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**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**

^{1,2}Santi M. Mandal, ¹Keshab C. Mondal, ²Satyahari Dey and ¹Bikas R. Pati

¹Department of Microbiology, Vidyasagar University,
Midnapore, West Bengal 721102, India

²Department of Biotechnology, Indian Institute of Technology,
Kharagpur, WB 721302, India

Abstract: Cultural and nutritional conditions were optimized for indole acetic acid (IAA) production by a newly isolated *Rhizobium* sp. from the root nodules of *Vigna mungo* (L.) Hepper. The isolate produced high amount of IAA (40 mg L⁻¹) when medium was supplemented with L-tryptophan over D-or DL-tryptophan isomer at pH 7.2. The effects of different carbon sources and nitrogen sources on indole acetic acid production were studied. IAA production increased upto 1.4 fold over control when the medium was supplemented with glucose (1%) and KNO₃ (0.2%). The tryptophan (1568±22.47 µg g⁻¹ fresh tissue) and IAA (6.34±0.18 µg g⁻¹ fresh tissue) levels were found to be higher in young and healthy root nodules than normal non-nodulated roots. The results indicated that the symbiont might provide high level of IAA to root nodules.

Key words: *Rhizobium* sp., indole acetic acid, root nodule, *Rhizobium*-legume symbiosis

INTRODUCTION

Leguminous plant is important both ecologically and agriculturally, since it is a major source of biological nitrogen fixation through root nodule formation (Sprent *et al.*, 1987). Root nodule is a unique and highly organized structure developed as a result of the symbiotic relationship between leguminous plants and bacteria of the genus *Rhizobium*. Different aspects of the physiology of legume-*Rhizobium* symbiosis and mechanism of nitrogen fixation have been well investigated. The development and persistence of a root nodule requires a high degree of regulation. It is postulated that plant hormones are involved in triggering the initiation of root nodules, nodule development, maintenance and senescence (Badenoch-Jones *et al.*, 1983). Earlier researchers, Thimann (1936) and Link (1937) mentioned that *Rhizobium* derived auxin might be the factor responsible for legume root nodule initiation and morphogenesis. It was evident that the indole-3-acetic acid (IAA) production by *Rhizobium* species and the production are stimulated by exogenous tryptophan pool in the culture medium (Bauer, 1981; Hunter, 1989). Several reports indicate that root nodules have greater auxin content compared with the roots (Fedorova *et al.*, 2000). Ferguson and Mathesius (2003) reviewed on the auxin level in roots and root nodules, which is highly relevant for the initiation of cortical cell divisions, nodule development and regulating nodule numbers. Furthermore, Rhizobia are the first group of bacteria, which are attributed to the ability of Plant Growth Promoting Rhizobacteria (PGPR) to release IAA that can help to promote the growth and pathogenesis in plants (Johri *et al.*, 2003).

Corresponding Author: Bikas Ranjan Pati, Professor, Department of Microbiology, Vidyasagar University, Midnapore (W), WB 721102, India Tel: (91) 3222 276555/477
Fax: (91) 3222 275329/264338

The aim of this study was therefore to investigate the indole-3-acetic acid (IAA) production in culture of a *Rhizobium* sp. isolated from the root nodules of common pulse plant *Vigna mungo* (L.) Hepper and regulation by various factor such as pH, tryptophan status along with different carbon and nitrogen sources.

MATERIALS AND METHODS

Organism and Growth Condition

The symbiont was isolated from the root nodules of common pulse plant *Vigna mungo* (L.) Hepper, isolation from fresh young nodules was made according to Vincent (1970) using yeast extract mannitol agar (YEMA) medium. Isolate was then grown in YEM broth to mid-logarithmic phase and after the addition of 1.5 M glycerol, dispensed in ampoules and stored in -80°C. 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). Axenic cultures of the bacteria were grown in 100 mL Erlenmeyer flask containing 30 mL of yeast extract mineral medium (Skerman, 1959) with 1% mannitol and 0.01% CaCl₂ at a pH of 7.0. The medium was supplemented with different isomer of tryptophan (L-, DL- or D-). The cultures were incubated in a temperature-controlled incubator at 30±2°C with shaking. Bacterial growth was determined using a spectrophotometer (Beckman Coulter, DU 640B) and taking optical density (O D) at 540 nm.

