Bulletin of Environment, Pharmacology and Life Sciences
Bull. Env. Pharmacol. Life Sci., Spl Issue [1] January 2023 : 163-165.

Online ISSN 2277-1808
Journal's URL:http://www.bepls.com
CODEN: BEPLAD

ORIGINAL ARTICLE **OPEN ACCESS**

PRODUCTION OF INDOLE ACETIC ACID IN TRANSPOSON INDUCED MUTANTS OF *RHIZOBIUM JAPONICUM* INFECTING *VIGNA RADIATA*

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ABSTRACT
In legume- Rhizobium symbiosis, both partners secrete several compounds which play an essential role in establishing successful symbiosis relationship. In this relationship rhizobia get encountered by many plant compounds such as phytohormone like Auxins. Many rhizoble strains are able to produce Auxins like indole-3 acetic- acid (IAA) but there exact role in symbiosis between legume plant & Rhizobium is still not very well known. In this view present work was undertaken with generation mutant library of 800 mutants of Rhizobium japonicum by transposon mutagenesis. The mutants show higher nitrogenase activity which also showed higher IAA production, clearly indicates that IAA has a distinct role in Rhizobium- Legume symbiosis.
***Key Words**: Rhizobium, Transposon mutagensis, IAA*

Received 12.10.2022 Revised 23.11.2022 Accepted 27.12.2022

INTRODUCTION

The symbiosis between Legume & *Rhizobium* involves coordination of signals between both partners in which early step is secretion of flavonoids by plants which are released via root exudates are detected by rhizobia via a membrane associated Nod D protein encoding synthesis of nod factors [1] Plant triggers nod factor to initiate nodule formation with induction of new meristems. Dividing bacteria penetrates the infection thread & gets surrounded by sacs of plant derived membrane in which bacteria differentiates into bacterios which converts atmospheric nitrogen into ammonia [2, 3]. Many rhizobial strains produces IAA [4]. The exact role of IAA in nodule function & formation is still not very well known but there are some publication which light on the role of Auxin in nodule formation [5]. IAA acts as signalling molecule in both IAA non-producing & producing species [1]. To detect role of IAA in nodulation this study was undertaken to produce library of mutants with variety of IAA production. The results of nitrogenase activity, nodulation compare with IAA production.

MATERIAL AND METHODS

Bacteria and growth Conditions:

Organism was isolated from locally grown root nodule of *Vigna radiata.* The isolate was identified by morphological, biochemical and physiological characteristics according to [6]. Pure cultures were maintained on Yeast Extract Mannitol Agar (YEMA) slants at 4^0C and frozen in 50% glycerol at -80^0C. Tn3 suicide vector used here was from Bangalore GeNei, India. *E.coli* was grown on Luria Bertani (LB) medium with antibiotics ampicillin (100 µg/mL), chloramphenicol (20 µg/ml) at temperature 37^0C. The minimal basal salts (M9) medium used for screening of transconjugants was supplemented with rifampicin (100 µg/ml) and ampicillin (100 µg/mL) with pH 7.2. The antibiotics and chemicals obtained from SRL Pvt. Ltd. (India).

Bacterial Mating:

The spontaneous rifampicin resistant mutants of *Rhizobium japonicum* were produced by streaking wild type culture suspension of 2.8 x 10^6 on YEMA medium containing rifampicin (100 µg/mL) and were maintained on the same medium containing rifampicin (25µg/mL).Mating between *Rhizobium japonicum* and *E.coli* was conducted by method of [7]. Donor cells were harvested by centrifugation at the late

exponential growth phase and recipient cells were harvested at stationary phase. Both the donor and recipient cells were washed three times with10 mM $MgSO_4$ and then mixed at a donor-to-recipient ratio of 1:1. Here donor cell is *E.coli* and recipient cell is *Rhizobium japonicum.* Then 0.1 mL of cell suspension was transferred to a filter membrane (0.45 µm pore size; 25-mm in diameter) placed on mating medium. Controls were kept as individual filtered portions of the above cells, after incubation at 8 hrs at 30^0C; the filter was transferred to 2.0 mL of L.B medium, diluted and plated on appropriate selective plates.

Nodulation assay:

The seeds of latika variety of mung bean were obtained and surface sterilized with 3% sodium hypochlorite as described by [6]. Three seeds each placed in 800 pots and overnight cultures of wild and mutant's strains were used for inoculation. Sterilized soil and water used for nodulation assay, after 30 days nodulation were observed in these pots.

Estimation of nitrogenase activity:

The measurement of nitrogenase activity was carried out at Vasantdada Sugar Institute, Manjri, Br. Pune, India by using the acetylene reduction method as described by [6]. and analyzed by Perkin Elmer Gas chromatography (with dual porpak N column of 2.0 M length) with standard flame ionization detector.

Stastical Analysis:

Results were expressed as mean ± SD for three replicates are significant at $p<0.05$; values followed by the same letter in column are not significantly different at 5% level of probability according to Duncan multiple range test

IIA Production:

The IAA production was quantified by using method of [8]. One mL strain was inoculated in Yeast Extract Mannitol Broth (YMB)+ 5mM tryptophan at 28^0C for 3-4 days . The bacterial suspension was centrifuged at 10,000 x g for 20 min. One mL of supernatant was mixed with 2 drops of phosphoric acid & 4 mL of salkowskis reagent & incubated at room temperature for 25 min. The pink auxin complex developed was read at 530 nm in spectrophotometer. The quantity of auxin in the cultures was estimated from a calibration curve using standard IAA values in µg/mL.

