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Production of Indole Acetic acid by *Rhizobium* sp. from Root Nodules of a Leguminous herb *Crotalaria saltiana* Andr. in Culture

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The root nodules of *Crotalaria saltiana* Andr., a roadside perennial weed, contained Indole Acetic Acid (IAA). The *Rhizobium* sp. isolated from the root nodules of the plant was identified by physio-biochemical characteristics. A tryptophan pool present in the nodule might serve as a source of IAA production. The isolated bacterial strain produced high amount of IAA (61 μg/ml) from L-tryptophan supplemented yeast extract mannitol (YEM) medium. Production of IAA and the growth of the bacteria started simultaneously, though the bacteria had a separate growth and production phase. At the stationary phase IAA production was maximum. Attempts were made to optimize the cultural requirement for growth and maximum IAA production. The IAA production was increased from 16 mg/ml to 212.5 mg/ml when a L-tryptophan (2 mg/ml) supplemented carbon-free mineral medium was enriched with mannitol (1.5%) as carbon source, biotin (1 μg/ml) as vitamin source and KNO_3 (0.1%) as nitrogen source. The possible role of the rhizobial production of IAA on the *Rhizobium*-legume symbiosis is discussed.

Key words: *Crotalaria saltiana, Rhizobium*, Indole acetic acid (IAA), root nodule.

The legume family is the third largest (after Orchidaceae and Asteraceae) family of angiosperms[1] with approximately 730 genera and over 19400 species worldwide[2].

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Legumes are second only to Poaceae (the grasses) in agricultural economic importance. Microorganisms producing root and stem nodules on legumes are represented by six genera- *Rhizobium, Azorhizobium, Mesorhizobium, Sinorhizobium*, *Allorhizobium*, and *Bradyrhizobium*[3]. Not all legumes bear nodules; only 3% of Caesalpinioideae are nodulated, though 90% of Mimosoideae and 97% of Papilionoideae nodulate[4].

Microbiologists showed great interest on increase in nitrogen contents by root nodule producing leguminous plants with an emphasis on the fixation of nitrogen and its supply to the host from symbionts for many years[5]. The fixation

of nitrogen and its supply to the host by *Rhizobium* in root nodules of legumes was thought to be the only function of nodules for many years among the scientists. Beside nitrogen fixation, the root nodules of leguminous plants contained appreciable amount of different phytohormones. The plant hormone production in nodules by the symbiont and transport it to the host[6] by the nodular symbionts has gained interest. *Rhizobium* spp. were known to produce IAA from tryptophan in culture[7,8,9]. Bacteria associated with the roots of greenhouse tropical orchids showed to produce indole-3-acetic acid (IAA) and to excrete it into the culture liquid[10].

Crotalaria saltiana Andr. is a roadside weed of subfamily Papilionaceae of family leguminosae (Fabaceae). It is a perennial herb, not climbing, found throughout West Bengal. The signs of *Crotalaria* poisoning after feeding of dry shoot to strains ASL mice were in appetence, dullness, dyspnea and recumbency. The main lesions were necrosis, portal fibroplasias and hemorrhage in the liver, pulmonary congestion and emphysema, fecal catarrhal enteritis and degeneration of the cells of the renal tubules[11]. The present study discussed the growth properties and IAA synthesizing capacity of the *Rhizobium* sp. isolated from the root nodules of the herb *Crotalaria saltiana* Andr., an attempt was made to optimize the cultural requirements by different supplements for maximum growth and IAA production.

MATERIAL AND METHODS

Microorganism, Medium and Growth condition

The bacteroid was isolated from fresh, healthy, surface sterilized and pink-coloured root nodules of *C. saltiana* and grown in pure culture. The medium selected for bacterial growth was yeast extract mineral medium of Skerman (1959)[12] with 1% mannitol (YEM) and having 0.01% $CaCl_2$, H_2O instead of NaCl and $CaCO_3$ at pH 7.0 and supplemented with different isomer of tryptophan (L-, DL-, D-tryptophan). The bacteria were incubated in 20 ml medium in 100 ml conical flasks in three replicates at 30±2°C on a rotary shaker. Growth of the bacteria was measured turbidimetrically by a colorimeter at 540 nm.

