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Production of Indole-3-Acetic Acid by *Rhizobium* Isolates from *Sesbania* Species

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Abstract: *Rhizobium* strains isolated from root (*Sesbania procumbens*) and stem nodules (*S. rostrata* and *S. procumbens*) of *Sesbania* species were tested for their ability to produce Indole-3-Acetic Acid (IAA). The isolates produced maximum amount of IAA in culture after 72 h of incubation and at 3.0 mg mL^{-1} L-tryptophan concentration. The effect of different carbon and nitrogen sources on IAA production was also studied and it revealed that, glucose (1%) and potassium nitrate (0.1%) were found to be best promoters for IAA production. An addition of EDTA (0.1 μg mL^{-1}) significantly increased the IAA production over controls and the production was maximum at 0.2 μg mL^{-1} EDTA concentration. Among the three isolates, maximum amount of IAA was produced by *Rhizobium* isolate from stem nodule of *S. procumbens*. The IAA was extracted, purified and identified by thin layer chromatography.

Key words: *Rhizobium* sp., indole acetic acid, *Sesbania* sp., *rhizobium*-legume symbiosis

INTRODUCTION

Plant Growth Promoting Rhizobacteria (PGPR) are considered to promote plant growth directly or indirectly. PGPR can exhibit a variety of characteristics responsible for influencing plant growth. The common traits include production of plant growth regulators (auxin, gibberellins and ethylene), siderophore, HCN and antibiotic production (Ahmad *et al*., 2005).

Indole Acetic Acid (IAA) is one of the most physiologically active auxins. IAA is a common product of L-tryptophan metabolism by several microorganisms including rhizobia (Datta and Basu, 2000; Ghosh and Basu, 2002, 2006). Furthermore, rhizobia are the first group of bacteria, which are attributed to ability of PGPR to release IAA that can help to promote the growth and pathogenesis in plants (Mandal *et al*., 2007).

Sesbania is one of the genera of Fabaceae with more than 70 species (Allen and Allen, 1981). Some species of *Sesbania*, like *Sesbania rostrata* was widely cultivated as green manure crop (Dreyfus and Dommergues, 1981). Eventhough the IAA production by *Azorhizobium* sp. associated with stem nodules of *S. rostrata* has been reported earlier (Pan *et al*., 1995), but comparative studies with other *Rhizobium* sp. associated with root and stem nodules of *S. procumbens* were not reported so far. Hence, the present research was taken up to study the IAA synthesizing capacity of *Rhizobium* isolates from root and stem nodules of *S. procumbens* and stem nodules of *S. rostrata*.

MATERIALS AND METHODS

Organism and growth conditions: The symbionts were isolated from the root and stem nodules of *Sesbania procumbens* and stem nodules of *S. rostrata* according to Vincent (1970), using Yeast Extract Mannitol Agar (YEMA) medium. Identification of the isolates was carried out on the basis of morphological, cultural and biochemical characteristics on YEM broth by standard method (Holt *et al*., 1994). The isolates were designated as SRS (stem nodule isolate of *S. rostrata*), SPR (root nodule isolate of *S. procumbens*) and SPS (stem nodule isolate of *S. procumbens*).

For IAA production, axenic cultures of the bacteria were grown in 100 mL Erlenmeyer flasks containing 25 mL of yeast extract mineral medium (Skerman, 1959) with 1% mannitol and 0.01% $CaCl_2$ at pH of 7.0 for 72 h (optimum time for IAA production). Bacterial growth was determined using colorimeter (Elico, CL 157) and taking Optical Density (OD) at 540 nm.

Estimation of IAA production: The IAA in cell free supernatant was estimated colorimetrically (Elico, CL 157) by the method adopted from Gordon and Weber (1951).

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Effect of incubation period was studied by inoculating *Rhizobium* isolates separately into L-tryptophan supplemented medium and incubated for 168 h at 30±2 °C. The samples were withdrawn every 24 h and the growth and IAA were estimated.

Different concentrations of L-tryptophan (0.5, 1.5, 2.5 and 3.0 mg mL^{-1}) were added to the basal medium to find out the maximum IAA production. Different carbon sources were also added to the tryptophan supplemented basal medium omitting mannitol. Then the EDTA was added to the tryptophan supplemented basal medium having most suitable carbon and nitrogen source. The effect of different concentrations of EDTA (0.1-0.5 μg mL^{-1}) on IAA production was also measured.

Extraction, purification and detection of IAA: The isolates were inoculated separately into 200 mL of YEM medium with most suitable substance and incubated at 28±2 °C for 3 d on rotary shaker. After incubation, the IAA was extracted according to the method described by Ahmad *et al.* (2005).

Partial purification of IAA from crude extract was done by using silica gel column chromatography 22×5 cm (E Merck, Germany) and fractions were collected with solvent system using ethyl acetate and hexane (20:80 v/v). Each fraction (10-20 μL) was tested on Thin Layer Chromatography (TLC) plates (E Merck, Germany) with solvent system (ethyl acetate and hexane, 2:8) and then developed with Salkowski reagent (Morales *et al*., 2003).

