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Studies on nodulation, biochemical analysis and protein profiles of *Rhizobium* isolated from *Indigofera* species

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ABSTRACT

Nodulation characteristics in five species of *Indigofera* viz., *I .trita*, *I. linnaei*, *I. astragalina*, *I. parviflora and I. viscosa* was studied at regular intervals on the plants raised in garden soil. Among the species studied, highest average number of nodules per plant of 23 with maximum sized nodules of 8.0 mm diameter was observed in *I. astragalina.* Biochemical analysis of root nodules of *I. astragalina* revealed that the leghaemoglobin content of nodules and nitrogen content of root, shoot, leaves and nodules were gradually increased up to 60 DAS, and then decreased with increase in age. *Rhizobium* isolates of five species of *Indigofera* were isolated and screened for enzymatic activities and total cellular protein profiles. All the five isolates showed nitrate reductase, citrase, tryptophanase and catalase activity while much variation was observed for enzymes like gelatinase, urease, caseinase, lipase, amylase, lysine decarboxylase and protease activities. Among the isolates studied, only the isolate from *I. viscosa* has the ability to solubilize the insoluble tricalcium phosphate. All the *Rhizobium* isolates exhibit similarity in protein content, except the isolate from *I. viscosa* which showed one additional protein band.

Keywords: Rhizobium, Indigofera species, biochemical analysis, SDS-PAGE

INTRODUCTION

Indigofera is one of the major nodulated genera in the family leguminosae. *Indigofera* was listed as one among the nine important legume genera used as green manure, with high nitrogen content of 5 and 40 kg N/ha in the leaves (Thonnissen *et al.*, 2000) in wetland rice cultivation (Allen and Allen, 1981). The species mainly used as green manure include *I. astragalina*, *I. hirsuta*, *I. suffruticosa*, *I. tinctoria*, *I. trita*, *I. tesymani* and *I. viscosa*. These legumes improve soil texture and tilth, and are important for soil conservation and reclamation.

Among plant-microbe interactions, legume–*Rhizobium* interactions are unique because they supply 80-90% of total nitrogen requirement of legumes. It involves a complex interaction among host, microbial symbiont and environment. Among nitrogen fixing systems, legume-rhizobium symbiosis is one of the most promising and the bacterial species of *Rhizobium* complex are very important (Sprent, 2001).

Out of the identified 700 species of *Indigofera,* only 225 species were reported to be nodulated so far. Though nodulation was reported in many species of *Indigofera*, the microsymbiont was isolated and characterized in only few species. One species of *Rhizobium indigoferae* (Wei *et al*., 2002) from three *Indigofera* sp. and a species of *Sinorhizobium terangae* and *Bradyrhizobium japonicum* from *I. brevidens* (Yates *et al.*, 2004) were reported so far.

In general, legumes' growing wild in any region show adoptability to the environment and fix the atmospheric nitrogen effectively than the cultivated legumes in that region. Recently, the use of effective rhizobia from wild non crop legumes as a bioinoculant to crop plants was proven to increase nodulation and nitrogen fixation (Zahran, 2001). Hence in the present study, five *Indigofera* species growing wild in our area were screened for effective rhizobial strain by comparing the data on nodulation; biochemical characteristics and total cellular protein profiles of the *Rhizobium* isolates.

MATERIALS AND METHODS

For nodulation studies, seeds of five *Indigofera* species viz., *I. trita*, *I. linnaei*, *I. astragalina*, *I. parviflora* and *I. viscosa* were raised in earthen pots using lateritic soil from the experimental plots of the university. Three pots were maintained for each species. The pots were watered regularly and each pot was maintained with at least 10 plants. Date of nodule initiation for all the five species was recorded by observing the root system of one plant for each species, daily after 5 days from the day of sowing. Five plants belonging to each species of *Indigofera* were gently uprooted for studies on nodulation characters like size, shape, color, number, distribution, fresh weight, dry weight and moisture content of each nodule. This nodulation data was recorded at 10 days intervals from 30 to 90 days after sowing.

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Among the five species, the species with highest number of nodules/plant and biomass was selected for biochemical studies. Biochemical constituents like leghaemoglobin, total nitrogen content of the nodules, leaves, root and shoot were estimated by collecting the plant samples at 10 days intervals from 30 to 90 days after sowing. Leghaemoglobin content was estimated according to the method described by Tu *et al.* (1970). Nitrogen content of the samples as percent dry weight was estimated using micro-Kjeldahl 'N' method given by ANON (1978). The data was statistically analyzed using ANOVA (one way classification technique).

