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Screening of rhizobia for indole acetic acid production

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ABSTRACT

The phytohormone auxins play a central role in plant growth and development as a regulator of numerous biological processes, from cell division, elongation and differentiation to tropic responses, fruit development and senescence. Auxins are employed to induce rooting, callus formation, flowering, parthenocarpy and so on. They can also prevent abscission of leaves, flowers and fruits. The action and interaction of some growth regulators like auxins regulate most of the physiological activities and growth in plants. Naturally occurring substances with indole nucleus possessing growth-promoting activity are referred to as auxins, chemically it is Indole acetic acid.(IAA) Not only plants but also microorganisms can synthesize auxins and cytokinins. The ability to synthesize phytohormone is widely distributed among plant-associated bacteria. 80% of the bacteria isolated from plant rhizosphere produce IAA. Many bacteria are known to produce IAA. Hence the present paper discusses isolation of rhizobia from the selected leguminous plants and its IAA production ability under laboratory conditions.

Key Words: Auxins, IAA, Rhizobia.

INTRODUCTION

As plant roots grow through soil they release water-soluble compounds such as amino acids, sugars and organic acids that supply food for the microorganisms. In return, the microorganisms provide nutrients for the plants. All this activity makes the rhizosphere the most dynamic environment in soil. Because roots are underground rhizosphere activity has been largely overlooked. The rhizosphere is a center of intense biological activity due to the food supply provided by the root exudates.

However, there are some microorganisms that do interact with specific plants. These interactions can be pathogenic, symbiotic, harmful, saprophytic or neutral. Interactions that are beneficial to agriculture include mycorrhizae, legume nodulation and production of antimicrobials compounds that inhibit the growth of pathogens. Rhizosphere microorganisms produce vitamins, antibiotics,

plant hormones and communication molecules that all encourage plant growth. Microbial population in rhizosphere may benefit the plant in a variety of ways including increased recycling and solubilization of mineral nutrients, synthesis of vitamins, amino acids, auxins, cytokinins and gibberellins which stimulate plant growth and antagonism with potential plant pathogens through competition and development of amensal relationships based on production of antibiotics.

The action and interaction of some growth regulators like auxins regulate most of the physiological activities and growth in plants. Naturally occurring substances with indole nucleus possessing growth-promoting activity are referred to as auxins. Chemically it is Indole acetic acid. The ability to synthesize phytohormone is widely distributed among plant associated bacteria. 80% of the bacteria isolated from plant rhizosphere are to produce IAA. [1] According to Halda-Alija [2], up to 74% of rhizobacteria identified and tested to produce IAA.

The plant growth regulator indole acetic acid (IAA) has long been postulated to play a role in one or more aspects of nodule growth and development and the detection of increased levels of IAA in nodule tissue supports this hypothesis [3]. The associative nitrogen-fixing bacteria tested produced IAA, especially with tryptophan as a precursor [4]

The phytohormone auxins plays a central role in plant growth and development as a regulator of numerous biological processes, from cell division, elongation and differentiation to tropic responses, fruit development and senescence [5]. Not only plants but also microorganisms can synthesize auxins and cytokinins. The role of phytohormone biosynthesis by microorganisms is not fully elucidated. But it was indicated that there might exist a symbiotic association between plants and microorganisms.

Hence the present study was undertaken to isolate organisms from nodules of leguminous plants and its rhizosphere and to study IAA production under laboratory condition.

MATERIALS AND METHODS

2.1 Isolation of *Rhizobium* species:

Leguminous plants selected for the present study are: Groundnut i.e. *Arachis hypogaea* L., Chickpea i.e. *Cicer arietinum* L., Fenugreek i.e. *Trigonella foenumgraecum* L., Lucerne i.e. *Medicago sativa* L. For isolation of Rhizobia from root nodules of leguminous plants following method was used: Healthy root nodules were selected, washed with tap water and surface sterilized by 1: 1000 $HgCl_2$, washed with sterile distilled water several times. These nodules were crushed in a drop of sterile water, inoculated on sterile yeast extract mannitol agar with Congo red [6-8]. Plates were incubated at room temperature for 48 hrs. Typical rhizobial colonies on YEMA were opaque, white and mucoid. Colony characters of a well-isolated colony were studied. Gram staining and motility was carried out. Isolates were identified as per Bergey's manual of Systematic bacteriology