Statistical analyses: The data on effect of carbon, nitrogen sources and EDTA concentrations were statistically analyzed using ANOVA (two way classification technique).

RESULTS AND DISCUSSION

Based on morphological, cultural and biochemical characteristics, the root and stem nodule isolates of *S. procumbens* and stem nodule isolate of *S. rostrata* were identified as species of *Rhizobium*. The identification was done following Bergey's Manual (Jordan, 1984). The IAA production by *Rhizobium* isolates started after 24 h and reached maximum after 72 h and then decreased slowly (Fig. 1). The decrease in IAA level might be due to the release IAA degrading enzymes such as IAA oxidase and peroxidase was reported earlier in *Rhizobium* sp. from *Cajanus cajan* (Datta and Basu, 2000). Among the three isolates maximum amount of IAA was produced by *Rhizobium* isolate from stem nodules of *S. procumbens* (30.2 μg mL^{-1}) after 72 h.

The isolates prefer L-tryptophan for maximum IAA production. The effect of different concentrations of L-tryptophan revealed that the maximum growth and IAA production were observed at 3.0 mg mL^{-1} L-tryptophan concentration in all the isolates (Fig. 2). That the *Rhizobium* sp. isolated from root nodules of *Roystonea regia* produced maximum amount of IAA at 3 mg mL^{-1} L-tryptophan concentration was reported earlier (Basu and Ghosh, 2001). While, the *Rhizobium* sp. isolated from root nodules of *Dalbergia lanceolaria* produced maximum amount of IAA at 2.5 mg mL^{-1} L-tryptophan concentration was reported earlier by Ghosh and Basu (2002). This indicates that *Rhizobium* strains differ in their utilization of different concentrations of L-tryptophan for IAA production.

Effect of carbon sources (1%) in the basal YEM medium by the replacement of 10 different carbon sources

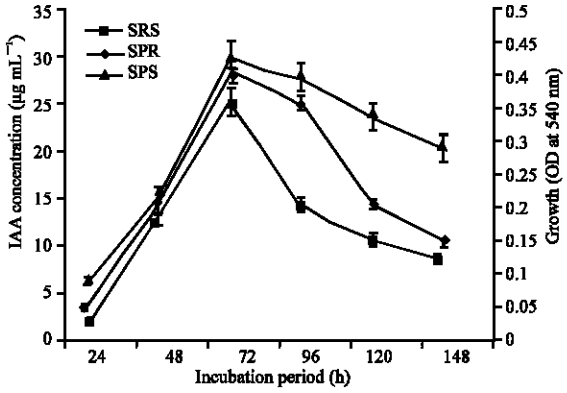

Fig. 1: Effect of incubation period on growth and IAA production by *Rhizobium* isolates. Data were means of three replicates. Bars at each point indicate ±SE

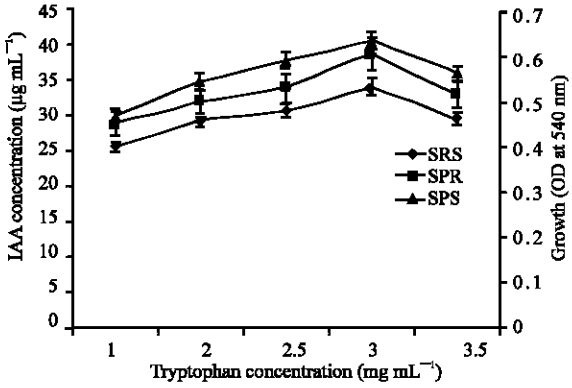

Fig. 2: Effect of different concentrations of tryptophan on growth and IAA production by *Rhizobium* isolates. Data were means of three replicates. Bars at each point indicates ±SE

Table 1: Effect of carbon sources on growth and IAA production by *Rhizobium* isolates

	SRS*		SPR*		SPS*	
Carbon source* (1%)	OD_{540}	IAA μg mL^{-1})	OD_{540}	IAA (μg mL^{-1})	OD_{540}	IAA (μg mL^{-1})
Control	0.20	6.2	0.22	6.9	0.24	9.8
Mannitol	0.39	25.9	0.42	29.0	0.44	30.2
Glucose	0.42	28.2	0.54	32.8	0.94	42.8
Galactose	0.24	9.2	0.30	14.4	0.32	13.8
Fructose	0.22	6.8	0.28	13.9	0.39	15.9
Sucrose	0.22	6.9	0.24	10.8	0.33	14.5
Ribose	0.32	18.8	0.22	9.2	0.23	14.0
Mannose	0.06	--	0.09	--	0.07	--
Lactose	0.15	2.0	0.12	1.0	0.14	1.9
Rhamnose	0.09	--	0.04	--	0.06	--
Raffinose	0.07	--	0.06	--	0.09	--
Xylose	0.12	1.2	0.11	1.0	0.10	0.9

* Significant at 1%. Between carbon sources (Fc = 52.3, Ft = 2.3), Between *Rhizobium* isolates (Fc = 4.2, Ft = 3.4)

Table 2: Effect of nitrogen sources on growth and IAA production by *Rhizobium* isolates

