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*Full Length Research Paper*

# Production of indole-3-acetic acid by *Rhizobium* isolates from *Sesbania* species

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***Rhizobium*** **isolates from root (*Sesbania procumbens*) and stem nodules (*S. rostrata* and *S. procumbens*) of *Sesbania* species were shown to produce indole-3-acetic acid (IAA) in culture supplemented with L-tryptophan. Production of IAA was maximal after 72 h of incubation when the bacteria reached stationary phase of growth. The cultural requirements were optimized for maximum IAA production. The effect of carbon (1%) and nitrogen sources (0.1%) revealed that glucose and potassium nitrate were best promoters for IAA production over controls. The effect of different concentrations of EDTA revealed that 0.2 gml$^{-1}$ EDTA increased IAA production. Among the three isolates, maximum amount of IAA was produced by the *Rhizobium* isolate from *S. procumbens*. The IAA from this isolate was extracted, purified and identified by thin layer chromatography.**

**Key words:** *Rhizobium* species, Indole acetic acid, *Sesbania* species, *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), siderophores, 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, 2006; 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* has been widely cultivated as a green manure crop (Dreyfus and Dommergues, 1981). Although IAA production by *Azorhizobium* sp. associated with stem nodules of *S. rostrata* has been reported earlier (Pan et al., 1995), comparative studies with other *Rhizobium* spp. associated with root and stem nodules of *S. procumbens* have not been reported so far. Hence, the present work was undertaken to study the IAA synthesis capacity of *Rhizobium* isolates from root and stem nodules of *S. procumbens*, and stem nodules of *S. rostrata*.

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## MATERIALS AND METHODS

### Organism and growth conditions

The symbionts were isolated from the root and stem nodules of *S. procumbens* and stem nodules of *S. rostrata* on yeast extract mannitol agar (YEMA) medium (Vincent, 1970). Identification of the isolates was carried out on the basis of morphological, cultural and biochemical characteristics on YEMA by standard methods (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 (YEM) medium (Skerman, 1959) with 1% mannitol and 0.01% $CaCl_2$ at pH 7.0 for 72 h (optimum time for IAA production).

### Determination of IAA production

The IAA concentration in cell -free supernatants were determined colorimetrically (Elico, CL 157) by the method adopted from Gordon and Weber (1951).

Effect of incubation period was studied by inoculating *Rhizobium* isolates separately into L-tryptophan-supplemented YEM medium and incubating for 168 h at 30 ± 2 $^{o}$C. Samples were withdrawn every 24 h and growth and IAA concentration were detrmined.

Different concentrations of L-tryptophan (0.5, 1.5, 2.5 and 3.0 mg.ml$^{-1}$) were added to the basal YEM medium to determine maximum IAA production. The effect of different concentrations of carbon sources (1%) was also studied by inoculating each isolate into the tryptophan-supplemented basal YEM medium omitting mannitol. The effect of nitrogen sources (0.1%) was studied in L-tryptophan-supplemented YEM medium, and IAA production was

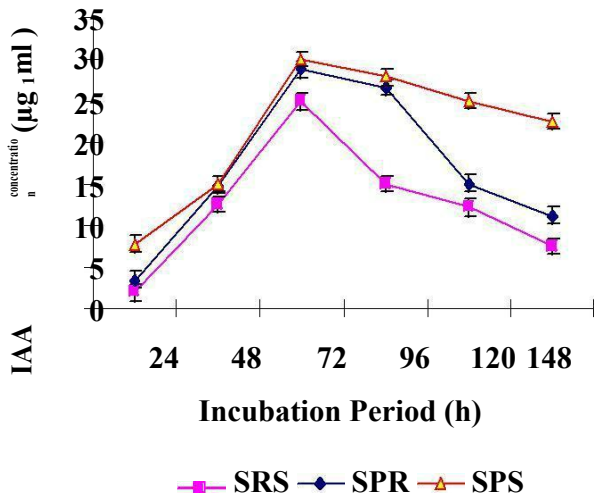


**Figure 1.** Effect of incubation period on IAA production by *Rhizobium* isolates. Data were means of three replicates. Bars at each point indicate ± SE.

