International Journal of Plant & Soil Science
10(3): 1-6, 2016; Article no.IJPSS.23340
ISSN: 2320-7035

SCIENCEDOMAIN *international*
www.sciencedomain.org

Morphological and Biochemical Activities of Root Nodule *Rhizobium* Isolated From Five Multipurpose Forest Species of India

K. K. Chandra[1*]

[1]*Department of Forestry, Guru Ghasidas Vishwavidhyalaya, Bilaspur - 495009 (C.G.), India.*

Author's contribution

The sole author designed, analyzed and interpreted and prepared the manuscript.

Article Information

DOI: 10.9734/IJPSS/2016/23340
Editor(s):
(1) Scd Victoria Anatolyivna Tsygankova, Department for Chemistry of Bioactive Nitrogen-Containing Heterocyclic Compounds, Institute of Bio-organic Chemistry and Petrochemistry, Plant Physiology, NAS, Ukraine.
Reviewers:
(1) Maria Angeles Calvo Torras, Autonomous University of Barcelona, Spain.
(2) Angelica Rodriguez Dorantes, Instituto Politecnico Nacional, Mexico.
Complete Peer review History: http://sciencedomain.org/review-history/13471

Original Research Article

Received 26th November 2015
Accepted 19th January 2016
Published 26th February 2016

ABSTRACT

Five leguminous tree species viz. *Dalbergia sissoo Albizia procera, Albizia labbeck, Pongamia pinnata* and *Leucaena leucocephala* were evaluated for their *rhizobial* activities in different growth medium through biochemical test and the *Rhizobium* strain isolated from different tree species were coded as Ds1(*D. sissoo*), Ap1(*A. procera*), Al1 *(A. lebbeck*), Pp1(*P. pinnata*) and Ll1(*L. leucocephala*) respectively. All strains of *Rhizobium* isolated showed variation in their morphology and biochemical test. The nodule in *A. procera* and *P. pinnata* mainly recovered from lateral roots while in *D. sissoo, A. lebbeck*, and *L. leucocephala* nodules were principally formed in tape roots. The isolated *Rhizobia* were rod shaped, gram negative, showed no growth in Hofer's alkaline medium and glucose peptone agar. The *Rhizobium* strain Ds1 exhibited fastest growth in YEMA and CRYEMA followed by Al1 strain as both isolates attained 6 to 11 mm diameter size within 3-4 days compared to other species under present investigation. In a salt tolerance test all the isolates found tolerant in 1% NaCl, however Ds1 and Ap1 strains both were showed their ability to grow even in 2% salt medium. The Pp1 strain was found the most active strain among all other isolates of tree species as this isolate rendered highest positive results in citrate utilization, carbohydrate catabolism, catalase activities, methyl red test and urease production test.

Keywords: Rhizobium; biochemical tests; nodule; leguminous tree.

**Corresponding author: E-mail: kkckvk@gmail.com;*

1. INTRODUCTION

Rhizobium – plant symbiosis is a natural gift of nature to leguminous species as through this interaction the most essential nitrogen nutrient is utilized by the host. Almost before two centuries (1679) a man named Malpighi observed *Rhizobia* in his drawing of a plant and later Herman Hellriegel with help from Wilfarth discovered *Rhizobium* inside the nodules in 1888. Another important scientist in the history of *Rhizobium* was Martinus Beijerinck who began to organize the *Rhizobium* in to their current species and symbiotic potential of *Rhizobium* was determined. He also standardized for the isolation technique of root nodule bacteria in pure culture. The actual name of *Rhizobium* was coined by Frank in 1988. The first known species of *Rhizobia*, *Rhizobium leguminosarum*, was identified in 1889, and all further species were initially placed in the genus *Rhizobium* under family *Rhizobia*ceae [1]. But Breed et al. [2] placed *Rhizobium* with *Agrobacterium* and *Chromobacterium* while in modern classification, the nodule forming bacteria is separated into 3 genera *Rhizobium, Bradyrhizobium and Azorhizobium* with characteristics of fast growing, slow growing and nodules in stem forming nature respectively. However, more advanced methods of analysis revised this classification, and now there were many in other genera.

