Research Journal of Microbiology

ISSN 1816-4935

Academic Journals Inc.

www.academicjournals.com

Research Journal of Microbiology 3 (4): 276-281, 2008
ISSN 1816-4935

Production of Indole-acetic-acid by *Rhizobium* Isolates from *Crotalaria* Species

M. Sridevi, N.C.S. Yadav and K.V. Mallaiah
Department of Microbiology, Acharya Nagarjuna University,
Nagarjuna Nagar-522510, Andhra Pradesh, India

Abstract: Cultural and nutritional conditions were optimized for Indole Acetic Acid (IAA) production by *Rhizobium* spp. isolated from root nodules of *Crotalaria juncea*, *C. retusa*, *C. laburnifolia*, *C. verrucosa* and *C. alata*. The isolates produced maximum amount of IAA after 72 h of incubation and at 2.5 mg mL^{-1} L-tryptophan concentration. The effect of different carbon and nitrogen sources on IAA production were also studied and it revealed that, mannitol and L-glutamic acid were the best promoters for IAA production. Addition of cell wall affecting agents increased the IAA production over controls. Among the five isolates of *Crotalaria* species, maximum amount of IAA was produced by isolate from *C. retusa*. The compound from *Rhizobium* sp. from *C. retusa* was extracted, purified and structurally confirmed as IAA.

Key words: *Rhizobium* species, indole acetic acid, *Crotalaria* species, *Rhizobium*-legume symbiosis

INTRODUCTION

The Plant Growth Promoting Rhizobacteria (PGPR) are involved in the production of diverse microbial metabolites like siderophores, plant growth enhancement through Indole Acetic Acid (IAA) production and HCN production (Ahmad *et al*., 2005). Rhizobia are the first group of bacteria, which are attributed to the ability of PGPR to release IAA that can help to promote the growth and pathogenesis in plants (Mandal *et al*., 2007).

Crotalaria is one of the largest genera of Fabaceae with more than 500 species commonly occurring in diverse climatological situations (Allen and Allen, 1981). Some *Crotalaria* species are of great agronomic interest, since they are used as green manure to improve soil fertility or control nematode populations in infested soils (Sy *et al*., 2001). Very few studies on IAA production by root nodule symbionts associated with this species. The objective of this study was therefore to investigate the IAA production by *Rhizobium* isolates of most commonly occurring *Crotalaria* species (*C. juncea* L., *C. laburnifolia* L., *C. retusa* L., *C. verrucosa* L. and *C. alata* Buch. Ham.) and regulation of various factors such as incubation time, tryptophan status along with carbon sources, nitrogen sources and cell wall affecting agents.

MATERIALS AND METHODS

Organism and Growth Conditions

The study was conducted in the Microbiology Department of Acharya Nagarjuna University, India, in January 2007. The symbionts were isolated from the root nodules of *Crotalaria* species

Corresponding Author: K.V. Mallaiah, Department of Microbiology, Acharya Nagarjuna University, Nagarjuna Nagar-522510, Andhra Pradesh, India

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 in YEM broth by standard method (Holt *et al*., 1994).

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 taking Optical Density (OD) at 540 nm.

Estimation of IAA Production

The IAA in cell free supernatant was estimated colorimetrically by the method adopted from Gordon and Weber (1951).

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, 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 different chemicals were added individually to the tryptophan supplemented basal medium having most suitable carbon and nitrogen source. The individual effect of the chemicals on IAA production was also measured.

Extraction of IAA

The *Rhizobium* sp. isolated from *C. retusa* was inoculated into 200 mL of YEM medium with most suitable substance and incubated at 28±2°C for 3 days on rotary shaker. After incubation, the IAA was extracted according to the method described by Sinha and Basu (1981).

Purification and Detection of IAA

Partial purification of IAA from crude extract was done by using silica gel column chromatography (22X5 cm) 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) with solvent system (ethyl acetate and hexane, 2:8) and then developed with Salkowski reagent (Morales *et al*., 2003).

Mass Spectrometry

The fraction that gave positive result with Salkowski reagent was collected and concentrated and analyzed by Liquid Chromatography-Mass Spectrometry (Agilent 1100 series, USA) equipped with Mass Selective Detector (MSD).

Statistical Analysis

The data were statistically analyzed by ANOVA (two way classification technique) using Statistica software.