The *Rhizobia* were isolated from the freshly collected healthy root nodules of all the five *Indigofera* species on to YEMA medium. The identity of the isolates as *Rhizobium* was established by characterization tests including Gram staining, growth on YEMA medium with Congo red, acid production, ketolactose test, growth in Hofer's alkaline broth, growth on Glucose–peptone agar and nodulation test (Holt *et al.*, 1994).. After confirmation as *Rhizobium* strains, pure cultures maintained on basal media were used in the study. All the five test isolates were screened for their ability to produce 13 different enzymes involved in biochemical reactions by following standard methods (Dubey and Maheshwari, 2002). In all the tests, the isolates were inoculated in to media (or) broth using a pre-sterilized inoculation loop.

Tests for enzymatic activity

Tryptophanase (4.1.99.1) activity was tested by inoculating the test isolates into 1% tryptone broth and incubated at 28 °C for 24-48 h. After incubation, Kovac's reagent was added, development of pink color is considered as positive result for tryptophanase activity/indole production and absence of color indicates negative result.

Protease (3.4.24.25) activity was studied by inoculating the test isolates into YEM broth and incubating at room temperature. During the 6 days of incubation, the protease activity was determined at every 24 h. To the 0.2 mL of culture suspension, 1 mL of 1% albumin was added and incubated for 1 h at room temperature. After incubation, 1 mL of 12% Tri-chloro acetic acid (TCA) solution was added and the mixture was cooled rapidly in the ice. The mixture was centrifuged and protein in the precipitate was estimated by Biuret method. Control was maintained by taking 1 mL of albumin added with 1 mL of 12% TCA. After mixing the contents thoroughly 0.2 mL of culture suspension was added. If the sample contains lower protein than the control, it indicates protease activity.

Nitrate reductase (1.7.99.4) activity was studied by inoculating 0.5 mL of isolate suspension in to 10 mL of the YEM broth containing 1% KNO_3 and incubated for 5 days at room temperature. After incubation, 0.1 mL of test reagent was added to the test tubes. Development of red color within minutes was considered as positive and absence of color indicates negative test.

Lysine decarboxylase (4.1.1.18) activity was studied by inoculating the test isolate test tubes containing 10 mL of Bromocresol purple Falkow medium followed by incubation at room temperature for 12 h. If the medium turns yellow in response to glucose fermentation in the first 12 h of incubation and remains bright purple, it was considered as positive test.

Amylase (3.2.1.1) activity was studied by growing the test isolates on Starch Agar Medium (SAM) plates. After incubation at 30 °C 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 (3.5.1.5) activity was tested by growing the test organisms on Urea medium plates (20% urea aqueous solution aseptically added to the basal medium after filtration through Whatman filter paper) containing phenol red as pH indicator. Change in color of medium from yellow to pink was taken as positive test for urease production.

For the *Rhizobium* isolates, catalase (1.11.1.6) 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 24–48 h the change in the color of the medium from green to blue indicates citrase (4.1.3.6) activity.

To test the gelatinase (3.4.24) activity test tubes containing 20 mL of gelatin medium (12%) were inoculated with 0.3 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. All the tests Rhizobia were inoculated on to Skim milk agar (HIMEDIA) plates and incubated at room temperature for 24–48 h for caseinase activity. Formation of clear zone around the bacterial growth after incubation was considered as positive test. To test the lipase (3.1.1.3) activity, plates with YEMA medium supplemented with 1% Tween-80 were inoculated with tested strains of *Rhizobia*. The inoculated plates were incubated at 28–30 °C for one week and observed for zone of hydrolysis around the colony growth. To test the phenylalanine deaminase (4.3.1.24) activity, *Rhizobium* isolates were inoculated on to plates with Phenylalanine agar medium and incubated. After incubation at 30 °C for 48 h, 10% $FeCl_3$ was added and examined for the change in colour. Appearance of green color indicates phenylalanine deaminase positive. To study the DNase (3.1.21.1) activity, media plates were prepared by using YEMA medium after adding 0.2% DNA at 50 °C. The test organisms were streaked on the YEMA medium containing 0.2% DNA. After incubation for 1–2 days at room temperature, when the plates were flooded with 1N HCl, DNase activity results in a clear zone surrounded by turbidity produced by the precipitate of the unaffected substrate.