Extraction & quantitative estimation of IAA and tryptophan from nodules

Mature, fresh nodules of *Crotalaria saltiana* Andr. were selected for all the experiments. Indole Acetic Acid (IAA) was extracted following Sinha and Basu (1981) [13] with slight modification. IAA was estimated spectrophotometrically following Gordon and Weber (1951)[14] using Salkowski reagent. The extract containing IAA was identified by thin layer chromatography (TLC). Tryptophan was extracted following Nitsch (1955)[15] and estimated colorimetrically following Hassan (1975)[16].

Quantitative estimation of IAA production

Cell free supernatant was used for IAA estimation. IAA was estimated spectrophotometrically at 530 nm following the method of Gordon and Weber (1951)[14] using a standard curve prepared from authentic IAA (Sigma, USA). The supernatant containing IAA was identified by thin layer chromatography (TLC).

Optimization of culture

To check maximum growth of bacteria and maximum IAA production by the bacteria different carbon sources (omitting mannitol), different nitrogen sources and different vitamin sources were used. Different chemicals were added individually to the tryptophan supplemented basal medium and the individual effect on growth and IAA production were measured. To establish the maximum growth and production of IAA by the symbiont in culture, the medium was enriched with the supplements that individually increased the growth and IAA production to the maximum.

Statistical analyses were done following Panse and Sukhatme (1985)[17].

RESULTS AND DISCUSSION

The symbiont was isolated in pure form fresh and healthy nodules of *Crotalaria saltiana* Andr. The symbiont isolated from the mature root nodules was identiûed as *Rhizobium* sp. by physio biochemical analysis, re-inoculation and nodule formation study according to the methods given in the Manual of Microbiological Methods[18] and Bergey's Manual of Systematic Bacteriology[3].

The nodules of the plant contained high amount of IAA. The amount of IAA was 11.75

µg/g (Table 1) fresh nodule tissues. The amount was much higher than the amount of IAA present in the nodule of *Lupinus luteus*[7], and the nodule of *Vicia faba*[19]. But the amount of IAA present in the nodule of *Crotalaria saltiana* was less than the amount of IAA present in the nodule of *Melilotus alba*[20]. According to many other early reports the amount of IAA present in the root was below the level of detection[20], but it was found that *Phaseolus mungo*, a herbaceous leguminous pulse, produced IAA in the young roots[21].

The *Rhizobium* sp. produced IAA in culture when yeast extract mannitol (1%) medium was supplemented with tryptophan (all three different isomers- D-, DL-, & L-tryptophan) as precursor of IAA (Table 2). The bacteria almost

Table 1. Content of IAA and tryptophan present in the root and nodule of *Crotalaria saltiana*

Plant parts	Amount of IAA (µg/g fresh tissue)	Amount of tryptophan (µg/g of fresh tissue)
Nodule	11.75	1325
Root	ND	875
Critical difference at P = 0.05	1.08	20.12

ND = Not Detected

Table 2. Effect of different isomers of tryptophan on growth of the *Rhizobium* sp. from *Crotalaria saltiana* and its IAA production in culture

Isomers of tryptophan	OD for growth at 540 nm	IAA production (µg/ml)	Specific productivity (IAA production/growth)
Control	0.98	02	2.04
D-tryptophan	1.26	23	18.25
DL-tryptophan	1.46	43	29.45
L-tryptophan	1.48	61	41.22
Critical difference at P= 0.05	0.11	1.12	-

Table 3. Effect of different carbon sources on growth and IAA production by the *Rhizobium* sp. from *Crotalaria saltiana* in culture