RESULTS AND DISCUSSION

Based on the morphological, cultural and biochemical characteristics, the isolates were identified as *Rhizobium* species. The identification was done following Bergey's Manual (Jordan, 1984). The *Rhizobium* isolates from *Crotalaria* species produced IAA after 24 h of incubation and reached maximum at 72 h and then decreased (Fig. 1). The decrease in IAA level might be due to the release of IAA degrading enzymes as reported earlier in *Rhizobium* sp. from *Cajanus cajan* (Datta and Basu, 2000). Among the five isolates of *Crotalaria* species, the isolate from *C. retusa* produced maximum amount of IAA (39.0 μg mL^{-1}) after 72 h.

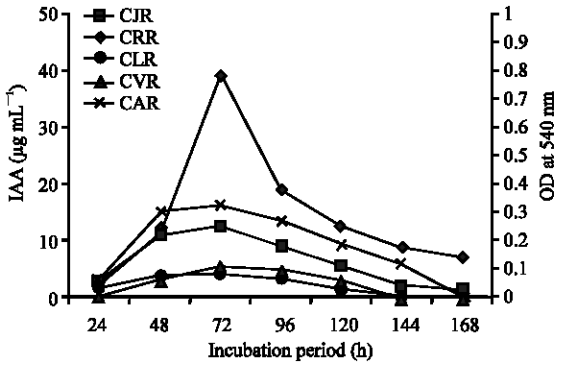

Fig. 1: Growth and IAA production by *Rhizobium* isolates from *Crotalaria* species

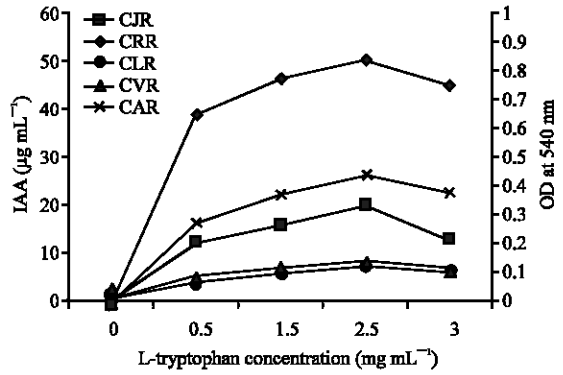

Fig. 2: Effect of different concentrations of L-tryptophan on growth and IAA production by *Rhizobium* isolates from *Crotalaria* species.

The effect of different concentrations of L-tryptophan revealed that the maximum growth and IAA production were observed in all the isolates at 2.5 mg mL^{-1} L-tryptophan concentration (Fig. 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^{-1} L-tryptophan concentration (Ghosh and Basu, 2002), while the *Rhizobium* sp. from root nodules of *Roystonia regia* produced maximum amount of IAA at 3 mg mL^{-1} L-tryptophan concentration (Basu and Ghosh, 2001).

Replacement of 10 different carbon sources (1 %) in the basal medium revealed that the *Rhizobium* isolates vary in their utilization and production of IAA. The isolates produced more amount of IAA when mannitol was used as carbon source (Table 1). But, *Rhizobium* sp. from *Cajanus cajan* produced maximum amount of IAA in glucose containing medium as reported earlier (Datta and Basu, 2000). The production of IAA was minimum when rhamnose, ribose, mannose and sorbitol were used as carbon source. But, ribose was reported to be the best promoter at 1.5% concentration by the *Rhizobium* species isolated from *Psophocarpus tetragonolobus* (Bhattacharya and Basu, 1992). Statistically significant was occurred in between *Rhizobium* spp. and in between carbon sources.

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 inorganic nitrogen sources like KNO_3, $(NH_4)_2SO_4$ and $NaNO_3$ supported maximum IAA production in all isolates, while amino acid glycine as additional nitrogen source reduced growth and IAA production (Table 2). Some amino acids

Table 1: Effect of carbon sources on growth and indole acetic acid production by *Rhizobium* isolates from *Crotalaria* species