In recent years when the cost of production is exponentially increasing in agriculture, horticulture and forestry plantations due high incidences of disease and insect, unscientific use of high doses of chemical fertilizers and also due to the effects of climatic changes. Moreover after tedious efforts of scientists, the maintenance of present productivity of major crops are a real challenge, some alternate technologies are in radar of scientific research for sustainable production, climate smart tree crops and management not only for 5Fs (Food, Feed, Fodder, Fibre and Fuel) production but also to mitigate the adverse effect of pollution and climate change. Now there is a need to promote biofertilizers technology which comprises *Rhizobium*, AMF (Arbuscular mycorrhizal Fungi) and other free living microorganisms which are most potential in enhancing the plant growth besides enriching the soil by fixing atmospheric N_2 or by mobilizing unavailable nutrients to available forms. Among plant microbe interactions, legume-*Rhizobium* are unique because they supply 80-90% of total nitrogen requirements of legume results better growth and establishment due to high N in host as this is very essential for photosynthesis and other metabolic activities [3,4]. In addition, the inoculation of *Rhizobia* induces a carbon sink on the root surface, which intern effects photosynthesis and carbon balance of the plant [5]. The legume crops are not only important in agriculture for its protein source but also in forestry this family has paramount importance as most of the legume trees with multipurpose in use, fast growing and soil improver and occupy in wide group of soils all over the world. The contribution of *Rhizobium* is well documented in agriculture [6,7] and in forest species [8,9]. They all have reported the varied response of *Rhizobium* with soil and host and also reported about different reactions of *Rhizobium* strains to different biochemical tests [10,11]. Among different factors the host plays a major role in symbiosis interaction thus the efficacy is also influenced as per characteristic of *Rhizobium* and host species. Therefore the present investigation was conducted to evaluate different morphological features and biochemical activities of nodule forming *Rhizobium* bacteria in most important five leguminous forest species.

2. MATERIALS AND METHODS

In the present study, five multipurpose tree species were selected on the basis of their importance used in forest plantations, agroforestry and wasteland development. The morphologically freshly, healthy and undamaged root nodules were collected from the roots of *Dalbergia sissoo, Albizia lebbeck, Albizia procera, Pongamia pinnata* and *Leucaena leucocephala. Rhizobium* was isolated from root nodules as per the method described by Somasegaran and Hoben [12]. First the collected nodules were immersed in 0.5% $HgCl_2$ solution for 5 minutes and washed thoroughly with distilled water. *Rhizobium* species was obtained by streaking the crushed root nodules on Yeast Extract Mannitol Agar, (YEMA) (pH 7) in 7.5cm media plates. The *Rhizobium* strain isolated from *D. sissoo, A. procera, A. lebbeck, P. pinnata, L. leucocephala* were coded as Ds1, Al1, Ap1, Pp1 and Ll1respectively. Seven plates for each tree species were stacked in YEMA media and incubated at 30℃. After 48 hours of incubation, colony morphology of isolates was recorded on YEMA plates. The observed colonies were characterized according to the method described by Aneja [13], it includes colonial color, size, shape, margin, elevation, opacity and consistency. The identity of the isolates as

Rhizobium was established by characterization tests including Gram staining, growth on Congo Red Yeast Exract Mannitol Agar (CRYEMA) medium, acid production, growth in Hofer's alkaline medium, growth on glucose peptone agar, motility and nodulation test [14].

After confirmation as *Rhizobium* strains, pure and effective cultures maintained on basal media were used in the study for different biochemical tests. Amylase activity was studied by growing the test isolates on Starch Agar Medium (SAM) plates. After incubation at 30℃ for 24 to 48 h, the plates were flooded with iodine solution and observed for starch hydrolysis around the colony growth e.g. the clear zone. Urease activity was tested by growing the test organisms on Urea medium plates (20% urea solution) containing phenol red as pH indicator. Change the color of medium from yellow to pink was taken as positive test for urease production. For the *Rhizobium* isolates, catalase activity was tested by growing them on YEMA medium. After incubation for 3 days, 3% H_2O_2 was added over the culture. Appearance of effervescence within 20 s indicates positive catalase activity. The test isolates were streaked across the Sodium Citrate amended YEMA medium plates (Mannitol in YEMA was replaced by 1% Sodium citrate) added with bromothymol blue indicator. After incubation at room temperature for 24 to 48 h the change in the color of the medium from green to blue indicates positive citrate utilization. To test the gelatinase activity test tubes containing 20 ml of test isolate suspension and incubated for one week. After incubation, the tubes were kept in a refrigerator for 30 min and observed for gelatin liquefaction. Caseinase activity was tested by inoculating *Rhizobium* isolates on to Skim milk agar plate. Formation of clear zone around the bacterial growth after incubation was considered as positive test. Other biochemical tests were performed: Organic acid production, carbohydrates metabolism, glucose, lactose, sucrose fermentation, Methyl red, Vogus-Proskauer test, H_2S production, isolates growth test in 1% and 2% NaCl. All the isolates were evaluated at 28℃ and 37℃ [15].

