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# Effect of Different Carbon and Nitrogen Sources on Growth and Indole Acetic Acid Production by *Rhizobium* Species Isolated from Cluster Bean [*Cyamopsis tetragonoloba* (L.)]

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***Authors' contributions***

*This work was carried out in collaboration between both authors. Both authors read and approved the final manuscript.*

***Original Research Article***

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## ABSTRACT

Five *Rhizobium* strains (Cb1, Cb2, Cb3, Cb4 and Cb5) were isolated from root nodules of cluster bean *[Cyamopsis tetragonoloba* (L.)] on yeast extract mannitol agar (YMA) medium. All the five *Rhizobium* isolates have shown the Indole Acetic Acid( IAA) production in culture medium supplemented with L-tryptophan. The IAA content in culture supernatant was estimated by using the colorimetric method (13). All the five *Rhizobium* isolates produced maximum amount of IAA in medium supplemented with 2.5mg/ml L-tryptophan concentration. Production of IAA was maximum at 72h of incubation when bacteria reached the stationary phase. The cultural conditions were optimized for maximum IAA production by using different carbon and nitrogen sources as well as changing the incubation period. Glucose and L-asparagine were found to be the best carbon and nitrogen sources, respectively for maximum IAA production. The cell wall affecting agent, penicillin increased the IAA production up to 77.95% in Cb4, 59.52% in Cb5 and 37.84% in Cb3 isolates, over the control. Among the five isolates studied, isolate Cb4 showed better performance in IAA production.

*Keywords: Rhizobium*; *Indole Acetic Acid (IAA); cluster bean*.

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## 1. INTRODUCTION

Application of Plant Growth Promoting Rhizobacteria (PGPR) is one of the important aspects in minimizing the usage of chemical fertilizers, pesticides and other supplements in agriculture. *Rhizobium* species are the best plant growth promoters among rhizobacteria [1], which increase the plant growth and yield either directly or indirectly. Some PGPR strains are able to improve the plant growth and development by interfering with the concentration of known phytohormones like Indole-3-acetic acid (IAA) [2,3]. The IAA helps the plant in root system development which in turn increase the plant nutrition status. Most of the rhizobacteria enable to produce IAA [4]. In order to produce IAA, the associative bacteria use L-tryptophan as precursor. *Rhizobium* species produce maximum amount of IAA in the presence of glucose as carbon source [5]. Nitrogen source L- asparagine caused an increase in IAA production by *Rhizobium* species [6]. It is well known that *Rhizobium* strains isolated from single host species vary in their cultural and biochemical characters [7]. Cluster bean [*Cyamopsis tetragonoloba* (L.)] is a drought tolerant annual legume grown in India and Pakistan [8]. Reports on the plant growth promoting characteristics of *Rhizobium* species isolated from cluster bean are meager. Hence, the present study was aimed at to investigate the IAA production by *Rhizobium* species *is*olated from root nodules of cluster bean and optimization of cultural conditions (incubation time, carbon nitrogen sources, and cell wall affecting agents) for maximum production of IAA.

## 2. MATERIALS AND METHODS

### 2.1 Isolation and Screening of *Rhizobium* Isolates for IAA Production

The fresh, healthy and pink colored nodules were collected from roots of *Cyamopsis tetragonoloba* (L.). Root nodules were surface sterilized with 0.1% (w/v) sodium hypochlorite for 3min and 0.1% mercuric chloride for 30 seconds and washed thoroughly 10 times with sterile distilled water. Bacterial suspension was prepared by taking the surface sterilized nodules in a sterile test tube and crushed with sterile glass rod after adding 1ml of sterile distilled water. A loopful of inoculum was streaked on to yeast mannitol agar medium (YMA) with congo red and petriplates were incubated at $30^0$ C for 3 days [9]. After incubation colonies with white, translucent, convex, mucilaginous and round or irregular margin were sub-cultured onto the same medium for pure culture maintenance. The pure culture isolates were used for this study.

A total of five *Rhizobium* strains were isolated from fresh, healthy root nodules of cluster bean plants raised in soils collected from different parts of Andhra Pradesh, on yeast extract mannitol agar (YMA) medium. These isolates were identified as *Rhizobium* species on the basis of morphological, cultural and biochemical characteristics as per Bergey's Manual of Determinative Bacteriology [10].The nodulation test [9] was also conducted for our isolates to confirm the identity of isolates as *Rhizobium* species. The isolates were designated as Cb1 (Guntur district soil), Cb2 (A.N.U campus soil), Cb3 (East Godavari district soil) Cb4 (Krishna district soil) and Cb5 (Srikakulam district soil). All the bacterial isolates were screened for their IAA production ability by following the method described by Dubey and Maheswari [11].