	SRS*		SPR*		SPS*	
Nitrogen source*	OD_{540}	IAA (μg mL^{-1})	OD_{540}	IAA (μg mL^{-1})	OD_{540}	IAA (μg mL^{-1})
Control	0.09	1.0	0.10	0.04	0.12	0.07
Potassium nitrate	0.50	28.9	0.55	32.9	0.99	44.9
Sodium nitrate	0.16	6.8	0.22	6.9	0.22	14.9
Sodium nitrite	0.22	7.2	0.25	10.9	0.29	15.8
Ammonium sulphate	0.20	6.2	0.22	9.2	0.20	12.0
L-asparagine	0.32	11.9	0.39	29.6	0.44	30.1
L-glutamic acid	0.34	14.2	0.44	30.6	0.49	32.9
Casamino acid	0.26	6.8	0.34	13.8	0.36	14.6
Tyrosine	0.12	--	0.09	--	0.06	--
Cystine	0.14	0.9	0.16	0.9	0.19	1.2

* Significant at 1%. Between nitrogen sources (Fc = 22.7, Ft = 2.5), Between *Rhizobium* isolates (Fc = 10.6, Ft = 3.6)

Table 3: Effect of different concentrations of EDTA on growth and IAA production by *Rhizobium* isolates

	SRS*		SPR*		SPS*	
EDTA concentration*	OD_{540}	IAA (μg mL^{-1})	OD_{540}	IAA (μg mL^{-1})	OD_{540}	IAA (μg mL^{-1})
Control	0.39	25.9	0.42	29.0	0.44	30.2
0.1	0.45	30.2	0.49	32.8	0.55	42.9
0.2	0.48	32.6	0.54	36.2	0.59	52.6
0.3	0.44	29.9	0.51	34.4	0.54	39.0
0.4	0.42	28.6	0.46	30.9	0.50	36.0
0.5	0.35	21.5	0.38	22.0	0.42	32.1

* Significant at 1%. Between EDTA concentration (Fc = 11.3, Ft = 3.8), Between *Rhizobium* isolates (Fc = 24.0, Ft = 4.4)

revealed that the *Rhizobium* isolates vary in their utilization and production of IAA (Table 1). The isolates produced more amount of IAA, when glucose was used as carbon source. The maximum IAA production in glucose containing medium was due to the better utilization of glucose than other carbon sources. *Rhizobium* sp. from *Cajanus cajan* also produced maximum amount of IAA in glucose containing medium as reported earlier by Datta and Basu (2000). The production of IAA was minimum in ribose, galactose, fructose, sucrose, lactose and xylose. This may be due to less utilization of these carbon sources than glucose. The IAA production was not observed, when mannose, rhamnose and raffinose were used as carbon sources. That the effect of carbon sources influenced the growth and IAA production was reported earlier in *Rhizobium* sp. isolated from *Roystonia regia* (Basu and Ghosh, 2001). The data on effect of carbon sources on IAA production by *Rhizobium* isolates were statistically analyzed using ANOVA and it was found that variations due to both carbon sources and *Rhizobium* isolates were found to be significant.

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 inorganic nitrogen sources like KNO_3 increased the IAA production followed by organic nitrogen sources like L-glutamic acid, L-asparagine and casamino acid. According to Jordan (1984), *Rhizobium* sp. could utilize several nitrogen compounds for growth. This might be responsible for the increased IAA production. Amino acid tyrosine as additional nitrogen source reduced the growth and IAA production (Table 2). Some amino acids were shown earlier to inhibit IAA production by

Rhizobium meliloti (Datta and Basu, 2000) due to inhibition of conversion of tryptophan to IAA. The *Rhizobium* isolates from root and stem nodules of *S. procumbens* and stem nodule isolate of *S. rostrata* showed maximum growth and IAA production in the medium amended with KNO_3. While, the *Rhizobium* sp. from root nodules of *C. cajan* produced maximum IAA, when L-glutamic acid was used as nitrogen source (Datta and Basu, 2000). Statistical analysis showed that the effect of nitrogen sources on IAA production was also significant.

Addition of cell wall affecting agent like EDTA revealed that 0.1 to 0.4 μg mL^{-1} EDTA increased the IAA production in all the three isolates, while the maximum IAA production was observed at 0.2 μg mL^{-1}. Above 0.2 μg mL^{-1} concentration, decrease in IAA production was observed (Table 3). Among the three isolates, the *Rhizobium* isolate from stem nodules of *S. procumbens* produced highest amount of IAA at 0.2 μg mL^{-1} EDTA concentration (52.6 μg mL^{-1}). Changes in cell wall or membrane by EDTA increased the availability of tryptophan to converting enzyme as well as increase the release of IAA from cell was reported earlier by Bhattacharya and Basu (1992). The effect of different concentration of EDTA on IAA production was also found to be statistically significant.

On the basis of IAA production level, different fractions collected from column chromatography were subjected to TLC. The TLC of the purified compound and standard IAA sprayed with Salkowski reagent showed almost the same Rf-values (0.88) which were identified under UV-light (254 nm). From this study, it is clear that *Rhizobium* isolates differ significantly in auxin production.

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