rhizobium* strains 6 and 18, 0.2 μg/ml of SDS in *Rhizobium* strains 12, 13, 16 and 50 IU of penicillin and lysozyme when compared to control (without surfactants) (Table 5). Changes in the cell well or membrane by these agents increased the release of tryptophan degrading enzymes as well as IAA production. Among the five *Rhizobium* strains, highest amount of IAA was produced by *Rhizobium* strain 16 (80.6 μg/ml) in the medium amended with lysozyme (50 IU). This *Rhizobium* strain was selected for characterization of IAA produced. The effect of surfactants on IAA production was statistically significant and different *Rhizobium* strains also differed significantly.

The *Rhizobium* strain 16 was inoculated into 200 ml of YEM medium with most suitable supplements and incubated at 28±2^{o}C for 3 days on rotary shaker. After incubation *Rhizobium* cells were separated from the supernatant by centrifugation at 10,000 rpm for 30 min. The supernatant was acidified to pH 2.5-3.0 with 1N HCl and extracted twice with ethyl acetate (Sinha and Basu, 1981). Partial purification of IAA from crude extract was done by using silica gel column chromatography (22x5 cm) and fractions were collected with solvent system ethyl acetate and hexane (20:80 v/v). Each fraction (10-20 μl) was tested on thin layer chromatography (TLC) with solvent system (ethyl acetate and hexane, 2:8) and then developed with Salkowski reagent (Morales *et al*. 2003). Spots with *Rf*-values (0.85) identical to authentic IAA were identified under UV-light (254 nm). The fraction that gave positive result with Salkowski reagent was collected , concentrated and analyzed by LC-MS. The pure indole was structurally confirmed as IAA by comparing with standard IAA present in compound library system of LC-MS (Figure 1). The Liquid chromatography-Mass spectrometry (LC-MS) of fraction revealed that it has a benzene moiety (m/z 77) in its chemical structure and its molecular weight may be 176.4.

A perusal of literature showed that the IAA production by *Rhizobium* strains from *Sesbania* species were very limited (Bhowmik and Basu, 1984; 1986; 1987) and it is only confined to single strain or species from a single legume host. From this study, it is clear that all the strains from a host were positive for IAA production, but the strains differ significantly in auxin

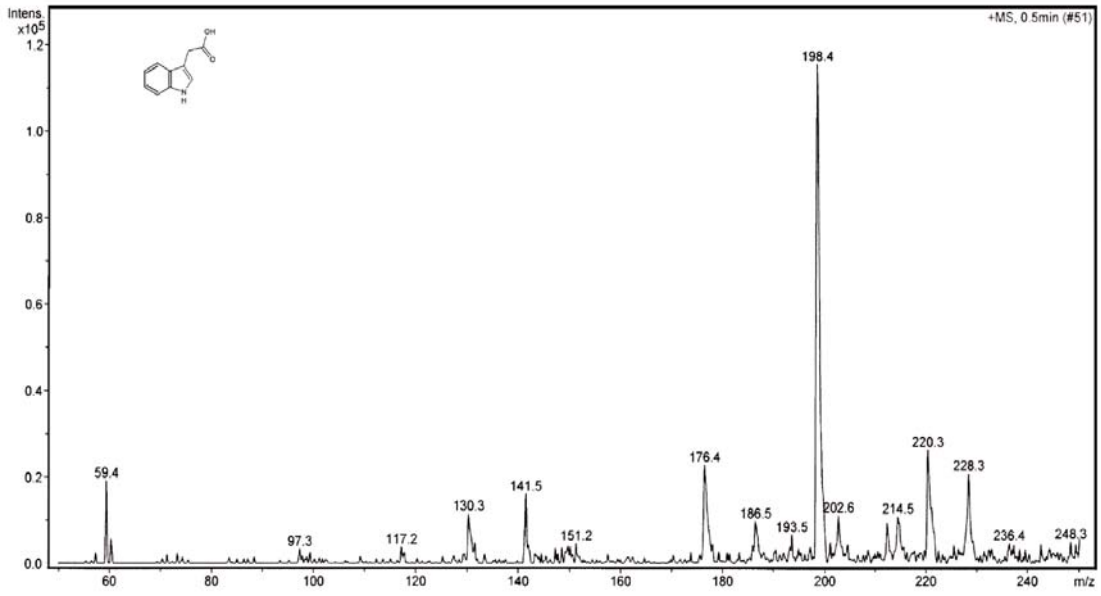

Figure 1. LC-MS of purified IAA from *Rhizobium* strain 16.

production depending upon the cultural conditions.

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