2.2 Screening for IAA production:

Both isolated organisms identified as *Rhizobium* sps. And organisms isolated from rhizosphere were screened for their ability to produce IAA [9]

Each inoculated plate was overlaid with nylon 6'6' membrane. Plates are incubated until colonies reached 0.5 to 2mm in diameter. After an appropriate incubation period the membrane was treated with salkowaski reagent [10] prepared as 2% 0.5 M $FeCl_3$ in 35% perchloric acid.

Reaction was allowed to proceed until adequate colour developed. All reagent incubations were carried out at room temperature. Bacteria producing IAA were identified by the formation of a characteristic red halo within the membrane immediately surrounding the colony. Known concentrations of IAA were also used to check the extent of red halo formed and also for the comparison for their ability to produce IAA.[11]

2.3 Preparation of standard graph of IAA:

Standard graph of IAA was prepared as mentioned by [10, 12] Different IAA concentrations are prepared as aqueous solution of IAA ranging from 10 microgram/ml to 100 micrograms/ml. To each 1 ml of the standard, 2ml of 2% 0.5 M FeCl_3 in 35% perchloric acid i.e. Salkowski reagent is added and readings are taken after 25 minutes at 530 nm by UV-Visible spectrophotometer SL Elico 159. Standard graph is prepared by plotting concentration of IAA in micrograms/ml Vs Optical Density at 530 nm.

2.4 Confirmation of IAA by using TLC:

Selected 4 rhizobial cultures were inoculated in YEMB amended with 5mM tryptophan. 1% inoculum of O.D.₆₀₀ 1.0 was used for inoculation. The inoculated broth was incubated at 28°C for 24 hrs. After 24 hrs of incubation, broth was centrifuged at 7000 rpm for 10 minutes. pH of broth brought to 3.0. 4:1 aliquots of liquid portion of centrifuged sample were extracted three times with ethyl acetate. The organic phase was concentrated to dryness and then diluted with 0.5 ml methanol. This solution along with the standard IAA was applied on silica gel G plate and TLC was run by using a solvent system chloroform: Ethyl acetate: Formic acid in 5:3:2 proportion and developed by using Salkowski reagent. Red colour spots were developed. R_f value of the standard and IAA produced by the selected isolates was calculated. [13, 14]

2.5 Effect of tryptophan concentration:

To check the effect of tryptophan on IAA production, YEMB amended with 1- 5mg/ml, as well as 5mM were inoculated with the selected isolates as 1% inoculum of O.D.₆₀₀ 1.0 and incubated at 28°C for 24 hrs. After incubation the broth was centrifuged at 7000rpm for 10 minutes. Supernatant was collected. To 1ml supernatant, 2ml of Salkowski reagent was added and extent of red colour i.e. IAA produced was measured spectrophotometrically at 530 nm.[15,16]

2.6 Effect of Carbon sources on IAA production:

IAA production was studied by replacing mannitol from YEMB by glucose, sucrose, lactose, Arabinose, Xylose and mannitol 1% w/v. supplemented with 2.5 mg/ml of tryptophan. IAA production was studied by using Salkowski reagent after 24, 48 and 72 hrs. Cultures selected for the study are RG and RH i.e. *Rhizobium* isolated from groundnut and chickpea respectively.

2.7 Effect of pH on IAA production:

To study the extent of IAA produced by the different isolates at different pH, YEMB with 2.5mg/ml of tryptophan is adjusted to different pH as 5, 6, 7, 8 and 9. Media were inoculated with 1% inoculum of O.D.₆₀₀ 1.0 and incubated at 28°C for 24 hrs. IAA production was studied by using Salkowski reagent after 24hrs.[17]

RESULTS AND DISCUSSION

3.1 Isolation and identification of Rhizobium:

White colored, mucoid, and like a drop of water colonies observed on YEMA with congo red were the characteristics of *Rhizobium* sp.. The representative colony used for further

biochemical chracterization also reflected the similar biochemical characteristics to that of *Rhizobium* sp.