RESULTS AND DISCUSSION:

Rhizobium japonicum AVR was successfully isolated from root nodules of mung bean plant and identification of same was done by physiological, morphological and biochemical characteristics. *Rhizobium japonicum*AVR1 a spontaneous rifampicin resistant mutant was grown on YEMA medium containing 100 µg/ml rifampicin this mutant nodulates mung bean plant as wild variety. *Rhizobium japonicum* AVR 1 formed colonies of 800 mutants by using transposon Tn3 on M9 medium with antibiotics rifampicin (100 µg/ml) and ampicillin (100 µg/ml). The transposition frequencies obtained in mating was 3.7×10^{-6}. These 800 mutants were subjected for nodulation assay. Seeds inoculated with control inoculum showed no nodulation on root system of plant. Out of 800 mutants screened 112 gave white coloured nodules, 80 gave no nodulation , 508 showed poor nodulation and 100 mutants formed pink coloured nodules on root system of mung bean plant. Among these 100 mutants, 10 mutants were selected on the basis of nodule number, fresh and dry weight of nodules and subjected for nitrogenase activity. Mutant *Rhizobium japonicum* AVR063 was depicted significant growth of nodule along with more plant biomass when compared with wild, control and other mutant inoculated plant. The maximum nitrogenase activity has been recorded in isolate AVR 063: : Tn3 was 19.4 µmol/hr/mg fresh weight of nodule while minimum nitrogenase activity was observed in isolate AVR1 which was 9.4 µmol/h/mg fresh weight of nodule. Highest IAA production was recorded in AVR 063:: Tn3 170.00 µg/ml greater than AVR1 116.20. We successfully developed transposon mutagenesis procedure for *Rhizobium japonicum* infecting *Vigna Radiata.* IAA helps in the elongation of root length with increase in number of root branches, root lateral & root hairs that aid in uptake of nutrients& minerals from surroundings [9]. IAA does not function as a hormone in microbial cells but it also involved in plant- microbe interaction. In present study, we report the ability of *Rhizobium* to be used for legume not only as N_2 fixers but also as plant growth promotor. IAA plays an important role in legume- *Rhizobium* interaction. Further studies are required to explore more on production of IAA by rhizospheric bacteria & study their effect on various legume plants.

Table 1. Effect of inoculation of selected rhizobial isolates on nodule number and shoot weight

Sr. No.	Isolate designation	Number of nodule / plant	Fresh weight of nodule (mg)	Nodule texture	Dry weight of nodule (mg)	IAA (µg/mL)	Nitrogenase activity (µmol/h/mg nodule)
1	**AVR**	14.0 ±3.55	139.9 ±3.00	Pink colour	14.5 ±1.45	103.00 ± 0.88	**9.61 ±0.02**
2	**AVR 1**	12.0 ±1.52	139.3 ±3.00	Pink colour	11.8 ±1.24	116.20 ± 0.58	**9.4 ±0.28**
3	**Control**	-	-	-	-		-
4	**AVR03**	16.0 ± 3.0	180.1 ±1.62	Pink colour	21.3 ±1.35	114.50 ± 0.40	10.2 ±0.15
5	**AVR07**	16.0 ±2.51	159.4 ±4.34	Pink colour	16.8 ±2.66	122.20 ± .50	10.5 ±0.24
6	**AVR018**	16.0 ±1.52	150.9 5.68	Pink colour	17.4 ±0.92	116.30 ± 0.58	9.82 ±0.051
7	**AVR022**	15.0 ±1.52	176.0 ±1.45	Pink colour	16.1 ±2.36	88.40 ± 0.60	11.2 ± 0.15
8	**AVR029**	19.0 ±2.81	176.0 ±1.45	Pink colour	20.8 ±2.27	102.90 ± 0.33	10.9 ± 0.24
9	**AVR030**	14.0 ±1.73	170.0 ±1.21	Pink colour	15.0 ±1.63	122.30 ± 0.60	11.2 0.15
10	**AVR036**	12.0 ±1.63	144.8 ±2.81	Pink colour	12.4 ±0.91	144.70 ± 1.20	11.2 0.15
11	**AVR040**	**21.0 ±2.86**	**183.3 ±2.69**	Pink colour	**24.0 ±1.31**	**156.00 ± 1.73**	**12.4 ±0.1**
12	**AVR044**	10.0 ±1.63	140.8 ±2.72	Pink colour	12.4 ±1.63	136.80 ± 0.32	11.5 ±0.25
13	**AVR063**	**22.0 ±1.73**	**184.3 ±2.72**	Pink colour	**20.4 ±6.53**	**170.00 ± 0.57**	**19.4 ±0.2**

ACKNOWLEDGEMENT
The Authors express their sincere thanks to Dr. D. Y.Patil & Biotechnology Institute and Krishna Institute of Medical Sciences Deemed To Be University, Karad, India for providing facilities

CONFLICT OF INTEREST:
There is no conflict of interest between authors & co-authors.

Ethics of Human and Animal Experimentation:
The Authors ensure that the study does not have any type of experiments on animals are humans.

Authors Contribution:
Dr. Abhay Ghatage did all experimental work in the present study and prepared manuscript. Dr.G.R. Pathade & Dr. Rachna Pandey did proof reading of manuscript.

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CITATION OF THIS ARTICLE
A. Ghatage, R. Pandey and G.R Pathade: Production of Indole Acetic Acid in Transposon induced mutants of Rhizobium japonicum infecting Vigna radiate, Bull. Env.Pharmacol. Life Sci., Spl Issue [1]: 2023:163-165