Table 1: Comparative account on root nodulation characteristics of five *Indigofera* species

Name of the Plant	DAS	Average Number of Nodules/Plant			Size in (mm)	Shape	Colour	*Weight of each nodule (mg)		*Moisture content (%)
		Tap root	Lateral root	*Total				Fresh weight	Dry weight	
I. trita	60	5	4	9	2.0-5.0	Elongated	Cream	10.4	3.6	65.3
I. linnaei	70	3	3	6	2.0-7.0	Aggregate	Cream	10.2	2.8	72.5
I. astragalina	70	12	11	23	2.0-8.0	Aggregate	Cream	16.7	5.1	69.4
I. parviflora	60	4	4	8	1.0-5.0	Globose	Pink	3.0	1.2	60.0
I. viscosa	70	4	7	11	2.0-5.0	Elongated	Cream	5.8	1.3	77.5

DAS: Days after sowing at which maximum no. of nodules were recorded.
Each data is an average of five plants.
* significant at 5% between the cultivars (Fc = 12.5 , Ft = 2.603)

Litmus milk reaction

Test isolates were inoculated into 10 mL of litmus milk medium and poured in separate tubes and incubated at 30 °C for 24–72 h. The characteristic reactions including acid production, alkali production, proteolysis and litmus reduction with serum zone formation were observed after incubation.

Phosphate solubilization test

All the five isolates of *Indigofera* species were also tested for solubilization of tri-calcium phosphate on Pikovskaya's medium using spot inoculation method (Pikovskaya, 1948). After 5–7 days of incubation the Pikovskaya's medium plates were observed for the formation of clear zone of phosphate solubilization around the colonies.

Analysis of total cellular proteins of rhizobia isolated from all the five *Indigofera* species was carried out by SDS-PAGE by method given by Laemmli (1970). After the electrophoresis, similarity index (percentage similarity between the isolates) was calculated dividing the total number of similar bands with total number of bands, multiplied by 100.

RESULTS AND DISCUSSION

All the *Indigofera* species studied showed variation in nodulation characteristics. Among the species studied, nodulation was initiated much earlier at 22 DAS in case of *I. trita* and *I. astragalina,* where as in *I. viscosa*, *I. parviflora* and *I. linnaei* nodulation started at 28, 30 and 35 DAS respectively. The nodulation in the present study can be referred as diffused type as they are distributed both on tap and lateral roots, with more on tap root than on lateral roots. Bhaduri and Sen (1968) classified the pattern of nodulation in *Phaseolus* and *Glycine max* in to three distinct types. Localized e.g. on the tap root only; diffused e.g. nodules diffused in tap and lateral roots and Mixed e.g. showing a tendency towards grouping of a few nodules in the tap root, the rest being diffused in the laterals. Similar pattern of diffused type of nodulation was reported in *Indigifera zollingeriana* by Anegbah *et al.* (2003) and in *Phaseolus* sp. by Bhaduri and Sen (1968).

Nodulation studies

The number of nodules increased with age of the plants and highest number of nodules was observed at 60 DAS in *I. trita* and *I. parviflora* and it was at 70 DAS in *I. linnaei*, *I. astragalina* and *I. viscose* (Table 1). Among the species studied the highest average number of nodules per plant was observed in *I. astragalina* (23) followed by *I. viscosa* (10) and a lowest of 6 was observed in *I. linnaei*. Much variation in nodule number was reported in *Indigofera* species. In *I. cardifolia*, *I. hochstetteri* and *I. linifolia* highest of only 1–5 nodules per plant reported from Pakistan (Athar and Shabbir, 2008). In *I. zollingeriana* the highest number of 25 nodules per plant was reported (Anegbah *et al*., 2003). In *Indigofera* sp (EAO 19), *I. parniculata* and *I. pulchra* the maximum number reported was 29, 24 and 19 nodules per plant, respectively (Ezedinma *et al*., 1978). In the present study, the size of the nodules ranged from (2.0–8.0 mm), the maximum nodule diameter observed was in *I. astragalina* followed by *I. linnaei* (7 mm) and in rest of the species it was observed as 5 mm. The root nodules of *Indigofera* are round to oval type at the early stage but became elongated with increase in age. The nodules are globose and pink in *I. parviflora*, while the nodules of *I. astragalina* and *I. linnaei* are cream and aggregated. The fresh weight of each nodule was more in *I. astragalina* (16.7 mg) and minimum in *I. parviflora* (3.0 mg). The dry weight of each nodule was also more in *I. astragalina* (5.1 mg), and minimum in *I. viscosa* and *I. parviflora* (1.3 mg and 1.2 mg, respectively). Maximum moisture content of 77.5% in the nodules was observed in *I. viscosa* while only of 60% was recorded in *I. parviflora.* The statistical analysis by taking cultivars as one treatment and the other parameters like no. of nodules, fresh and dry weight of the nodule as the second treatment reveals the F-calculated (Fc) value was greater that F-tabulated (Ft) value. If Fc>Ft then it is considered as significant. Therefore significance exists between cultivars and other parameters.