From the results of morphological, cultural and biochemical characters and the host from which it was isolated, the isolates were identified as *Rhizobium leguminosarum* from groundnut, *Rhizobium loti* from chickpea, *Rhizobium meliloti* from Lucerne, *Rhizobium meliloti* from Fenugreek(Trigonella), abbreviated as RG, R H, RL and RT respectively.(**Table 1**)

Table 1: Results of Biochemicals

CHARACTERISTICS	RG	RH	RL	RT
Root nodules produced	+	+	+	+
Fast growth on YEMA	+	+	+	+
Growth at 39-40 ⁰ C	-	-	+	+
Growth in presence of 2% NaCl	-	-	-	+
H ₂ S production	-	-	-	+
Growth at PH				
3.5	-	-	-	-
4.0	-	-	-	-
4.5	-	-	-	-
5.0	+	+	-	-
8.0	+	+	+	+
9.0	+	+	+	+
9.5	-	+	+	+
Alkaline phosphate activity	+	+	+	+
Starch hydrolysis	-	+	-	-
Casein hydrolysis	-	-	-	-
Utilization of sugars				
Glucose	+	+	-	+
Sucrose	+	+	-	+
Lactose	-	+	-	-
Maltose	+	+	-	+
Mannitol	+	+	-	+
Xylose	+	+	+	+
Fructose	+	+	+	+
Rhmnose	+	+	+	+
Arabinose	+	+	+	+
Raffinose	+	+	+	+
Dulcitol	+	+	+	+
Antibiotic sensitivity				
Penicillin	+	+	-	-
Streptomycin	+	+	+	+
Tetracycline	+	+	+	+

RG—*Rhizobium* isolated from groundnut

RH-- *Rhizobium* isolated from Chick Pea

RL--*Rhizobium* isolated from Lucerne

RT-- *Rhizobium* isolated from Trigonella

3.2 Screening of IAA production:

Both isolated organisms identified as *Rhizobium* sps. and colonies obtained from rhizosphere soil were screened for their ability to produce IAA. Colour reaction of various isolates of *Rhizobia* with Salkowaski reagent resulted in the appearance of red colour. All isolated rhizobia showed red colour reaction with salkowaski reagent indicating their ability to produce IAA.

Out of 60 colonies selected from rhizosphere of groundnut, 40 colonies from rhizosphere of groundnut and 22 colonies from rhizosphere of Lucerne showed red colour with Salkowaski reagent on nylon 6'6' membrane indicating production of IAA by the organisms. Salkowaski reagent method was highly sensitive which can detect 50 pmoles of IAA. Among the rhizosphere soil, dominant IAA producers were identified as *Pseudomonas*, *Azotobacter* and *Bacillus* sps.

Comparison between the isolated Rhizobia and their rhizosphere soil organisms for their ability to produce IAA, it was observed that isolated Rhizobia from root nodules good producers of IAA than the remaining organisms. (Fig 1)



Fig 1: Screening on Nylon 6'6' membrane From left to right RG, RH, RL, RT.

3.3 Standard graph of IAA:

Straight-line graph indicates direct proportion between concentrations of IAA and the extent of red colour developed. R^2 value of the graph was found to be 0.9724 that showed the validity of the graph. Rf values of the standard IAA produced and IAA produced by the selected isolates showed same value. Hence production of IAA by the organisms was confirmed (Fig 2).

