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Isolation and Characterization of *Rhizobium* Associated with Root Nodules of *Dalbergia sissoo*

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A B S T R A C T

A study was conducted to isolate and characterize the rhizobia from the root nodule of *Dalbergia sissoo* from five different sites of Himachal Pradesh (Bilaspur, Solan, Hamirpur, Kangra and Una) and Uttrakhand (Rishikesh, Nainital, Haridwar, Dehradun and Udham Singh Nagar). A total of 79 bacterial isolates were isolated from root nodule of *Dalbergia sissoo* on YEMA media after 3 days of incubation at 37˚C by serial dilution method. Out of 79 isolates only 38 isolates were identified as rhizobia on the basis of authentication test (Congo red test, Bromothymol blue test and Plant infection test). Thirty eight strains were characterized on the basis of biochemical characterization. On the basis of morphological properties all the isolates were round and slimy white with raised elevation and smooth surface indicating rhizobia. In Gram's reaction they were pink, rod in shape indicating gram negative bacteria. Only two rhizobial isolates DBD1 and DRT5 were selected on the basis of plant growth promoting traits (P-solubilization, Nitrogen fixation, Siderophore production, HCN production and Biocontrol activity). These two rhizobial isolates may be useful to increase the symbiotic nitrogen fixation in legume tree and can be used as potential biofertilizer owing to their plant growth promoting characters.

Introduction

Low fertility is an important problem in establishing vegetation on the degraded lands. The nitrogen is generally deficient in these lands; the fertility of such soils can be maintained by supplementing fertilizers especially the nitrogen. The nitrogen fertilizer is not only costly and scarce, but also lost rapidly due to leaching, runoff and volatilization, causing water and atmospheric pollution.

Biological Nitrogen Fixation is a natural process where certain bacteria and leguminous plants with nodules in their root systems are able to convert the nitrogen gas into a form that is usable for plant life. The ability to fix atmospheric nitrogen into a form that can be used for plant growth is confined

to bacteria and cyanobacteria. Plants fix nitrogen only by virtue of associations with these simple organisms. The best-known associations are the symbioses of *Rhizobium* bacteria with legumes. Nitrogen fixing leguminous plants not only supports plant growth independent of mineral nitrogen in the soil but also improve soil nitrogen status for associated crops by the residues of these plants.

Bacteria involved in symbiotic nitrogen fixation belong to genera *Rhizobium* and *Bradyrhizobium*. Rhizobia forms tumor like swellings called nodules on the root surface of host plant. Rhizobia inside the nodules absorb air from the soil and convert gaseous nitrogen into ammonia. This association between the host plant and rhizobia is mutually beneficial.

The process of BNF is strongly related to the physiological status of host plant and rhizobial strains. The different responses of rhizobial strains to various factors considered as basic criteria for differentiation and identification of these bacteria (Zahran *et al.*, 2012). Rhizobia adapt themselves in different environment including soil, rhizosphere and grow within legume roots, where they fix nitrogen (Ghosh and Maiti, 2016). They can trigger leguminous host plants to form root nodules.

Biofertilizer enhance plant growth and productivity and has globally been acknowledged as a substitute of chemical fertilizer. Rhizobacteria efficiently inhabit plant root and boost plant growth by production of various plant growth hormones, P-solubilizing activity, N2 fixation and biological control activity (Deshwal *et al.*, 2003).

A well conventional practice for maintaining soil fertility has been the cultivation of leguminous plants which replenish atmospheric nitrogen through symbiosis with rhizobia in rotation with non leguminous plants.

Materials and Methods

Sample collection and sowing

Seeds were collected from 15 to 25 year old matured and healthy trees of *Dalbergia sissoo* from different geographic locations in Himachal Pradesh (Bilaspur, Solan, Hamirpur, Kangra and Una) and Uttarakhand (Rishikesh, Nainital, Haridwar, Dehradun and Udham Singh Nagar).

In each location three representative sites were selected and three plant samples were collected from each site. Seeds from different sources were kept in germinator at 25 - 30°C for germination and then sown in the poly bags (mixture of soil, sand and farm yard manure in 2:1:1 ratio). The seeds from different locations were sown in nursery intended for evaluating their growth.

Isolation of *Rhizobium* from root nodules

Healthy nodules were isolated from the six month old *D. sissoo* seedlings grown under net house conditions. The nodules were washed in tap water to remove the adhering soil particles on its surface. Nodules were dipped in 0.1 % mercuric chloride ($HgCl_2$) solution for 30 second and then washed successively eight to ten times with sterilized distilled water to remove the traces of $HgCl_2$.