Carbon sources (1%)	OD for growth at 540 nm	IAA production (µg/ml)	Specific productivity (IAA production/growth)
Control	0.76	16	21.05
Fructose	0.94	18	19.15
Lactose	1.12	23	20.54
Myo-inositol	1.16	23	19.83
Glucose	1.16	26	22.41
Arabinose	1.16	31	26.17
Mannose	1.60	53	40.77
Galactose	1.42	56	39.44
Sucrose	1.62	56	34.57
Mannitol	1.48	61	41.22
Critical difference at P= 0.05	0.17	2.38	-

failed to produce IAA without supplementation of tryptophan in medium. The bacteria preferred L-tryptophan for both IAA production and growth (Table 2). The specific productivity was also high (41.22) with L-tryptophan than the two other isomers (Table 2). The bacteria reached its stationary phase of growth at 20h of incubation (Fig. 1) and produced maximum IAA when the medium was supplemented with 2 mg/ml of L-tryptophan (Fig. 2). Production of IAA was decreased when tryptophan concentration was increased above 2 mg/ml (Fig. 2). The IAA produced by the *Rhizobium* sp. was identified with the help of thin layer chromatography (TLC) comparing the Rf value with authentic sample. The similar greater preference of L-tryptophan for IAA production observed by many authors[22-25].

The production of IAA by the *Rhizobium* sp. in L-tryptophan supplemented yeast extract mannitol (1%) medium was started from the very beginning simultaneously with the log phase of growth (Fig. 1). After a gradual increase of IAA production by the *Rhizobium* sp. with their growth, maximum production of IAA was 59.0 mg/ml occurred after 20 h of incubation (Fig. 1). IAA production by the *Rhizobium* sp. was much smaller than the amount of L-tryptophan added. This is probably due to the utilization of this essential amino acid partly in protein synthesis and partly for the formation of other indole compounds in addition with IAA in culture[26].

Replacement of mannitol from the basal yeast extract mannitol medium of Skerman (1959)[12], eight different carbon sources (1%) and mannitol (1%) also were added with supplementation of L-tryptophan (2 mg/ml). Productions of IAA by the *Rhizobium* sp. and its growth with the change of different carbon sources of basal medium were checked. The maximum IAA production (61 mg/ml) was observed with mannitol (Table 3) though maximum growth was observed in sucrose. The specific productivity

Table 4. Effect of different nitrogen sources on growth and IAA production by the *Rhizobium* sp. from *Crotalaria saltiana* in culture

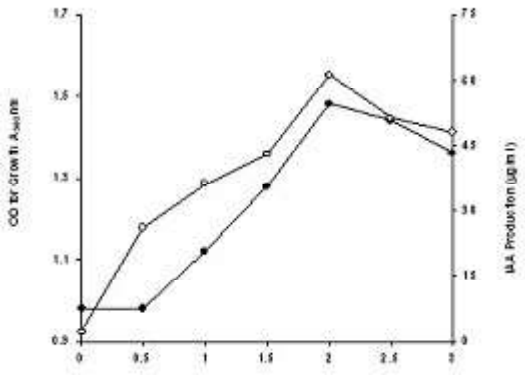
Nitrogen sources (0.1%)	OD for growth at 540 nm	IAA production (µg/ml)	Specific productivity (IAA production/growth)
Control	1.48	61.0	41.22
Glycine	6.11	56.0	9.17
NH_4Cl	1.60	58.0	36.25
L-asparagine	6.98	61.0	8.74
$NaNO_3$	4.37	162.5	37.19
KNO_3	7.42	202.5	27.29
Critical difference at P= 0.05	2.11	09.81	-

Table 5. Effect of different concentration of most preferred nitrogen source (KNO_3) on growth and IAA production by the *Rhizobium* sp. from *Crotalaria saltiana* in culture

Concentration of KNO_3 (%)	OD for growth at 540 nm	IAA production (mg/ml)
Control	1.51	57.5
0.1	7.51	190.0
0.2	7.34	157.5
0.3	7.34	182.5
0.4	7.17	90.0
0.5	7.15	102.5
Critical difference at P=0.05	1.45	05.34

(41.22) was also high with mannitol (Table 3). The percentage of the most preferred carbon source (mannitol) was also tested. A gradual increase of the percentage value of mannitol from 0.5% to 2.5% as carbon source with a control set devoid of any carbon source was prepared for checking the maximum growth and IAA production with the concentration of mannitol. It was found that maximum IAA production (63 mg/ml) (Fig. 3) by the *Rhizobium* sp. as well as its growth was occured at 1.5% of mannitol. The production of IAA by the bacteria kept its stationary state upto 2% of mannitol but decreased when the concentration of mannitol became increased above 2% (Fig. 3), though the growth of the bacteria decreased when the concentration of mannitol became increased above 1.5% (Fig. 3). The effect of carbon sources in the rhizobial IAA production was reported earlier by many authors[8,23].