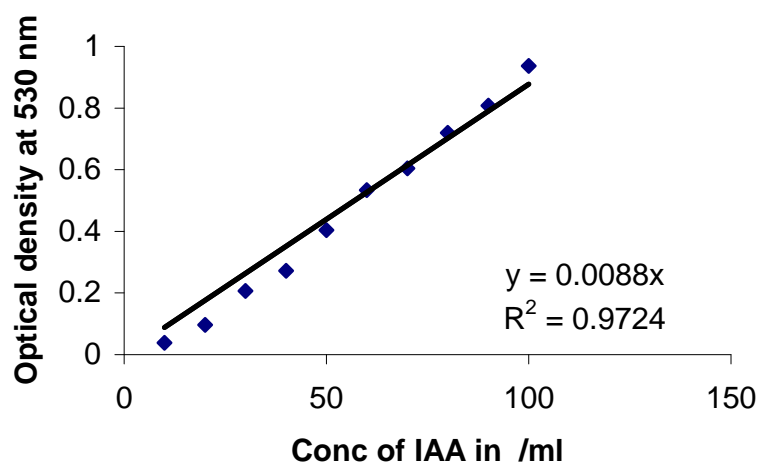


Fig 2 Standard graph of IAA

3.4 Confirmation of IAA by TLC: Silica gel thin layer chromatography (TLC) was found to be a powerful technique in purification, separation and possible identification of natural and synthetic indole derivatives.[13] separation showed R_f value of standard IAA 0.59 The same R_f value was obtained from IAA produced by the isolates. Standard IAA showed R_f value of .57. [13]

3.5 Effect of tryptophan concentration:

Most of the organisms produce IAA in presence of tryptophan. In present study it was observed that as the concentration of tryptophan in the medium increases, the amount of IAA produced increased. Both *Rhizobium leguminosarum* and *Rhizobium loti* was found to be the efficient producer of IAA amongst all the isolates. Using 5mM concentration of tryptophan in the medium, IAA production within 24 hrs was found to be 313.63µg/ml, 319.31 µg/ml, 242.04 µg/ml and 198.86 µg/ml respectively by RG, RH, RL and RT. (Fig 3) IAA production was found to be decreased in 72 hrs. This may be due to its degradation by the isolate. Under variable tryptophan concentration, *Rhizobium leguminosarum* (RG), *R. loti* (RH), *R. meliloti* from Trigonella i.e. Fenugreek showed proportionate increase in IAA production with increased tryptophan concentration. *R. meliloti* from Lucerne showed maximum production at 3mg/ml of tryptophan concentration after which amount of IAA produced decreased. *R. loti* gave graded response to tryptophan concentration. The same organism showed maximum IAA production at all concentrations. No organism showed IAA production in absence of tryptophan, which concludes the requirement of tryptophan as a precursor for the synthesis of IAA. Various researchers reported variable IAA production ability of bacteria. Yasmin S. et al reported 20-90.8mg/L of IAA where as *Azotobacter chroococcum*, *Azotobacter vinelandii*, *Pseudomonas aeruginosa*, *Pseudomonas putida*, *Serratia* sp. And *Klebsiella pneumoniae* strains shown to produce IAA from 3.5 mg/ml to 32.2mg/ml.[13] *Azospirillum brasilense* produced 26.1µg/ml of IAA. [18] The bacteria produced a high amount(107 microg/ml) of IAA in culture from tryptophan supplemented yeast extract mannitol medium. [19] IAA production reached maximum using 3mg/ml of L- Tryptophan.[20] Selected cultures showed much higher IAA production than reported.