3. RESULTS AND DISCUSSION

3.1 Morphological Characters of Nodules

All the leguminous species studied showed variation in nodulation characteristics like size, color and shape. Among the species studied, nodulation occurred as diffused type as they are distributed both on tape and lateral roots. It was also noticed that in *A. procera* and in *P. pinnata* nodule formed more in lateral root than tape root as compared to other species where higher number of nodules recorded on tape root. The pattern of nodulation in *Phaseolus* sp. and *Glycine max* were classified in localized, diffused and mixed group [16] showing a tendency towards grouping of a few nodules in the tape root, the rest being diffused in the laterals. Similar pattern of diffused type of nodulation was reported in five *Indigofera* species [17]. The root nodules of *D. sissoo* were round to oval in shape, pink color and smallest in size compared to other legume species studied while in *A. procera, A. lebbeck* and *P. pinnata* nodules were formed in groups, dichotomously branched tips initially with light pink color but later they turned into white leathery in appearance. But in *L. leucocephala* the young nodules were transparent whitish and older ones were active whitish with pink color tip.

3.2 Growth Activities of *Rhizobium* Isolates

From the characterization tests it is evident that all the isolates from five leguminous species were gram negative, rod shaped, acid producers and showed no growth in Hofer's alkaline medium and glucose peptone agar and were confirmed as *Rhizobia* by the nodulation test for strain effectiveness in Leonard jar under aseptic condition. All isolates were grown well on YEMA (Fig. 1) and CRYEMA and did not show chromogenesis (Table 1). The isolates grown in CRYEMA were developed in a punctiform (< 1mm dia to 2 mm dia) indicates their ability to grow fast. The *Rhizobium* strain Ds1exhibited highest growth followed by Al1 as these both attained 6 to 11 mm diameter size within 3-4 days compared to other species under present investigation. In the present study it was found that, samples give positive results in motility test, grow well between 30℃ to 37℃ while almost unable to grow when temperature dropped at or below 28℃ temperature. All five isolates have salt tolerant ability when inoculated in 1% NaCl whereas merely Ds1 and Ap1 both were capable to grow in 2% NaCl medium; this indicates their importance to improve growth and development under higher salinity condition. This was according to the study of Patil et al. [18] who have reported that some isolates of *Rhizobium* from legume plant tolerated high salt concentration and have advantages to improve saline soil by using such *Rhizobium* strains.

Moreover, several other results [8,19,20] revealed that the growth and nutrient uptake of plant adversely affected with the present of high exchangeable sodium in soil and inoculation of salt tolerance *Rhizobia* and host in such site increases plant survival and growth.

3.3 Biochemical and Enzymatic studies

The utilization of pattern of various carbon sources by the isolates was examined and tabulated in Table 2. It was found that almost all the isolates utilized glucose, sucrose and lactose however the variation in quantitative utilization was observed among the isolates for the enzymatic activities like amylase production, citrate utilization, casein hydrolysis and urease production. The variation in enzymatic activities of *Rhizobium* isolates was reported by various authors [17,18]. Salve and Gangawanae [21] reported that out of the 13 isolates studied by them some are positive and some are negative. All the isolates showed positive amylase production however, *Rhizobium* strains of Pp1 and Ll1were reported to produce high amylase than other species of the present study. The Pp1strain was observed biologically more significant in comparison to other species as the isolate rendered highest positive test results in citrate utilization, carbohydrate catabolism, catalase activities, methyl red test and urease production test. Thus *Rhizobium* of *P. pinnata* was considered best for nodulation and nitrogen fixation due to highest biochemical activities and may plays important role during nodule formation and nitrogen fixation. In case of gelatin hydrolysis Ap1 showed highest positive result followed by Pp1 and Ds1 but in H_2S production test, only Ds1 and Ap1 showed positive while other strains of *Rhizobium* expressed negative result. These results confirmed that the isolated bacterial strains are *Rhizobium* species as in previous studies reported by Erum [22] and Shahzad et al. [23] who found positive results of *R. meliloti* in sugar, citrase and catalase test. The variation in biochemical test results revealed due to the differences may be in N_2 fixation by different hosts and this potential of leguminous tree species should be employed for success of plantation especially in nutrient deficient soils.