### 2.2 Estimation of IAA

For IAA production, axenic cultures of bacteria were grown separately in 100ml Erlenmeyer flasks containing 25ml of yeast extract mineral broth (YMB) medium [12] containing 1%

mannitol and L- tryptophan. The bacterial culture medium was incubated in the dark at 30±2ºC for 144h on shaker at 200 rpm. After incubation, the culture samples were withdrawn from flasks for every 24h to measure the growth and IAA. The growth was measured in terms of O.D values by using spectrophotometer at 540 nm. The IAA content was measured by following the standard method of Gordon and Weber [13]. The culture sample withdrawn from the flasks was centrifuged at 8000g for 20 min. then the cell free supernatant was used for IAA determination. To 1ml of this supernatant, 2ml of 2% Salkowsky's reagent (0.5M $FeCl_3$ in 35% Perchloric acid) was added and the absorbance was measured after 25 min of incubation at 530 nm by UV-Visible spectrophotometer. The content of IAA was calculated from the standard graph prepared by using the IAA.

## 2.3 Optimization of Cultural and Nutritional Conditions for IAA Production

### 2.3.1 Determination of L- tryptophan effect on IAA production

To study the effect of different levels of L-tryptophan on IAA production, YMB supplemented with different concentrations (0.1, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/ml) of L-tryptophan, as well as inoculated with *Rhizobium* isolates ($10^9$ cells/ml) separately and incubated at 30±2ºC for 72h. on rotary shaker at 200 rpm. After incubation, IAA was extracted and estimated.

### 2.3.2 Determination of incubation period effect on IAA production

Effect of incubation period (24h to144h) on IAA production was studied by inoculating the five bacterial isolates separately into YMB medium containing L-tryptophan (2.5mg/ml) and incubated at 30±2ºC for 144h on rotary shaker at 200 rpm. Then IAA was determined after incubation.

### 2.3.3 Determination of carbon sources effect on IAA production

Different carbon sources namely, glucose, fructose, lactose, sucrose, maltose, arabinose, xylose, mannitol, inositol, raffinose, starch and cellulose, were added separately at 1% concentration to the basal YMB medium supplemented with L-tryptophan of 2.5mg/ml, replacing mannitol. After 72h of incubation, IAA was estimated.

### 2.3.4 Determination of nitrogen sources effect on IAA production

Different inorganic nitrogen sources (ammonium sulphate, sodium nitrate) and organic nitrogen sources (L-asparagine, aspartic acid, glycine, cysteine, proline, tyrosine and phenylalanine) were added separately at 0.1% concentration to the basal YMB medium containing L-tryptophan (2.5mg/ml), replacing yeast extract. The medium was inoculated with *Rhizobium* isolates and incubated at 30± 2ºC for 72h on rotary shaker at 200 rpm and estimated the IAA content.

### 2.3.5 Determination of cell wall affecting agents effect on IAA production

Different cell wall affecting agents (EDTA, SDS and Penicillin) were added to basal YMB medium supplemented with L-tryptophan to assess the effect of cell wall affecting agents on IAA production by *Rhizobium* isolates. The IAA concentration was measured at the end of 72h incubation.

All experiments were set in triplicate and the data represents the average of three. The data was statistically analyzed using ANOVA (Factorial analysis).