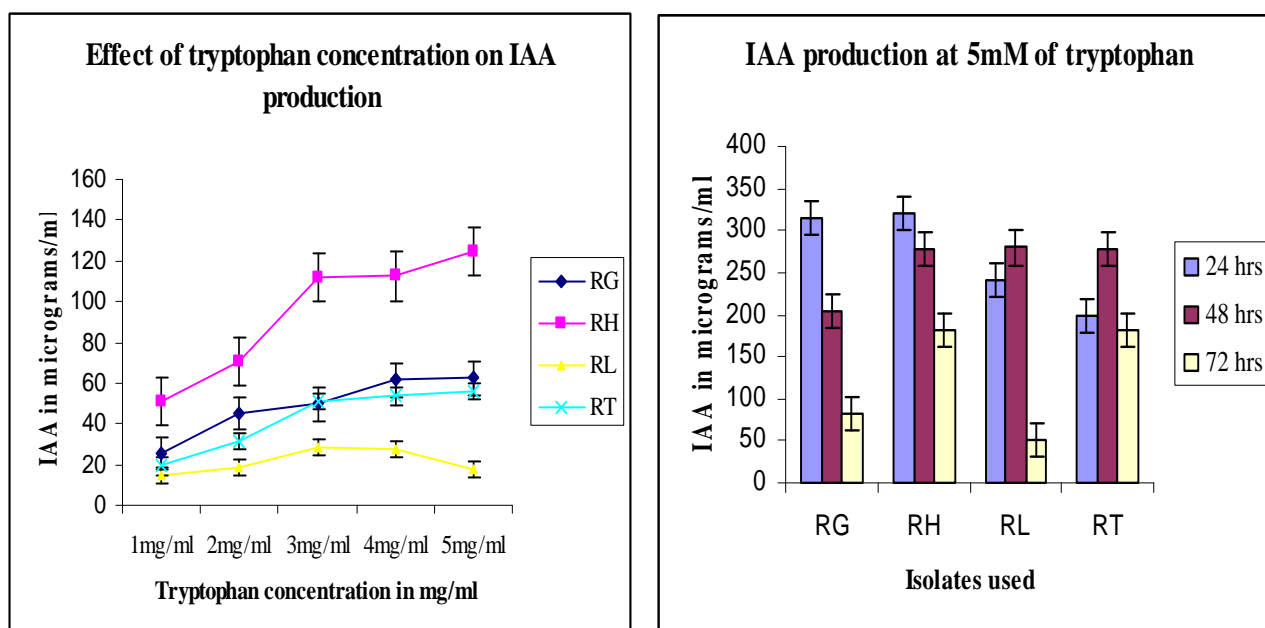


Fig 3: Effect of tryptophan concentration on IAA production

3.6 Effect of Carbon sources on IAA production:

For finding out the most favourable carbon source giving maximum IAA production mannitol from YEMB is replaced by different sugars. Rhizobial isolates responded in varied manner to different carbon sources. *Rhizobium leguminosarum* and *R. loti* showed maximum IAA production in presence of lactose, mannitol in 48 hrs. In 24 hrs *Rhizobium leguminosarum* gave maximum production of IAA in presence of glucose. For the study of effect of carbon source on IAA production *R. leguminosarum* (RG) & *Rhizobium loti* (RL) is selected as these organisms are found to be the efficient producers amongst all. *R. leguminosarum* shows maximum IAA production in presence of glucose followed by sucrose, mannitol, Arabinose, Xylose and lactose in descending order. Basu and Ghosh reported 1% glucose as a preferred carbon source for IAA production. *Rhizobium loti* uses mannitol as the best carbon source as it gives maximum IAA production in presence of mannitol as compared to all carbon sources (Fig 4 A & B)