Fig. 1. Growth of different isolates of root nodulating bacteria in YEMA media

Table 1. Growth activities of different strains of *Rhizobium* isolated from different leguminous tree species

S. No.	Name of test	Name of the Isolates				
		Ds1	**Al1**	**Ap1**	**Pp1**	**Ll1**
01	Growth on YEMA	3+	3+	2+	2+	2+
02	Growth on glucose agar	-	-	-	-	-
03	Growth on Congored mannitol agar medium(CRYEMA)	Cream translucent	Cream translucent	Cream translucent	Slightly pinkish	Cream translucent
04	Growth in Hofer's alkaline medium	-	-	-	-	-
05	Gram's Reaction	-	-	-	-	-
06	Motility	+	+	+	+	+
07	Growth in 1% NaCl	+	+	+	+	+
	2% NaCl	+	-	+	-	-
08	Growth at 28℃	-	-	-	-	-
09	Growth at 37℃	+	+	+	+	+

(-) Negative/ absent/ no utilization, (+) positive utilization below 10%, (2+) Positive utilization above 10to 30% , (3+) positive utilization above 30 to 50%

Table 2. Enzymatic and biochemical activities of different *Rhizobium* strains isolated from leguminous tree species

S. No.	Name of test	Code of the Isolates				
		Ds1	Al1	Ap1	Pp1	Ll1
1	Glucose fermentation	+	+	+	+	+
2	Sucrose fermentation	+	+	+	+	+
3	Lactose fermentation	+	+	+	+	+
4	Amylase production	3+	2+	2+	4+	4+
5	Organic acid production	+	2+	2+	3+	+
6	Citrate Utilization	+	3+	2+	4+	+
7	Casein hydrolysis	2+	5+	3+	4+	+
8	Carbohydrate catabolism	3+	3+	2+	4+	2+
9	Methyl Red Test	-	-	-	+	-
10	Vogus-Proskauer test	+	+	+	-	+
11	Catalase activity	2+	2+	3+	4+	2+
12	Gelatin hydrolysis	3+	2+	4+	3+	2+
13	Urease test	4+	+	3+	4+	3+
14	H_2S production	+	-	+	-	-

(-) Negative/ absent/ no utilization, (+) positive utilization below 10%, (2+) Positive utilization above 10to 30% , (3+) positive utilization above 30 to 50%, (4+) positive utilization above 50 to 70%, (5+) positive utilization above 70%

4. CONCLUSION

The symbiosis between the root nodule bacteria of the genus *Rhizobium* and legumes is of special significance to leguminous tree species where no external nitrogen input is provided after plantation. The effective strains of *Rhizobium* could meet the partial nitrogen requirement of legumes and hence reduction in dependence of external nitrogen inputs. The results obtained in the present study are concluded as the most number of nodules formed in lateral roots in *A. procera* and *P. pinnata* while in tape roots of *D. sissoo, A. lebbeck* and *L. leucocephala*. Ds1 and Al1 showed prominent, white colonies in YEMA whereas Pp1 showed pinkish colonies. All isolated *Rhizobium* strains were found to be gram negative whereas strains of Ds1 and Ap1 showed prominent growth even in 2% NaCl which indicates their ability to salt tolerance as compared to other strains of *Rhizobium* under present study. This result can imply in the selection of *D. sissoo* and *P. pinnata* for saline soil. All strains of *Rhizobium* showed better growth at 37℃ while no growth at below 28ºC. Among all strains of *Rhizobium*, Pp1 was found to be the best in amylase production, organic acid production, citrate utilization and carbohydrate catabolism and catalyses activity tests.

COMPETING INTERESTS

Author has declared that no competing interests exist.

REFERENCES

1. Fred BE, Baldwin IL, McCoy E. Root nodule bacteria and leguminous plants No. 5. University of Wisconsin Press, Madison, Wisconsin; 1932.
2. Breed RS, Murrey EGD, Smith NR. Bergey's manual of determinative bacteriology. 7th edition London Baltimore Tindal and Lox; 1957.
3. Bais HP, Weir TL, Perry LG, Gilroy S, Vivanco JM. The role of root exudes in rhizosphere interactions with plants and other organism. Ann Rev Pl Biol. 2006; 57:233-266.
4. Nishita G, Joshi NC. Growth and yield response of Chick pea (*Cicer arietinum*) to seed inoculation with *Rhizobium* species. Nature Sci. 2010;8(9):231-236.
5. Schulze J, Poschel G. Bacterial inoculation of maize affects carbon allocation to roots and carbon turnover in the rhizosphere. Pl and Soil. 2004;267:235-241.
6. Akhtar MS, Siddiqui ZA. Effects of PSB and *Rhizobium* species on the growth, nodulation yield and root rot disease complex of Chikpea under field condition. Af J Biotech. 2009;8(15):3489-3496.
7. Xiurong W, Qiang P, Fengxian C, Xiaolong Y, Hong L. Effects of co-inoculation with arbuscular mycorrhizal fungi and *Rhizobia* on soybean growth as related to root architecture and availability of N and P. Mycorrhiza. 2011;21:173-181.