## 3. RESULTS AND DISCUSSION

Isolated bacterial strains were identified as *Rhizobia* on the basis of morphological, cultural and bioochemical characteristics. *Rhizobium* colonies of white, transluscent, mucilaginous, elevated with circular or irregular margin were appeared. The isolates were positive to the biochemical tests characteristic for *Rhizobium* viz., oxidase, citrate utilization, catalse, acid production and nitrate reduction tests. All the five bacterial strains isolated showed the ability of IAA production. All the *Rhizobium* isolates showed maximum IAA production at 2.5mg/ml concentration of L-tryptophan. With an increase in L-tryptophan concentration, the IAA production also increased (Fig. 1). The maximum amount of IAA production at 2.5 mg/ml L-tryptophan was also reported earlier in *Alysicarpus vaginalis* [14], *Dalbergia lanceolaria* [6] and *Sesbania sesban* [15]. Among the *Rhizobium* isolates studied, Cb4 produced maximum (Fig.1) amount of IAA at 2.5mg/ml of L-tryptophan concentration. In the present study, our *Rhizobium* isolates produced higher content of IAA in culture media than in earlier reports [16,17,18]. However, the variations in the IAA production at different concentrations of L-tryptophan by the *Rhizobium* isolates indicate the intrinsic ability of the isolates towards the IAA production. Though the bacteria were able to produce IAA in the absence of L-tryptophan, they produced higher amount of IAA in culture media supplemented with tryptophan [12]. L-tryptophan is generally considered as IAA precursor, because its addition to IAA producing bacterial culture media enhanced an increase in IAA biosynthesis [19]. The production of IAA was much less as compared to the amount of L- tryptophan applied. This was 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 to IAA [20,7]. Maximum of IAA production was recorded with *Rhizobium* isolate Cb4 followed by Cb1 at 72h of growth (Table 1). In our experiment, *Rhizobium* isolates reached stationary phase at 72h of incubation (data not shown). In all the bacterial isolates, the growth as well as IAA production increased up to 72h of incubation and there after gradually decreased. This observation was found to be consistent with earlier studies [18,5,14,6,15]. The decrease in IAA content after the stationary phase (72h) may be due to the release of IAA degrading enzymes such as IAA oxidase and IAA peroxidase as reported earlier [7]. The maximum production of IAA at the stationary phase may be due to more availability of L-tryptophan from the dead cells of bacteria as reported in *Pseudomonas* by Unyayar et al. [21]. ANOVA (Factorial analysis) results revealed that carbon sources, nitrogen sources, cell wall affecting agents and *Rhizobium* isolates and their interactions significantly affected IAA production.

Replacement of mannitol in basal yeast extract mineral broth (YMB) medium with 12 different carbon sources (1.0% each) revealed that *Rhizobium* isolates vary in their carbon utilization capacity and IAA production (Table. 2). Among the 12 carbon sources tested, glucose was found to be the best carbon source for IAA production followed by starch and mannitol. *Rhizobium* isolates viz., Cb4 and Cb1 produced higher amount of IAA (55.8 µg/ml) in the presence of glucose. The maximum production of IAA in the presence of glucose was earlier reported in *Rhizobium* sp. isolated from *Cajanus cajan* [5] and in *Rhizobium leguminosarum* [22]. Maximum amount of IAA production in glucose containing medium may be due to the better utilization of glucose when compared to the other carbon sources. In the present study, carbon sources like fructose, lactose, maltose, raffinose and xylose showed varied levels of IAA production depending on the isolate. Out of the 12 carbon sources

tested, only three carbon sources such as glucose, starch and mannitol stimulated the IAA production over the control in all the isolates.