Five different nitrogen sources were used in the yeast extract mannitol (1%) medium supplemented with 2 mg/ml L-tryptophan to check the maximum IAA production and growth. Among the five different nitrogen sources, the maximum growth and IAA production (202.5 mg/ml) was found in the culture supplemented with KNO_3 (Table 4). All the nitrogen sources were applied at a definite concentration (0.1%) with a control set devoid of any nitrogen sources (Table 4). The specific productivity was 27.29 (Table 4) with KNO_3 as nitrogen source was also remarkable. It was found that maximum growth and IAA production by the *Rhizobium* sp. occurred at 0.1% of KNO_3 (Table 5), but here the specific productivity was lower than that obtained for other nitrogen sources. According to Vincent (1974)[27] the *Rhizobium sp*. can utilize several nitrogenous compounds for growth which might responsible for the increased IAA production.

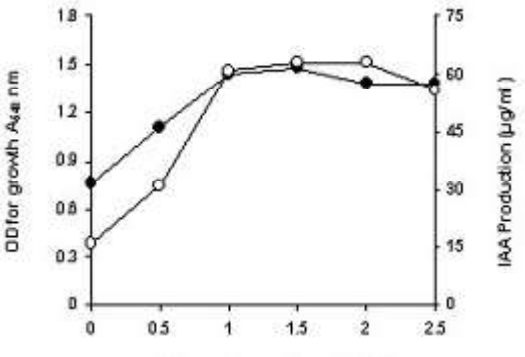


conc. of L-tryptophan

Fig. 2. Growth (•) and IAA production (o) by the *Rhizobium* sp. in YEM medium containing L-tryptophan of different concentrations

Time (hours)

Fig. 1. Growth (•) and IAA production (o) by the *Rhizobium* sp. in culture.
The bacteria were grown in YEM medium containing 2 mg/ml of L-tryptophan



conc. of mannitol (%)

Fig 3. Growth (•) and IAA production (o) by the *Rhizobium* sp. in yeast extract mineral medium containing different concentration of mannitol

To check the preferred vitamin source by the *Rhizobium* sp. for maximum IAA production, five different vitamin sources were used with a control set devoid of any vitamin source (Table 6). The basal media used for checking preferred vitamin source was yeast extract mannitol (1%) medium supplemented with 2 mg/ml L-tryptophan. Each and every vitamin source used at a definite concentration (1 mg/ml) in the axenic medium. Maximum IAA production (65 mg/ml) was estimated when the media supplemented with d-biotin (Table 6). The specific productivity was 48.15 (Table 6), though maximum growth occurred when the medium was supplemented with riboflavin. The increased production of IAA by some vitamins suggested that the organism required a number of vitamins as co-factor as suggested by Vincent (1974)[27] and Alexander (1977)[28].

To obtain a maximum production of IAA by the *Rhizobium* sp. in culture, the most effective supplements that increase the IAA production maximally were added to the medium gradually (Table 7). The *Rhizobium* sp. initially produced 16 mg/ml IAA when L-tryptophan (2 mg/ml) was supplemented with carbon free basal yeast extract mineral medium. IAA production reached its maximum by the *Rhizobium* sp. when mannitol (1.5%) as preferred carbon source, KNO_3 as preferred nitrogen source, and d-biotin (1 mg/ml) as preferred vitamin source were added in L-tryptophan supplemented yeast extract mineral medium. Maximum IAA production was 212.5 mg/ml with gradual increase of growth (Table 7), which was enormous increase through cultural optimization.