8. Bisht R, Chaturvedi S, Srivastava R, Sharma AK, Johri BN. Effect of arbuscular mycorrhizal fungi, *Pseudomonas fluorescens* and *Rhizobia leguminosarum* on the growth and nutrient status of *Dalbergia sissoo.* Roxb. Trop Eco. 2009; 50(2):231-242.
9. Moreira FMS, Carvalho TSD, Siqueira JO. Effect of fertilizers, lime and Inoculation with *Rhizobia* and mycorrhizal fungi on the growth of four leguminous tree species in a low fertility soil. Bio. Fertil Soils; 2010. DOI: 10.1007/s00374-010-0477-5
10. Ghorbanian D, Harutyunyan S, Mazaheri D, Rejali F. Effects of mycorrhizal symbiosis and different levels of phosphorus, macro and micro elements on yield of *Zea mays* L. under water stress condition. Af J Agri Res. 2011;6(24):5481-5489.
11. Lalitha S, Rajeshwaran P, Senthil Kumar, Deepa S. Role of AM fungi and *Rhizobial* inoculation for reclamation of phosphorus deficient soil. Asian J Pl Sci. 2011;10:227-232.
12. Somasegaran P, Hoben HJ. Handbook for *Rhizobia*: Methods in legume – *Rhizobium* technology. Springer- verlag, New York; 1994.
13. Aneja KR. Experiments in microbiology plant pathology and biotechnology. 4th edition, New Age Int Pub. New Delhi, India; 2003.
14. Holt JG, Kreieg NR, Sneath PHA, Staley JT, Williams ST. Bergey's manual of determinative bacteriology. 9th Edition Williams and Wilkins, A Wavely Company, Baltimore. 1994;787.
15. Lowe GH. The rapid detection of lactose fermentation in paracolon organism by demonstration of 6 – D-galactosidase. J Med Lab Tech. 1994;19:21-25.
16. Bhaduri PN, Sen R. Distribution pattern of nodules in *Phaseolus* species and glycine max. Ind J Genetics Pl Breed. 1968; 28:287-296.
17. Kumari BS, Ram MR, Mallaiah KV. Studies on nodulation, biochemical analysis and protein profiles of *Rhizobium* isolated from Indigofera species. Malaysian J Microbiol. 2010; 6(2):133-139.
18. Patil SM, Patil DB, Patil MS, Gaikwad PV, Bhamburdekar SB, Patil PJ. Isolation, characterization and salt tolerance activity of *Rhizobium* species from root nodules of some legumes. Int J Cur Microbiol Appl Sci. 2014;3(5):1005-1008.
19. Abdelmoumen H, Filali-maltour A, Neyra M, Belabed A, Idrissi MME. Effect of high salt concentrations on the growth of rhizobia and responses to added osmotica. J. Appl Microbiol. 1999;86(6):889-898.
20. Shaharani TS, Shelta ND. Evaluation of growth, nodulation and nitrogen fixation of two Acacia species under salt stress. World Appl Sci J. 2011;13(2):256-265.
21. Salve PB, Gangwanae LV. *Rhizobium* from wild legumes and nitrogen fixation in ground nut. In: Biofertilizers Technology Transfers. Associated Publications Co, New Delhi. 1992;95-100.
22. Erum S, Asghari B. Variation in phytohormone production in *Rhizobium* strains at different altitudes of northern areas of Pakistan. Pak Int J Agri Biol. 2008;10:536-540.
23. Sahzad FM, Shafee F, Abbas S, Babar Tariq MM, Ahmad Z. Isolation and biochemical characterization of *Rhizobium* meliloti from root nodules of Alfalfa (*Medico sativa*). J Animal Pl Sci. 2012; 22(2):522-524.

Peer-review history:
The peer review history for this paper can be accessed here:
http://sciencedomain.org/review-history/13471