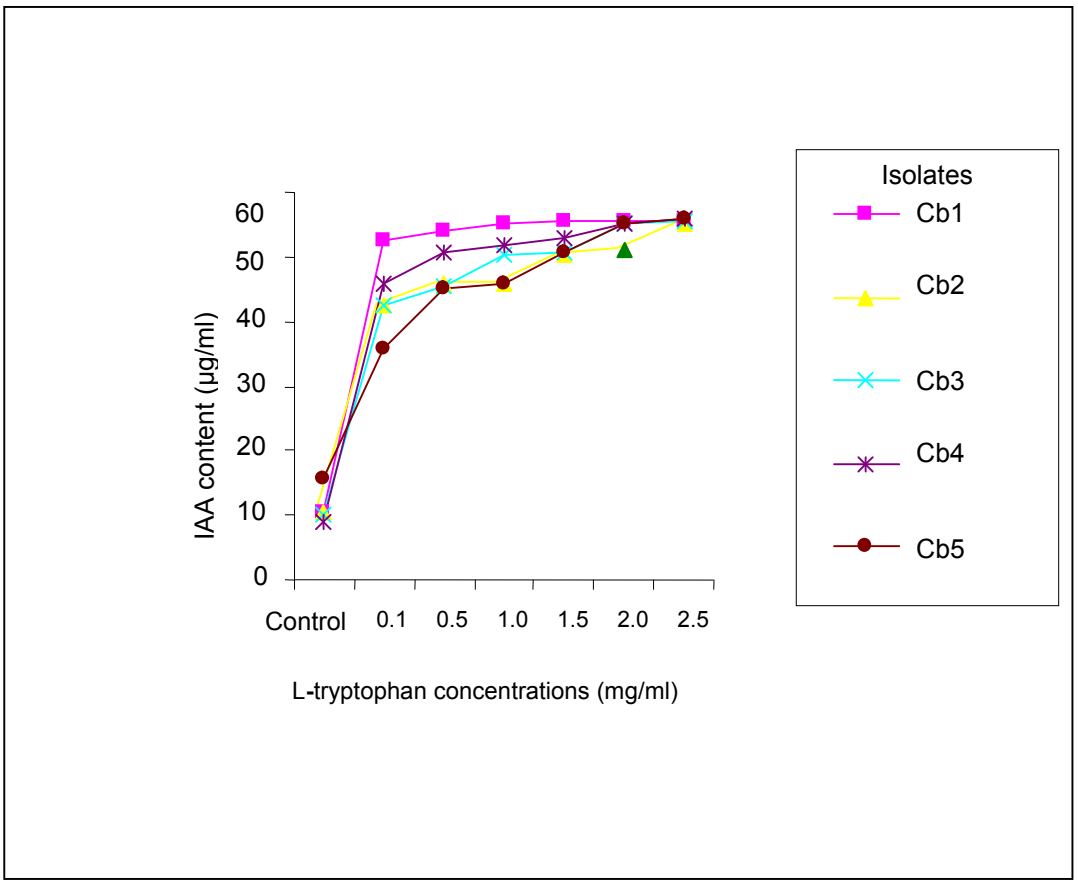


**Fig. 1. Effect of different concentrations of L- tryptophan on IAA production by Rhizobium species associated with cluster bean**

The present data on carbon sources also revealed that the *Rhizobium* isolates, Cb3 and Cb5 efficiently utilized most of the (8 of 12) carbon sources in stimulating the IAA production over the control, when compared to other isolates. Ghosh and Basu [6] reported 1% glucose as the best carbon source for IAA production by symbiont from *Dalbergia lanceolaria*. A similar observation was also made by Bhattacharya and Patil [14] in *Rhizobium* sp. from *Aleysicarpus vaginalis*.

Among the various organic and inorganic nitrogen sources (0.1%) tested (Table.3), L-asparagine was proved to be the best nitrogen source followed by lysine for IAA production by all the five *Rhizobium* isolates.

**Table 1. Effect of incubation period on growth (OD) and IAA (µg/ml) production**

| Isolate name | Incubation period | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 24 hrs | | 48 hrs | | 72 hrs | | 96 hrs | | 120 hrs | | 144 hrs | |
| | OD at 540 nm | IAA | OD at 540 nm | IAA | OD at 540 nm | IAA | OD at 540 nm | IAA | OD at 540 nm | IAA | OD at 540 nm | IAA |
| Cb1 | 0.512 | 10.9 | 0.874 | 20.3 | 1.172 | 55.6 | 1.025 | 40.4 | 0.655 | 30.3 | 0.641 | 20.1 |
| Cb2 | 0.432 | 15.8 | 0.603 | 19 | 1.081 | 52.5 | 0.581 | 25.6 | 0.542 | 15 | 0.47 | 10.6 |
| Cb3 | 0.761 | 15.6 | 0.919 | 20.3 | 1.286 | 52.4 | 1.073 | 25.3 | 0.962 | 20.5 | 0.422 | 10.9 |
| Cb4 | 0.759 | 20.6 | 0.866 | 25.4 | 1.307 | 55.8 | 1.148 | 30.6 | 0.761 | 25.4 | 0.347 | 15.2 |
| Cb5 | 0.781 | 15.3 | 0.912 | 20.6 | 1.605 | 35.6 | 0.972 | 30.3 | 0.781 | 21.7 | 0.523 | 15.4 |

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

| Carbon source (1%) | Name of the isolate | | | | |
|---|---|---|---|---|---|
| | Cb1 | Cb2 | Cb3 | Cb4 | Cb5 |
| | | (IAA | µg/ml) | | |
| Control** | 20.3 | 22.0 | 18.0 | 20.8 | 18.4 |
| Glucose | 55.6 | 52.5 | 52.4 | 55.8 | 35.6 |
| Fructose | 20.5 | 20.9 | 15.9 | 20.6 | 20.0 |
| Xylose | 15.6 | 10.0 | 24.0 | 10.1 | 20.1 |
| Arabinose | 16.1 | 18.0 | - | 25.4 | 10.0 |
| Maltose | 33.2 | 35.6 | 20.4 | 20.3 | 12.3 |
| Lactose | 15.9 | 15.5 | 19.2 | 20.3 | 20.0 |
| Sucrose | 25.1 | 15.4 | - | 15.2 | - |
| Mannitol | 25.7 | 32.2 | 41.1 | 25.4 | 25.2 |
| Inosital | 15.5 | - | - | 15.8 | 15.4 |
| Raffinose | 10.0 | 5.0 | 40.3 | 50.6 | 19.0 |
| Starch | 50.9 | 45.3 | 50.6 | 55.2 | 30.2 |
| Cellulose | - | - | 30.3 | 20.0 | 25.6 |