Analytical Assay

Cells were removed from the tryptophan-supplemented culture medium by centrifugation at 10,000xg for 5 min and supernatant was used for IAA estimation following the method of Glickmann and Dessaux (1995). Briefly, 2 mL of Salkowski reagent (4.5 g of FeCl₃ per liter in 10.8 M H₂SO₄) was added to 1 mL of supernatant and the mixture was left in dark for 30 min at room temperature. Development of a pink color indicates IAA production. OD was read at 530 nm by spectrophotometer. Yield of IAA were calculated by using authentic IAA (Sigma, USA) as standard. Nodular tryptophan were extracted and estimated following Nitsch (1955) and Hassan (1975), respectively.

Extraction of Crude IAA

Cell free supernatant was used for IAA extraction according to Ahmad *et al.* (2005). The supernatant was acidified to pH 2.5 to 3.0 with 1 N HCl and extracted twice with ethyl acetate at double the volume of the supernatant. Extracted ethyl acetate fraction was evaporated to dryness in a rotary evaporator at 40°C. The extract was dissolved in methanol and kept at -20°C.

High Performance Thin Layer Chromatography (HPTLC)

On the basis of IAA production level, culture filtrates were used to extract IAA for HPTLC characterization. Methanol dissolved ethyl acetate fractions (10-20 µL) were applied on silica gel TLC plate using an applicator (LINOMAT 5, CAMAG) and developed with the solvent system benzene: n-butanol: acetic acid (70:25:5) (Ahmad *et al.*, 2005). Spots with R_f values were analyzed by high performance thin layer chromatography (CAMAG TLC Scanner 3, Switzerland).

Effect of pH

An overnight bacterial culture was prepared by inoculating in 20 mL YEM broth with an aliquot of bacterial stock and incubating at 30±2°C with 180 rpm, treated as inoculum. The pH was adjusted to different values ranging from 6.5 to 8.0 before introducing the inoculum into the L-tryptophan supplemented medium.

Impact of Carbon and Nitrogen Sources

Different carbon sources were added individually to the L-tryptophan supplemented yeast extract mineral medium except control, to determine the effect of IAA production by the isolate. Impact of nitrogen sources on IAA production was studied by subsequently addition of various nitrogenous chemicals to the tryptophan supplemented basal medium having the most suitable carbon source. Individual effect of each chemical on IAA production was checked colorimetrically.

RESULTS AND DISCUSSION

Based on the morphological, cultural and biochemical characteristics [positive results in catalase assay, indole production, nitrate reduction, congo red absorption but showed negative results in 3-ketolase production and citrate utilization (data not shown)], the isolate was identified as a member of the genus *Rhizobium* and the strain designated as VMA301. The identification of the bacteria was done according to Manual of Microbiological Methods (Conn *et al.*, 1957) and also following Bergey's Manual (Jordan, 1984). Bacteria were grown in YEM broth with different isomers of tryptophan (Fig. 1) and culture without tryptophan used as control. Results indicated that L-tryptophan was more active for IAA production than other tryptophan isomers (D-or DL-) though bacteria were able to produce IAA in absence tryptophan. Dullaart (1970) in research on root nodules from *Lupinus luteus*, postulated L-tryptophan as IAA precursor of rhizobial symbiont. D-amino acids are present in a small amount practically in all living organisms despite of L-amino acids are normally used for protein