Surface sterilized nodules were crushed in sterilized distilled water by glass rod to obtain a milky suspension of bacteriods. The suspension was streaked on YEMA medium and incubated at 28±2°C for 2-5 days. The growth on YEMA medium was counted and expressed as cfu/g. Isolates obtained from nodules of *Dalbergia sissoo* were purified on YEMA medium by streak plate method.

Authentication

Congo red test

All the purified rhizobial isolates were streaked on CRYEMA medium and were observed for absorption of congo red dye (Vincent, 1970).

Bromothymol blue test

The YEMA medium containing bromothymol blue was streaked with isolated strains and was observed either for yellow colour due to production of acids or blue colour due to production of alkali (Norris, 1965).

Hofer's alkaline test

This test is based on the fact that *Rhizobium* is unable to grow at higher pH 11.0 on yeast extract mannitol broth (Hofer, 1935).

Ketolactose agar test

Ketolactose agar plates were streaked with isolated microbes. After incubation for 4-6 days at 28±2°C, the plates were flooded with Benedict's solution. This test is based on the fact that *rhizobium* is unable to utilize the lactose (Bernaerts and Deley, 1963).

Plant infection test

The different isolates were tested for their ability to nodulate *Dalbergia sissoo* plants grown in plastic pots. Seeds of *Dalbergia sissoo* were inoculated with *Rhizobium* isolates by soaking seeds. Plants were carefully uprooted after 75 days and observed for nodulation.

Morphological characterization

Morphological characteristics of isolates including colony color, opacity, form, elevation, margin, surface, texture, motility, shape, arrangement, gram's reaction, endospore staining, capsular staining were determined by observing the colonies on YEMA plates

Physiological characterization

Separate experiments were performed which include pH, temperature, NaCl concentration, incubation period for optimization conditions for growth of selected rhizobial isolates.

Biochemical characterization

Indole test, Methyl red test, Citrate utilization, Starch hydrolysis, Casein hydrolysis, Gelatin hydrolysis, Hydrogen sulphide production, Catalase test, Voges Proskauer test, Urease test, Carbohydrates fermentation, Ammonia production test, Cellulase test.

Results and Discussion

Isolation of Rhizobia from root nodules of *Dalbergia sissoo*

The isolation of rhizobia was carried out from the root nodules of six month old *D. sissoo* seedlings grown under net house conditions. The growth on YEMA medium was counted and expressed as cfu/g. A total of 38 bacterial isolates were isolated from 5 different sites of Himachal Pradesh. A great variation in the rhizobial population colonizing the roots of *D. sissoo* seedlings were noticed (Table 1). The maximum rhizobial count (8.53×10^3 cfu/g root) and (8.14×10^3 cfu/g root) on YEMA medium were observed with Hamirpur and Haridwar seed sources, respectively. Whereas, minimum rhizobial count (6.85×10^3 cfu/g root) and (6.73×10^3 cfu/g root) were recorded in Solan seed source of Himachal Pradesh and Rishikesh seed source of Uttrakhand, respectively. The variation in population may arise due to difference in soil

physio-chemical properties, environmental conditions, root exudates and the plant age (Wieland *et al.,* 2001). Balota and Chaves (2011) and Zhou *et al.,* (2017) have also reported similar results.

Authentication of rhizobia isolated from *Dalbergia sissoo* root nodules

A total of 38 rhizobial isolates from *Dalbergia sissoo* root nodules of Himachal Pradesh were isolated and screened for different authentication tests *viz*. congo red test, bromothymol blue test, growth in Hofer's alkaline broth, ketolactose medium and plant infection test. All the 38 isolates were found negative for congo red test on congo red YEMA medium. The results are supported by Mahmood and Athar (2008) who reported negative results for congo red medium. None of the isolate showed growth on Hofer's alkaline broth and did not utilize lactose and peptone after incubation period at 28±2°C. Out of these 38 isolates, 28 isolates from Himachal Pradesh showed yellow colored acid producers colonies enriched with bromothymol after incubation for 3-5 days at 28±2°C. Fast grower takes 3-5 days of incubation to grow. The plant infection test also revealed that only 26 out of 38 isolates nodulated the *Dalbergia* seedlings after 75 days of inoculation and these isolates were tentatively confirmed as *Rhizobium* spp. (Table 2). Similar results were observed in the findings of Agrawal *et al.,* (2012); Deshwal and Chubey (2014) and Dipta *et al.,* (2017) reporting absence of congo red dye absorption, absence of growth in Hofer's alkaline broth and did not utilize lactose or peptone.