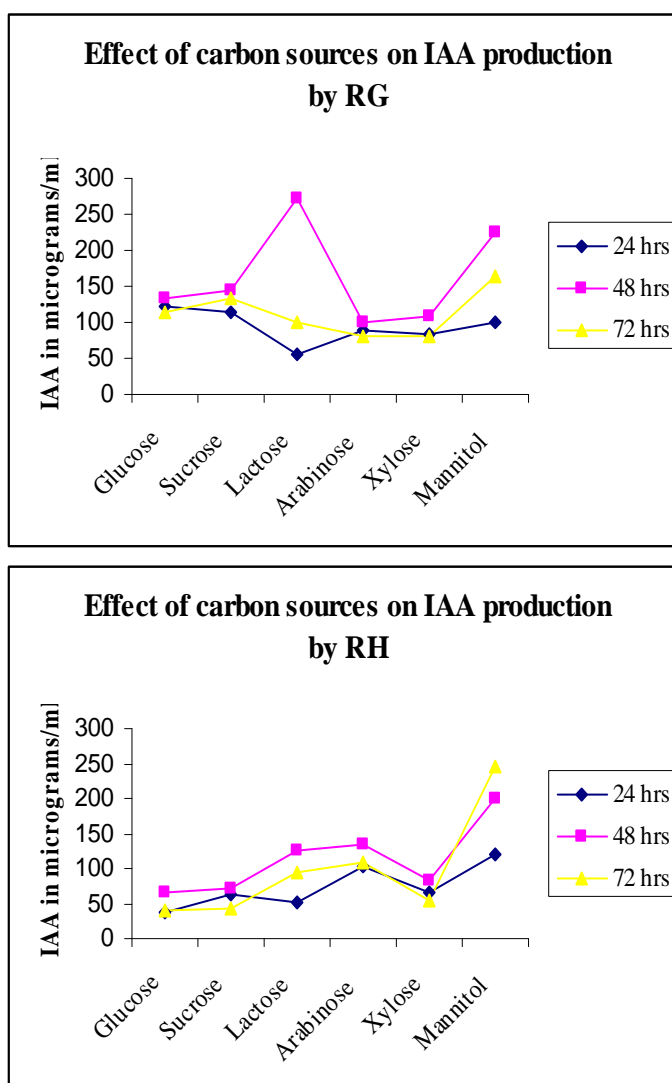


Fig 4 [A] and [B] Effect of carbon sources on IAA production

3.7 Effect of pH on IAA production:

To decide the optimum pH for IAA production, the isolates are inoculated in YEMB amended with 2.5 mg/ml of tryptophan having different pH such as 5, 6, 7, 8, 9. (Fig 5) All rhizobia showed no or little amount of IAA production at pH 5 whereas maximum production found at pH 7. IAA production decreased at pH 9. *Pantoea agglomerans* produced maximum IAA

production at pH 7. [21] *Rhizobium leguminosarum* and *R.meliloti* from Lucerne did not show IAA production at pH 5 and 6 respectively. All isolates showed maximum IAA production at pH 7 except *R.loti*, which showed peak at pH 8. Hence optimum pH for IAA production was found to be 7

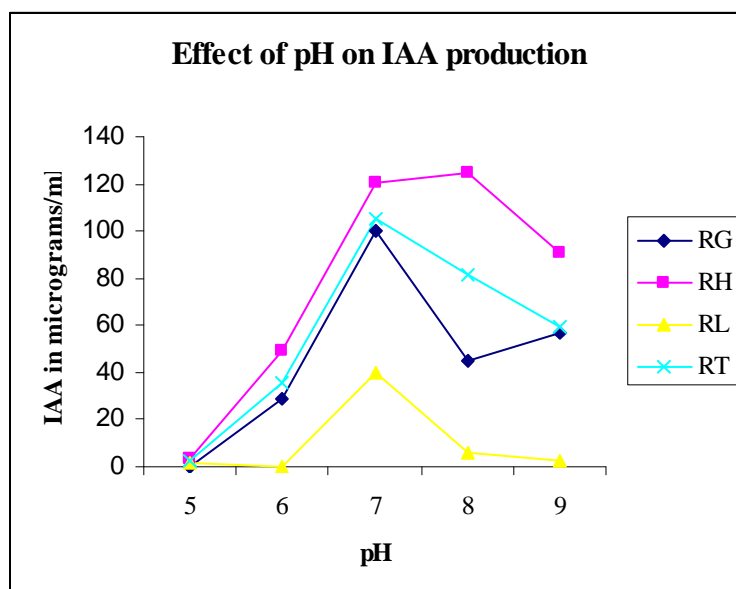


Fig 5: Effect of pH on IAA production

CONCLUSION

The isolated organisms were identified as *Rhizobium leguminosarum* from groundnut, *Rhizobium loti* from chickpea, *Rhizobium meliloti* from Lucerne, *Rhizobium meliloti* from Fenugreek. It could be concluded that the IAA produced by the organisms could be used as sprays for plant growth promotion. Out of the selected Rhizobia, *R.loti* was found to be the best to produce IAA. Co- inoculation of rhizobia with other plant growth promoting bacteria can be done for growth promotion. All the isolates produce only IAA in the culture medium and no other interfering substance. *Rhizobium loti* showed maximum IAA production at all concentrations of Tryptophan. No organism produced IAA in absence of Tryptophan. Optimum PH for IAA production was found to be 7.

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