Indole acetic acid (IAA) is produced by the *Rhizobium* sp. in the nodule tissues and supplied to the host. IAA is involved in host plant growth and development after metabolism. IAA is also produced by the bacteria in culture when the organism grown in laboratory with tryptophan supplemented basal yeast extract mannitol medium. This particular hormone, which has an importance in plant science, is being also to be made industrially with the help of large fermentor. Here attempt is to be made to optimize the cultural condition at which maximum IAA production

Table 6. Effect of different vitamin sources on growth and IAA production by the *Rhizobium* sp. from *Crotalaria saltiana* in culture

Vitamin sources (1µg/ml)	OD for growth at 540 nm	IAA production (µg/ml)	Specific productivity (IAA production/growth)
Control	1.49	57.5	38.59
L-ascorbic acid	1.39	45.0	32.37
Riboflavin	1.42	52.5	36.97
Thiamine hydrochloride	1.16	57.5	49.57
Nicotinic acid	1.36	57.5	42.28
D-Biotin	1.35	65.0	48.15
Critical difference at P=0.05	0.78	11.18	-

Table 7. Increase in the growth of the *Rhizobium* sp. and its IAA production using the most preferred supplements

Supplements	Growth		IAA production		Specific productivity (IAA production / growth)
	OD at 540 nm	Percent increase over control	(mg/ml)	Percent increase over control	
Yeast extract mineral (C)	0.76	-	16.0	-	21.05
(C) + mannitol(1.5%)	1.50	97.37	63.0	293.75	42.00
(C) + mannitol(1.5%)+	1.48	94.74	70.0	937.50	47.30

with definite concentrations of preferred carbon, nitrogen as well as vitamin sources used. Earlier reports showed that the symbiont, *Rhizobium meliloti* produced 20 mg/ml of IAA [29], *R. leguminosarum* produced 2.0 mg/ml of IAA [30], *Rhizobium* sp. from a climbing herb produced 17 mg/ml of IAA in culture [24], *Rhizobium sp* of *Phaseolus mungo* produced 138 mg/ml of IAA in culture [21].

Again, all the supplements, which increased the IAA production in culture, might be available to the bacteria within the nodules. The host plant might further induce the symbiont to produce excess IAA for its own benefit. Earlier work by Hunter (1989)[6] *Rhizobium* spp. were known to produce IAA from tryptophan in culture[7,8,9]. There were also some phosphate solubilising strains of bacteria (phosphobacteria – CP01, RP07), which were able to produce phytohormones like IAA and GA3 under *in vitro* condition[31]. Bacteria associated with the roots of greenhouse tropical orchids showed to produce indole-3-acetic acid (IAA) and to excrete it into the culture liquid[10]. Hunter (1989) [6] demonstrated that *Bradyrhizobium japonicum* clones that produced large amounts of IAA in culture also formed nodules that accumulated large amounts of IAA. He also showed that nodules that accumulated large amount of IAA contained bacteroids with an enhanced IAA producing capacity. These correlations suggested that bacteroids of this legume also produced IAA symbiotically and that IAA accumulated in the nodules of this plant. The production of IAA by the nodule bacteria might have important physiological implications as it seemed reasonable to suspect that IAA, alone or in conjunction with other plant hormones, might be involved in several stages of the symbiotic relationship and might be transported to the other parts of the plant as several authors evidenced that phytohormones from the nodules were transported to the other parts of plant[19]. The nodule bacteria either released hormones or stimulated the activities of endogenous hormones in the host root and helped in nodulation (Hopkins 1995)[32]. During the period of infection the IAA produced by the *Rhizobium* sp. could trigger cell division of the cortical cells in roots and thus initiate the nodule development[19]. The application of IAA to plant root increased the number of nodules, their mass and amount of nitrogen fixed[32]. Genes induced by IAA were probably involved in the execution of vital cellular functions and development processes[33]. Verma *et al.* (1992) [34] suggested that hormones might be one of the signal molecules returned by *Rhizobium* spp. for nodulation.

From the data presented here and from the works of other authors [21] it seems that if the nitrogen fixation in the nodules and supply to the host be taken as first-line of symbiosis, IAA content in the nodule and supply to the host may be taken as a second-line of symbiosis in the root nodule-*Rhizobium* association.

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