Morphological characterization of selected rhizobial isolates

The results presented in (Table 3) the colony morphology, Gram's reaction and cell shape of selected rhizobial isolates. The isolates produced opaque, nearly round and gummy colonies with raised elevation and smooth surface, which indicated it as rhizobia spp. The Gram's reaction indicated them to be gram negative rods. They were motile, non endospore forming capsulated microbes.

Table.1 Rhizobial population from root nodules of *D.sissoo*

	Sites	Rhizobial population (10^3 cfu/g soil)
Himachal Pradesh	**Solan**	6.85
	Bilaspur	7.34
	Kangra	7.16
	Hamirpur	8.53
	Una	7.87
	CD(0.05)	7.76
Uttrakhand	**Babu Gate FRI**	7.45
	Haridwar	8.14
	Rishikesh	6.73
	Pantnagar	8.02
	Nainital	7.73
	CD(0.05)	4.76

Table.2 Authentication of rhizobia isolated from root nodules of *Dalbergia sissoo*

Himachal Pradesh						
S. No	**Isolates**	**Congo Red Test**	**Bromothymol Blue Test**	**Growth in Hofer's alkaline broth**	**Ketolactose Test**	**Infectivity test (Nodule formation)**
1	**DSN_1**	-	Alkaline producer	No growth	-	Absent
2	**DSN_2**	-	Acid producer	No growth	-	Present
3	**DSN_3**	-	Acid producer	No growth	-	Present
4	**DSN_4**	-	Acid producer	No growth	-	Present
5	**DSN_5**	-	Acid producer	No growth	-	Absent
6	**DSN_6**	-	Acid producer	No growth	-	Absent
7	**DSN_7**	-	Acid producer	No growth	-	Present
8	**DSN_8**	-	Alkaline producer	No growth	-	Present
9	**DHL_1**	-	Acid producer	No growth	-	Present
10	**DHL_2**	-	Acid producer	No growth	-	Present
11	**DHL_3**	-	Acid producer	No growth	-	Absent
12	**DHL_4**	-	Alkaline producer	No growth	-	Absent
13	**DHL_5**	-	Acid producer	No growth	-	Present
14	**DHL_6**	-	Alkaline producer	No growth	-	Present
15	**DKA_1**	-	Alkaline producer	No growth	-	Present
16	**DKA_2**	-	Acid producer	No growth	-	Present
17	**DKA_3**	-	Acid producer	No growth	-	Present
18	**DKA_4**	-	Acid producer	No growth	-	Absent
19	**DKA_5**	-	Acid producer	No growth	-	Absent
20	**DKA_6**	-	Alkaline producer	No growth	-	Present
21	**DKA_7**	-	Acid producer	No growth	-	Present
22	**DKA_8**	-	Acid producer	No growth	-	Present
23	**DBD_1**	-	Acid producer	No growth	-	Present
24	**DBD_2**	-	Acid producer	No growth	-	Absent
25	**DBD_3**	-	Acid producer	No growth	-	Absent
26	**DBD_4**	-	Acid producer	No growth	-	Present
27	**DBD_5**	-	Acid producer	No growth	-	Present
28	**DBD_6**	-	Acid producer	No growth	-	Present
29	**DBD_7**	-	Acid producer	No growth	-	Present
30	**DUB_1**	-	Acid producer	No growth	-	Present
31	**DUB_2**	-	Alkaline producer	No growth	-	Absent
32	**DUB_3**	-	Acid producer	No growth	-	Present
33	**DUB_4**	-	Acid producer	No growth	-	Absent
34	**DUB_5**	-	Acid producer	No growth	-	Present
35	**DUB_6**	-	Alkaline producer	No growth	-	Present
36	**DUB_7**	-	Acid producer	No growth	-	Present
37	**DUB_8**	-	Alkaline producer	No growth	-	Present
38	**DUB_9**	-	Alkaline producer	No growth	-	Absent

Table.3 Morphological, Biochemical characterization and Optimization of selected rhizobial isolates