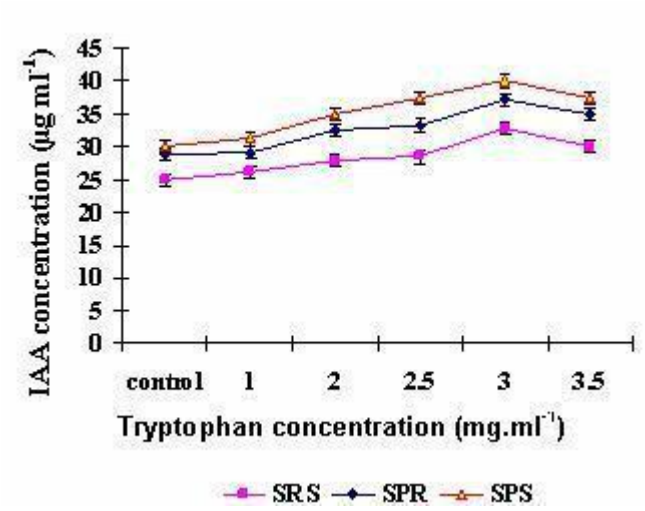


**Figure 2.** Effect of different concentrations of tryptophan on IAA production by *Rhizobium* isolates. Control consists of without tryptophan. Data were means of three replicates. Bars at each point indicates ± SE.

determined after 72 h of incubation at 30 ± 2 $^{o}$C.

The effect of different concentrations of EDTA (0.1, 0.2, 0.3, 0.4 and 0.5 gml$^{-1}$) was studied by inoculating each isolate into L-tryptophan-supplemented basal YEM medium containing the most suitable carbon and nitrogen source and incubated at 30 ± 2 $^{o}$ C for 72 h. After incubation, the IAA production by the isolates was determined.

#### Extraction, purification and detection of IAA

The isolates were inoculated separately into 200 ml of YEM medium, containing the most suitable carbon and nitrogen source, and incubated at 28 ± 2 $^{o}$ C for 72 h on a rotary shaker. After incubation, the IAA was extracted according to the method described by Ahmad et al. (2005).

Partial purification of IAA from crude extracts was done using silica gel column chromatography (22 x 5 cm) (Merck, Germany), and fractions were collected with a solvent system comprising ethyl acetate and hexane (20:80 v/v). Each fraction (10 - 20 µl) was tested on thin layer chromatography (TLC) plates (Merck, Germany) with a solvent system (ethyl acetate and hexane, 2:8) and then developed with Salkowski reagent (Morales et al., 2003).

#### Statistical analyses

Data regarding the effect of carbon, nitrogen sources and EDTA concentrations on IAA production were statistically analyzed using ANOVA (two way classification technique) (Statistica software 6.0).

## 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 according to Bergey's Manual of Determinative Bacteriology (Jordan, 1984). The IAA production by *Rhizobium* isolates started after 24 h and reached a maximum after 72 h when the bacteria reached stationary phase of growth, and then decreased slowly (Figure 1). The pattern of growth was similar in all these isolates, but they differed only in IAA production. The decrease in IAA production after 72 h might be due to the release IAA-degrading enzymes such as IAA oxidase and peroxidase, as has been reported earlier in *Rhizobium* sp. from *Cajanus cajan* (Datta and Basu, 2000) . Among the three isolates the maximum amount of IAA was produced by the *Rhizobium* isolate from stem nodules of *S. procumbens* (30.2 gml$^{-1}$) after 72 h. This could be due to better utilization of medium components for IAA production by this isolate compared to the other isolates.

The isolates preferred L-tryptophan for maximum IAA production. The effect of different concentrations of L-tryptophan revealed that maximum growth and IAA production were observed at 3.0 gml$^{-1}$ L-tryptophan for all the isolates (Figure 2). Similarly, a *Rhizobium* sp. isolated from root nodules of *Roystonea regia* has been reported to produce a maximum amount of IAA at 3 gml$^{-1}$ L-tryptophan (Basu and Ghosh, 2001), while a *Rhizobium* sp. isolated from root nodules of *Dalbergia lanceolaria* produced a maximum amount of IAA at 2.5 gml$^{-1}$ L-tryptophan (Ghosh and Basu, 2002). This indicates that *Rhizobium* isolates differ in their utilization of different concentrations of L-tryptophan for IAA production.

The effect of carbon sources (1%) on IAA production in YEM medium was studied by replacing mannitol with other carbon sources and it revealed that the *Rhizobium* isolates vary in their utilization and production of IAA (Table 1). The isolates produced the most IAA, when glucose was used as carbon source. The maximal IAA production in glucose- containing medium may have

**Table 1.** Effect of carbon sources on IAA production by *Rhizobium* isolates.

| Carbon source* (1%) | SRS* (IAA gml$^{-1}$) | SPR* (IAA gml$^{-1}$) | SPS* (IAA gml$^{-1}$) |
|---|---|---|---|
| Control | 6.2 | 6.9 | 9.8 |
| Mannitol | 25.9 | 29.0 | 30.2 |
| Glucose | 28.2 | 32.8 | 42.8 |
| Galactose | 9.2 | 14.4 | 13.8 |
| Fructose | 6.8 | 13.9 | 15.9 |
| Sucrose | 6.9 | 10.8 | 14.5 |
| Ribose | 14.8 | 9.2 | 14.0 |
| Mannose | -- | -- | -- |
| Lactose | 2.0 | 1.0 | 1.9 |
| Rhamnose | -- | -- | -- |
| Raffinose | -- | -- | -- |
| Xylose | 1.2 | 1.0 | 0.9 |

*Significant at 1 % (Between carbon sources, p = 0.01).