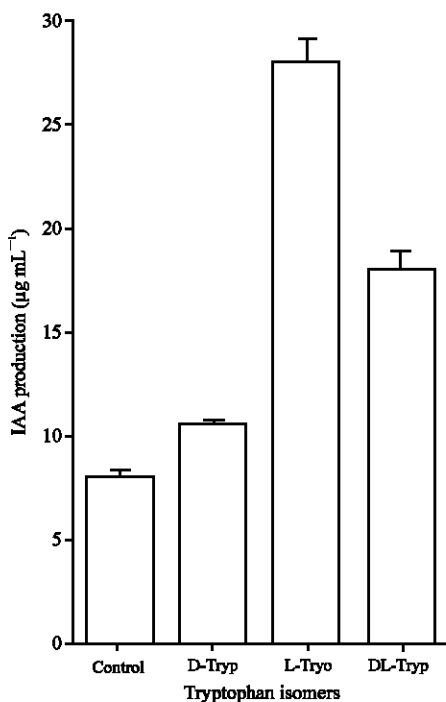


Fig. 1: Effect of tryptophan isomers on IAA production by the strain VMA301 in culture. Bacteria were grown in tryptophan (1 mg mL⁻¹) supplemented YEM medium (pH 7.0) for 24 h at 30±2°C. Control was without tryptophan. Data are the mean of triplicates and bar at the points indicate±SE

biosynthesis and interconversion of D-and L-amino acids is widespread in microorganisms (Rekoslavskaya *et al.*, 1999). In recent, Ahmad *et al.* (2005) mentioned that there are numerous microflora involved in the synthesis of auxin in pure culture in the presence or absence of suitable precursor such as L-tryptophan. L-tryptophan at a 1.5 mg mL⁻¹ concentration was best for IAA production by the isolate, whereas at higher concentration of tryptophan exerts the adverse effects on production (data not shown).

IAA production was found maximum at pH 7.2 (Fig. 2). Acidic and high alkaline pH was not suitable for IAA production. A significant correlation was also observed between bacterial growth and IAA production. pH affect the function of enzyme systems and also affects the solubility of many substances that bacteria need for their proper growth. Earlier reports showed that the synthesis of highest IAA level was determined in cultures grown in alkaline medium at pH 7.5 (Yurekli *et al.*, 2003). The strain VMA301 reached its stationary phase of growth after 28 h in yeast extract mannitol medium. Production of IAA by the strain was started at the beginning of its growth and reached maximum at starting of stationary phase (26 h) (Fig. 3). After that, IAA production started to declined which might due to the release of IAA degrading enzymes like IAA oxidase, peroxidase in the medium by the bacteria (Hunter, 1989).

Among the carbon sources glucose was found the best carbon source for IAA production followed by mannitol (Fig. 4). IAA production was also studied at various concentration of glucose and it was found that maximum production occurred at 1% (w/v) glucose (data not shown). Earlier workers described that IAA synthesis requires depletion of carbon source from the growth medium in batch culture of *Azospirillum brasilense* (Ona *et al.*, 2005) and it relevant to IAA production in *Rhizobium* sp. (Yoshida and Yatazawa, 1973). Nitrogen source is another growth impacting factor for IAA production of the strain. Among the tested nitrogen sources (Fig. 5), KNO₃ was best nitrogen source for IAA production whereas (NH₄)₂SO₄ and NH₄Cl retarded IAA production in respect to control and 0.2% KNO₃ was the optimum for highest IAA production (data not shown). It has been reported that *Rhizobium* species could utilize several nitrogen compounds for their growth,