Carbon source* (1 %)	*Rhizobium* isolates*									
	Rhizobium sp. (*C. juncea*)		*Rhizobium* sp. (*C. retusa*)		*Rhizobium* sp. (*C. laburnifolia*)		*Rhizobium* sp. (*C. verrucosa*)		*Rhizobium* sp. (*C. alata*)	
		IAA		IAA		IAA		IAA		IAA
	OD_{540}	($\mu g\ mL^{-1}$)	OD_{540}	($\mu g\ mL^{-1}$)	OD_{540}	($\mu g\ mL^{-1}$)	OD_{540}	($\mu g\ mL^{-1}$)	OD_{540}	($\mu g\ mL^{-1}$)
Control	0.15	11.0	0.34	20.2	0.10	2.0	0.12	4.8	0.22	14.9
Mannitol	0.20	12.5	0.80	39.0	0.12	4.0	0.20	5.3	0.30	16.0
Glucose	0.34	11.0	0.39	36.2	0.14	2.0	0.15	4.2	0.31	14.2
Maltose	0.20	4.2	0.20	12.9	0.12	1.0	0.18	3.9	0.22	6.9
Galactose	0.32	11.9	0.38	28.6	0.15	3.2	0.15	2.6	0.33	12.2
Lactose	0.28	6.2	0.30	14.4	0.12	1.6	0.18	3.0	0.29	11.8
Sucrose	0.14	4.2	0.22	12.2	0.06	1.0	0.12	1.5	0.20	10.9
Fructose	0.24	10.0	0.20	14.4	0.05	-	0.06	-	0.15	6.9
Rhamnose	0.10	1.0	0.04	-	0.04	-	0.08	1.0	0.10	2.8
Ribose	0.08	-	0.09	1.0	0.08	0.6	0.04	-	0.04	-
Mannose	0.06	-	0.12	1.2	0.06	-	0.04	-	0.05	-
Sorbitol	0.20	6.3	0.28	12.3	0.15	2.8	0.09	1.2	0.04	1.9

*: Significant at 1% (Between *Rhizobium* sp.: Fc = 4.7, Ft = 2.0; Between carbon sources: Fc = 12.8, Ft = 2.6)

Table 2: Effect of nitrogen sources on growth and indole acetic acid production by *Rhizobium* isolates from *Crotalaria* species

Nitrogen source* (0.1 %)	*Rhizobium* isolates*									
	Rhizobium sp. (*C. juncea*)		*Rhizobium* sp. (*C. retusa*)		*Rhizobium* sp. (*C. laburnifolia*)		*Rhizobium* sp. (*C. verrucosa*)		*Rhizobium* sp. (*C. alata*)	
		IAA		IAA		IAA		IAA		IAA
	OD_{540}	($\mu g\ mL^{-1}$)	OD_{540}	($\mu g\ mL^{-1}$)	OD_{540}	($\mu g\ mL^{-1}$)	OD_{540}	($\mu g\ mL^{-1}$)	OD_{540}	($\mu g\ mL^{-1}$)
Control	0.12	1.80	0.14	6.0	0.10	2.0	0.09	2.0	0.09	1.0
KNO_3	0.39	14.50	0.45	40.2	0.20	4.9	0.22	5.9	0.46	16.2
$(NH_4)_2SO_4$	0.30	12.20	0.38	38.6	0.14	12.4	0.18	14.2	0.30	12.0
$NaNO_3$	0.25	10.90	0.30	36.2	0.14	11.0	0.20	15.2	0.32	14.2
$NaNO_2$	0.28	12.20	0.28	34.9	0.22	2.9	0.20	5.1	0.30	12.1
L-asparagine	0.29	10.60	0.32	26.9	0.28	4.9	0.28	8.9	0.32	14.1
L-glycine	0.06	2.00	0.12	2.6	0.06	-	0.09	-	0.39	14.5
Casamino acid	0.30	12.60	0.38	26.2	0.32	10.2	0.30	9.2	0.32	14.0
L-glutamic acid	0.40	11.60	0.56	44.2	0.40	14.4	0.34	10.9	0.40	16.9
Cystine	0.42	13.30	0.22	20.6	0.22	3.0	0.30	9.0	0.22	10.9
Tyrosine	0.16	3.00	0.20	18.6	0.20	2.5	0.16	3.0	0.20	8.2