**Table 2.** Effect of nitrogen sources on IAA production by *Rhizobium* isolates.

| Nitrogen source* (0.1%) | SRS* (IAA gml$^{-1}$) | SPR* (IAA gml$^{-1}$) | SPS* (IAA gml$^{-1}$) |
|---|---|---|---|
| Control | 1.0 | 0.04 | 0.07 |
| Potassium nitrate | 28.9 | 32.9 | 44.9 |
| Sodium nitrate | 6.8 | 6.9 | 14.9 |
| Sodium nitrite | 7.2 | 10.9 | 15.8 |
| Ammonium sulphate | 6.2 | 9.2 | 12.0 |
| L-asparagine | 11.9 | 29.0 | 30.1 |
| L-glutamic acid | 14.2 | 30.6 | 32.9 |
| Casamino acid | 6.8 | 13.8 | 14.6 |
| Tyrosine | -- | -- | -- |
| Cysteine | 0.9 | 0.9 | 1.2 |

* Significant at 1% (Between nitrogen sources, p = 0.001).

been due to the better utilization of glucose compared to the 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). In contrast to minimal production of IAA in medium containing ribose, galactose, fructose, sucrose, lactose or xylose, no IAA production was observed when mannose, rhamnose or 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 regarding the effect of carbon sources on IAA production by the *Rhizobium* isolates were statistically analyzed using ANOVA and it was found that variations due to carbon sources was significant (p = 0.01).

The effect of different nitrogen sources (0.1%) was studied by inoculating each isolate into the original YEM medium supplemented with L-tryptophan and 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* spp. can utilize several nitrogen compounds for growth. This might be responsible for the increased IAA production. The amino acid tyrosine, as additional nitrogen source, reduced 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 the stem nodule isolate of *S. rostrata* showed maximum growth and IAA production in the medium amended with $KNO_3$. A *Rhizobium* sp. from root nodules of *C. cajan* was reported to produce maximum IAA when L-glutamic acid was used as nitrogen source (Datta and Basu, 2000). Statistical analyses showed that the effect of nitro-gen sources on IAA production was also significant (p value = 0.001).

Addition of cell wall-affecting agent like EDTA revealed

**Table 3.** Effect of different concentrations of EDTA on IAA production by *Rhizobium* isolates.

| EDTA concentration* ( gml$^{-1}$) | SRS* (IAA gml$^{-1}$) | SPR* (IAA gml$^{-1}$) | SPS* (IAA gml$^{-1}$) |
|---|---|---|---|
| Control | 25.9 | 29.0 | 30.2 |
| 0.1 | 30.2 | 32.8 | 42.9 |
| 0.2 | 32.6 | 36.2 | 52.6 |
| 0.3 | 29.9 | 34.4 | 39.0 |
| 0.4 | 28.6 | 30.9 | 36.0 |
| 0.5 | 21.5 | 22.0 | 32.1 |

*Significant at 1% (Between *Rhizobium* isolates, p = 0.009).

that 0.1 to 0.4 gml$^{-1}$ EDTA increased IAA production in all three isolates, while maximum IAA production was observed at 0.2 gml$^{-1}$. At a concentration above 0.2 gml$^{-1}$, a decrease in IAA production was observed (Table 3). Among the three isolates, the *Rhizobium* isolate from stem nodules of *S. procumbens* produced the highest amount of IAA at 0.2 gml$^{-1}$ EDTA (52.6 gml$^{-1}$). Changes in the cell wall or membrane by EDTA has been reported to increase the availability of tryptophan to converting enzymes, as well as increased release of IAA from the cell (Bhattacharya and Basu, 1992) . The effect of different concentration of EDTA on IAA production among isolates was found to be statistically significant (p value = 0.009).

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).

## Conclusions

From this study it is clear that *Rhizobium* isolates differ significantly in auxin production. The ability of *Rhizobium* isolates to produce IAA in tryptophan-supplemented medium suggested the possibility that the symbiont was responsible for higher IAA content of root nodules.

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