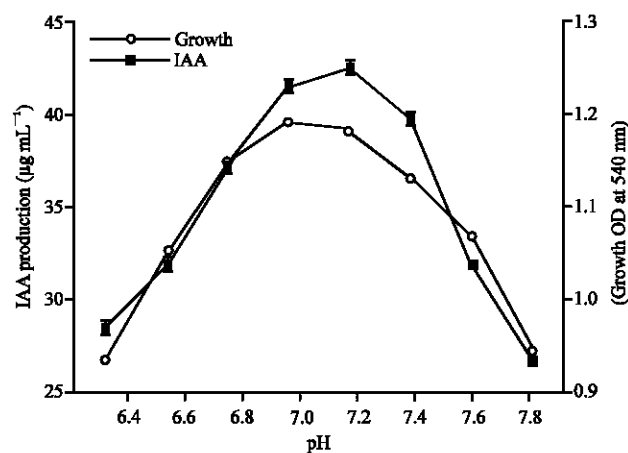


Fig. 2: Effect of pH on IAA production by the strain VMA301 in culture. Bacteria were grown in L-tryptophan (1.5 mg mL⁻¹) supplemented YEM medium with various range (pH adjust using IN HCl/IN Na OH) for 24 h at 30±2°C. Data are the mean of triplicates and bar at the points indicate ±SE

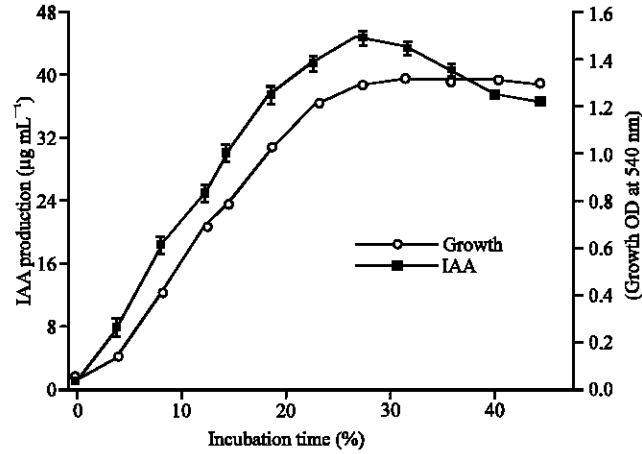


Fig. 3: IAA production with growth of the symbiont VMA301 in culture. Bacteria were grown in L-tryptophan (1.5 mg mL^{-1}) supplemented YEM medium (pH 7.2) at $30 \pm 2^\circ\text{C}$. Data are the mean of triplicates and bar at the points indicate \pm SE

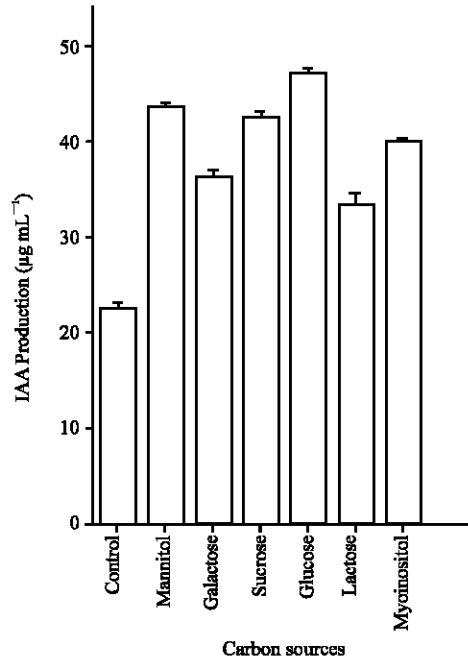


Fig. 4: Effect of nitrogen sources on IAA production with incubation by the symbiont VMA301 in culture. Bacteria were grown in L-tryptophan (1.5 mg mL^{-1}) supplemented yeast extract basal medium containing individual carbon source (1 mg mL^{-1}) except control and incubate for 26 h. Other conditions were same as in Fig. 3

which might be responsible for increased IAA production (Jordan, 1984). The production of IAA was also been confirmed by HPTLC. Chromatograms of culture spots and standard IAA observed under UV light and showed same R_f value 0.88 (Fig. 6).