*: Significant at 1% (Between *Rhizobium* sp.: Fc = 4.5, Ft = 2.1; Between nitrogen sources: Fc = 30.2, Ft = 2.6)

were shown earlier to inhibit IAA production by *Rhizobium meliloti* (Garcia-Rodriguez *et al*., 1981) due to inhibition of conversion of tryptophan to IAA. The *Rhizobium* isolates from *C. retusa*, *C. laburnifolia* and *C. alata* showed maximum growth and IAA production in the medium amended with L-glutamic acid (44.2, 14.4 and 16.9 $\mu g\ mL^{-1}$). That the L-glutamic acid was the most effective nitrogen source for growth and IAA production were also reported in *Rhizobium* sp. isolated from root nodules of *Cajanus cajan* (Datta and Basu, 2000). The effect of different nitrogen sources on IAA production was also found to be statistically significant.

Addition of cell wall affecting agents or surfactant revealed that, all the five isolates produced highest amount of IAA in medium amended with different concentrations of EDTA and SDS. But, penicillin and lysozyme have generally negative effect on IAA production (Table 3). IAA production was maximum at 0.2 $\mu g\ mL^{-1}$ EDTA and 0.1 $\mu g\ mL^{-1}$ SDS concentrations. Among the five isolates, the isolate from *C. retusa* produced maximum amount of IAA (50.9 $\mu g\ mL^{-1}$) at 0.2 $\mu g\ mL^{-1}$ EDTA concentration. Changes in the cell well or membrane by these agents increased the availability of

Table 3: Effect of cell wall affecting agents on growth and indole acetic acid production by *Rhizobium* isolates from *Crotalaria* species

Cell wall effecting agents	Concentration	*Rhizobium* isolates*									
		Rhizobium sp. (*C. juncea*)		*Rhizobium* sp. (*C. retusa*)		*Rhizobium* sp. (*C. laburnifolia*)		*Rhizobium* sp. (*C. verrucosa*)		*Rhizobium* sp. (*C. alata*)	
		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	-	0.20	12.5	0.80	39.0	0.12	4.0	0.15	5.3	0.30	16.0
EDTA	0.1 ($\mu g\ mL^{-1}$)	0.32	14.2	0.38	48.2	0.19	3.9	0.25	5.6	0.34	16.2
	0.2 ($\mu g\ mL^{-1}$)	0.30	15.2	0.42	50.9	0.22	4.2	0.20	5.9	0.32	14.0
	0.3 ($\mu g\ mL^{-1}$)	0.22	10.0	0.36	44.0	0.15	2.1	0.16	4.2	0.28	12.8
SDS	0.1 ($\mu g\ mL^{-1}$)	0.28	12.9	0.36	44.0	0.16	2.4	0.20	4.9	0.33	16.0
	0.2 ($\mu g\ mL^{-1}$)	0.22	10.2	0.32	41.9	0.11	1.9	0.16	5.5	0.32	16.2
	0.3 ($\mu g\ mL^{-1}$)	0.18	6.2	0.28	38.5	0.10	1.2	0.14	3.4	0.28	12.0
Penicillin	25 IU	0.25	11.2	0.36	22.6	0.11	2.2	0.19	4.9	0.38	16.0
	50 IU	0.20	8.2	0.34	20.9	0.10	2.0	0.16	2.0	0.28	14.2
	100 IU	0.16	4.2	0.32	16.4	0.08	-	0.08	-	0.25	12.0
Lysozyme	25 IU	0.30	11.8	0.36	20.9	0.10	2.2	0.20	4.9	0.35	15.5
	50 IU	0.20	12.0	0.32	14.6	0.08	-	0.18	4.0	0.30	12.8
	100 IU	0.18	9.2	0.28	12.9	0.04	-	0.11	2.9	0.26	11.9

* Significant at 1% (Between *Rhizobium* sp.: Fc = 2.07, Ft = 2.01; Between surfactant sources: Fc = 44.0, Ft = 2.5)

tryptophan to converting enzyme as well as increase the release of IAA from the cell was reported earlier by Bhattacharya and Basu (1992). The effect of surfactant on IAA production was statistically significant but in different *Rhizobium* isolates production of IAA was not significant.

As the *Rhizobium* isolate from *C. retusa* produced highest amount of IAA (50.9 $\mu g\ mL^{-1}$) in the medium amended with 0.2 $\mu g\ mL^{-1}$ of EDTA, this *Rhizobium* sp. was selected for characterization of IAA produced.