Morphological Characters											
Rhizobial Isolates	**Colony colour**	**Opacity**	**Form**	**Surface**	**Texture**	**Motility**	**Shape**	**Arrangement**	**Colour**	**Gram's reaction**	**Endospore staining**
DBD_1	White	Opaque	Circular	Granular	Slimy	+	Rods	Cluster/single	Pink	-	-
Biochemical characters of selected rhizobial isolates											
	Indole test	**Methyl-Red test**	**Voges Proskauer test**	**Hydrogen sulphide production**	**Catalase test**	**Citrate test**	**Starch hydrolysis**	**Cellulase Test**	**Casein test**	**Urease test**	**NH_3 production**
DBD_1	-	+	-	-	+	-	-	-	-	+	++
Carbohydrate test	Xylulose	Fructose	Sucrose	Galactose	Maltose	Mannitol	Lactose	Glucose	Xylulose		
DBD_1	+	+	+	+	+	+	-	+	+		
Optimization of conditions for selected rhizobial isolates											
pH	**DBD_1**	**Temperature(°C)**	**DBD_1**	**Incubation period (h)**	**DBD_1**	**NaCl concentration (%)**	**DBD_1**				
4.0	-	20	+	0	-	0	+				
5.0	+	25	++	12	-	0.25	+				
6.0	+	30	++	24	+	0.5	+				
7.0	++	35	+	48	++	0.75	+				
8.0	+	40	+	72	++	1	+				
9.0	-	45	-	96	+	2	+				
				120	+	3	+				
						4	+				
						5	-				

Biochemical characterization of selected isolates

The results presented in (Table 3) indicated that the rhizobial isolate was positive for methyl red test, catalase test, urease test, ammonia production test, and negative for Indole test, voges Proskauer test, hydrogen sulphide production, citrate test, cellulase and casein test. In case of carbon utilization, the isolate was able to ferment xylulose, sucrose, galactose, maltose, mannitol fructose except lactose.

The present results are in confirmatory with those of Menna *et al.*, (2006) also characterized strains based on morpho-physiological characters. Kumar *et al.*, (2017a) who also observed similar morphological characteristic for the genus *Rhizobium*. Surange *et al.*, (1997) characterized *Rhizobium* strain with respect to carbon utilization (including sugars). Batzli *et al.*, (1992) also characterized and identified 11 isolates as *Rhizobium* based upon their morphological characters. The utilization of diverse carbon sources by bacterial isolates might indicate their metabolic and ecological diversity suggesting their use as biofertiliser/ biocontrol agent (Deng *et al.*, 2011; Fitriyah *et al.*, 2013; Mantilla-Afanador *et al.*, 2017).

Physiological characterization of selected isolates

The results presented in Table 3 indicated the growth of selected isolate at a pH 4.0-9.0, temperature of 20-45ºC, incubation period of 0-120 hrs and NaCl concentration of 0-5 per cent. The isolate was able to grow at pH

ranges between 5.0 and 8.0. However, was unable to grow at high acidic pH of 4.0 and alkaline pH at 9.0. The isolate was able to grow at the temperature range of 20-35º C and was unable to grow at temperature of 45º C. The suitable salt concentration for its growth was found to be at most 5 per cent. Similar morphological, physiological and biochemical characters of rhizobial isolates have been reported by Lalitha and Immanuel (2013) and Rasool *et al.,* (2015). Pawar *et al.,* (2014) reported that nodulating bacteria isolated from soybean root nodules showed good growth at temperature 36°C and pH 7.0. Datta *et al.,* (2015) reported *Rhizobium* strains isolated from root nodules of *Trifolium, Vigna radiate, Glycine max* and *Lens culinaris* grew well at 6.0 and 7.0 pH, 34°C temperature and 4 per cent salt concentration.

On the basis of morphological, physiological, biochemical characteristics and Bergey's Manual of Systematic Bacteriology (Claus and Berkeley, 1986) the isolate DBD_1 was tentatively identified as *Rhizobium* species. Similar morphological, physiological and biochemical characteristics of rhizobial isolates have reported by Lalitha and Immanuel (2013), Rasool *et al.,* (2015).

In conclusion, in the present study we isolated *Rhizobium* spp. from the root nodules of *Dalbergia sissoo* and resolute their ability for growth promoting factors. We obtained only two Rhizobium strains those are capable for fixing higher amount of nitrogen along with different growth promoting factors. Thus these strains may be applied in proficient nodulation of *Dalbergia sissoo* for biological nitrogen fixation as well as in afforestation programme.

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N/A

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