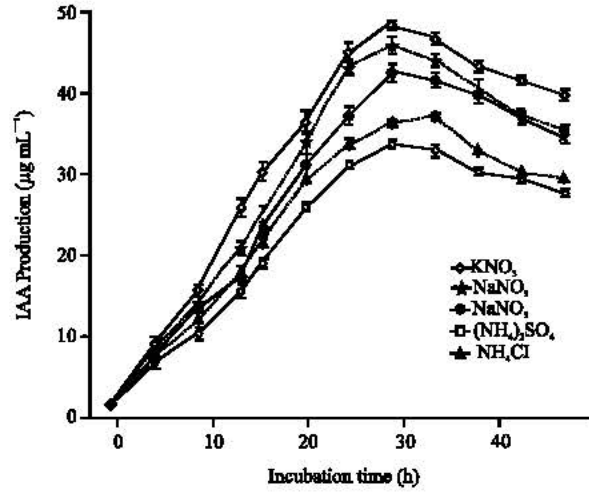


Fig. 5: Effect of nitrogen sources on IAA production with incubation by the symbiont VMA301 in culture. Bacteria were grown in L-tryptophan (1.5 mg mL^{-1}) supplemented yeast extract basal medium continue glucose (1 mg mL^{-1}) and individual nitrogen sources at 0.1% level. Other conditions were same as in Fig. 4

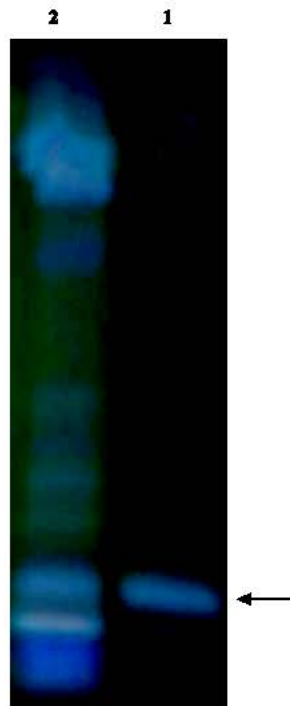


Fig. 6: HPTLC chromatogram of ethyl acetate fraction (lane 2) extracted from bacterial culture supplemented with L-tryptopuan (1.5 mg mL^{-1}) and authentic IAA (lane 1 right side arrow). Others conditions are same as Fig. 3

Table 1: Tryptophan and IAA content in root nodules and non-nodulated normal roots of *V. mungo*

Plant parts ^a	Tryptophan ($\mu\text{g g}^{-1}$ fresh tissue) ^b	IAA ($\mu\text{g g}^{-1}$ fresh tissue) ^b
Root nodules	1568 \pm 22.47	6.34 \pm 0.180
Normal roots	436 \pm 8.12	\leq 2.00

^aPlant parts were used in this study was young and fresh, pulled out after three weeks from seedling. ^bData are the mean of triplicates \pm SE

The root nodules of *V. mungo* contained high amount of tryptophan and IAA than normal roots (Table 1). Isolated bacteria *Rhizobium* VMA301 from the root nodules of the plant has the ability to produced a high amount of indole acetic acid in culture which might correlating for high level of IAA in root nodules. Moreover, the preferred supplements that increased the IAA production in culture might be available in the root nodules. Thus the symbiont enhanced the IAA level in root nodules, which is essential for development. The high level of tryptophan pool in root nodules might serve as IAA precursor. Initially seemed to suggest that rhizobia could provide the hormones that subsequently stimulate nodule formation (Phillips and Torrey, 1972) and IAA, in alone or in conjunction with other plant hormones, might be involved in several stages of the symbiotic relationship (Hunter, 1989). Reports are available on IAA deficient mutant TN 3 of *Bradyrhizobium elkanii* USDA 31 showed less number of nodule on soybean cv. Enrei root which was recovered by the exogenous application of IAA (Fukuhara *et al.*, 1994). The role of auxin in nodulation is tightly linked to the development of other root structures like lateral roots and root primordia. Auxin transport is required for lateral root induction (Bhalerao *et al.*, 2002) and auxin appears to accumulate not only in nodule but also lateral root primordia (Himanen *et al.*, 2002). Thus, the isolate *Rhizobium* sp. VMA301 have the ability to produce higher amount of IAA that might have physiological implications in nodulation to the specific host *V. mungo*.

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