TLC of chromatogram of purified compound and standard IAA sprayed with Salkowski reagent showed almost the same Rf-values (0.85) with all ithentic JAA, which were identified under UV-light (254 nm).

The fraction that gave positive result with Salkowski reagent was collected and 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. The LC-MS of fraction revealed that it has a benzene moiety (m/z 77) in its chemical structure and its molecular weight may be 175.4. From this study, it is clear that all the isolates from *Crotalaria* sp. were positive for IAA production, but the isolates differ significantly in auxin production depending upon the cultural conditions.

REFERENCES

Ahmad, F., I. Ahmad and M.S. Khan, 2005. Indole acetic acid production by the indigenous isolates of *Azotobacter* and fluorescent *Pseudomonas* in the presence and absence of tryptophan. Turk. J. Biol., 29: 29-34.

Allen, O.N. and E.K. Allen, 1981. The Leguminosae, a Source Book of Characteristics, Uses and Nodulation. University of Wisconsin Press, Masison.

Basu, P.S. and A.C. Ghosh, 2001. Production of indole acetic acid in cultures by a *Rhizobium* species from the root nodules of a monocotyledonous tree, *Roystonea regia*. Acta Biotechnol., 21: 65-72.

Bhattacharya, R.N. and P.S. Basu, 1992. Bioproduction of indole acetic acid by a *Rhizobium* sp. from root nodules of leguminous Climber, *Psophocarpus tetragonolobus* DC. Ind. J. Exp. Biol., 30: 632-635.

Datta, C. and P.S. Basu, 2000. Indole acetic acid production by a *Rhizobium* species from root nodules of a leguminous shrub, *Cajanus cajan*. Microbiol. Res., 155: 123-127.

Garcia-Rodriguez, T., A.M. Gutierrez-Navarro, R. Jimenez and J. Perez Silva, 1981. Effects of legume root exudates in indole acetic acid production by *Rhizobium meliloti*. Pol. J. Soil Sci., 14: 45-52.

Ghosh, A.C. and P.S. Basu, 2002. Growth behaviour and bioproduction of indole acetic acid by a *Rhizobium* sp. isolated from root nodules of a leguminous tree *Dalbergia lanceolarea*. Ind. J. Exp. Biol, 40: 796-801.

Gordon, S.A. and R.P. Weber, 1951. Colorimeteric estimation of indole acetic acid. Plant Physiol., 26: 192-195.

Holt, G.J., N.R. Krieg, H.A. Sneath, J.T. Staley and S.T. William, 1994. Bergey's Manual of Determinative Bacteriology, 9th Edn., Baltimore, William and Wilkins.

Jordan, D.C., 1984. Bergey's Manual of Systematic Bacteriology. Krieg, N.R. and J.G. Holt (Eds.). (Williams and Wilkins, Baltimore), pp: 234.

Mandal, S.M., K.C. Mondal., S. Dey and B.R. Pati, 2007. Optimization of cultural and nutritional conditions for indole-3-acetic acid (IAA) production by a *Rhizobium* sp. isolated from root nodules of *Vigna mungo* (L.) Hepper. Res. J. Microbiol., 2: 239-246.

Morales, L.J.M., L.S. Urzua, B.E. Baca and J.A.S. Ahedo, 2003. Indole-3-butyric acid (IBA) production in culture medium by wild strain *Azospirillum brasilense*. FEMS Microbiol. Lett., 228: 167-173.

Sinha, B.K. and P.S. Basu, 1981. Indole-3-acetic acid and metabolism in root nodules of *Pongamia pinnata* L. Pierre. Biochem. Physiol. Pflanzen, 176: 218-227.

Skerman, V.B.D., 1959. A Guide to Identification of the Genera of Bacteria. Williams and Wilkins Co., Baltimore, 2: 189-191.

Sy, A., E. Girand, P. Jourand, N. Garcia, A. Willems, P. de Lajudie, Y. Prin, M. Neyra, M. Gillis, C. Boivin-Masson and B. Dreyfus, 2001. Methylotrophic *Methylobacterium* bacteria nodulate and fix nitrogen in symbiosis with legumes. J. Bacteriol., 183: 214-220.

Vincent, J.M., 1970. A manual for the Practical Study of the Root Nodule Bacteria. Blackwell Scientific Publications, Oxford.