** Significant at 1% (between carbon sources: Fc=1751.9, Ft=1.75, between Rhizobium species: Fc =267.04,Ft=2.37*

**Table 3. Effect of nitrogen sources on IAA production by *Rhizobium* isolates**

| Nitrogen source (0.1%) | Name of the isolate | | | | |
|---|---|---|---|---|---|
| | Cb1 | Cb2 | Cb3 | Cb4 | Cb5 |
| | | (IAA | µg/ml) | | |
| Control | 8.0 | 6.0 | 4.0 | 8.4 | 7.2 |
| Ammonium sulphate | 10.8 | - | - | - | 9.0 |
| Sodium nitrate | 9.0 | 15.0 | 6.8 | 7.2 | 14.9 |
| L-Asparagine | 25.7 | 32.8 | 21.5 | 27.0 | 26.4 |
| Cysteine | 20.3 | 25.9 | 20.6 | 15.6 | 5.0 |
| Lysine | 20.9 | 30.8 | 7.0 | 25.0 | 20.4 |
| Glycine | 20.6 | 10.0 | - | 20.3 | 8.0 |
| Tyrosine | - | - | - | 25.8 | 25.4 |
| Proline | 23.0 | 28.0 | 10.6 | 14.0 | 17.9 |
| Aspartic acid | 21.4 | 22.0 | 20.5 | 17.6 | 10.8 |
| Phenyl alanine | 9.0 | 14.9 | 6.5 | 11.8 | 15.0 |

**Significant at 1% (between nitrogen sources: Fc=839.86, Ft= 1.98; between Rhizobium species: Fc=401.96, Ft=2.49)*

L-asparagine was also reported as effective nitrogen source for IAA production in *Rhizobium* sp. isolated from *Cajanus cajan* [23], in *Rhizobium* isolates from different legumes [24] Among the five isolates studied, isolate Cb2 produced highest amount (32.8µg/ml) of IAA in the presence of L- asparagine, which is 46% over the control. Besides L-asparagine and lysine, other nitrogen sources namely proline, aspartic acid and phenylalanine also induced the IAA production by isolates over the control. Amino acid cysteine supplementation resulted in increase of IAA production over the control by all isolates studied, except Cb5. whereas, tyrosine and ammonium sulphate retarded the IAA production in some isolates. Inhibition of IAA production by some amino acids in *Rhizobium meliloti* was also reported earlier [25]. It has been reported that *Rhizobium* isolates could utilize several nitrogen sources for growth, which might be responsible for increased IAA production [26,9].

In the present study, the effect of cell wall affecting agents on IAA production varied with the *Rhizobium* isolates (Table 4).

**Table 4. Effect of cell wall affecting agents on IAA production by *Rhizobium* isolates**

| Cell wall affecting agent | Name of the isolate | | | | |
|---|---|---|---|---|---|
| | Cb1 | Cb2 | Cb3 | Cb4 | Cb5 |
| | | (IAA | µg/ml) | | |
| Control | 25.7 | 33.2 | 41.1 | 25.4 | 25.2 |
| EDTA | - | 15.6 | 25.2 | 25.6 | 25.4 |
| SDS | 5.0 | 30.3 | 35.8 | 25.9 | - |
| Penicillin | 25.4 | 45.1 | 30.3 | 45.2 | 40.1 |

**Significant at 1% (between cell wall affecting agents: Fc=7482.72, Ft=2.86, between Rhizobium species: Fc= 4559.59, Ft=2.63)*

Among the cell wall affecting agents, penicillin increased IAA production up to 77.95%, 59.52% and 35.84% in Cb4, Cb5 and Cb2 *Rhizobium* isolates, respectively over the control. The cell wall affecting agents being as surfactants change the integrity of the cell wall or cell membrane to increase the availability of tryptophan to convert as well as to increase the release of IAA from the cell [17].

## 4. CONCLUSION

From the present study, it can be concluded that all the *Rhizobium* isolates studied were able to produce maximum amount of IAA, in the presence of L-tryptophan (2.5mg/ml conc.) during 72h after inoculation. Glucose and L-asparagine supported maximum IAA production in all the *Rhizobium* isolates, as carbon and nitrogen sources, respectively. Among the cell wall affecting agents, penicillin induced maximum IAA production by Cb2, Cb4 and Cb5 *Rhizobium* isolates studied. As the *Rhizobium* isolates of our present study are potential for IAA production, it needs further study on these isolates to explore the chances for commercial production of IAA.

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## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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