In all the *Indigofera* species studied, nodulation was initiated 3 weeks after seed germination and the number increased with age of the plant up to 70 DAS. That the number of nodules mainly increased during vegetative phase (30 to 70 d) and decline during flowering and pod

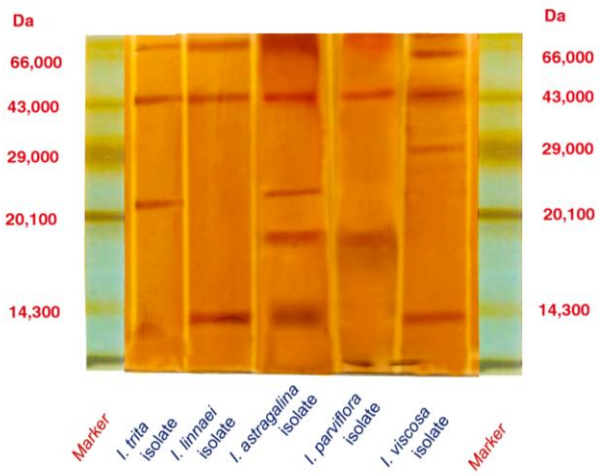

Figure 1: Electrophoretic banding pattern of total cellular proteins of five *Rhizobium* isolates from *Indigofera* species

setting phase, was also reported by Arya and Singh (1996) in horse gram.

Biochemical studies

The leghaemoglobin content of *I. astragalina* nodules gradually increased with age of the plant from 30 DAS and reached maximum of 512 µg/g at 60 DAS (Table 2). The leghaemoglobin content then showed a sudden decrease and reached the minimum of 171 µg/g at 90 DAS. A gradual increase in nitrogen content was also observed along with increase in leghaemoglobin content of the nodules and reached the maximum at 60 DAS. Similar type of positive correlation was also reported by Sidhu *et al.* (1967) in 6 legumes and Subba Rao and Chopra (1967) in soyabean. Raghava *et al.* (1993) reported that leghaemoglobin content of the nodules was peaked at 40–45 days. However, in the present study the peak was observed at 60 DAS.

Among the different plant parts analyzed, the maximum nitrogen content recorded was 4.76% in nodules, followed by leaves with 1.40% and shoot 1.26 % at 60 DAS. The nitrogen content of nodules was 3 times higher than that recorded in other plant parts at 30 DAS and showed a rapid increase in nitrogen content with increase in age. In all the aerial parts, the nitrogen content increased gradually from 30 DAS and reached maximum at 60 DAS and showed gradual decrease afterwards. Ezedinma *et al.* (1978) reported that among the 15 species of *Indigofera* studied, *Indigofera* sp. (EAO 19) gave the highest nitrogen concentration in the leaves (4.27%) followed by *I. simplicifolia*, *I. hirsuta* and *I. parniculata*. In the present study, relatively high nitrogen content was observed in nodules and leaves than in shoot, supporting the green manure status of *I. astragalina*. The statistical analysis by taking nitrogen at different DAS as one treatment and N content in other plant parts as the second treatment reveals the F-calculated (Fc) value was greater that F-tabulated (Ft) value. If Fc>Ft then it is considered as significant. Therefore significance exists between nitrogen contents and different plant parts.

Table 2: Biochemical analysis of different plant parts of *Indigofera astragalina* at different growth stages

DAS	Leghaemo-globin content (µg/g)	*Nitrogen content (%)			
		Nodules	Leaves	Root	Shoot
30	312	1.68	0.56	0.56	0.56
40	328	2.52	1.12	0.70	3.08
50	424	3.36	1.12	0.84	1.40
60	512	4.76	1.40	0.98	1.26
70	390	3.92	1.26	0.70	0.84
80	216	1.26	0.84	0.56	0.56
90	171	1.40	0.56	0.56	0.42

DAS days after sowing
Each data is an average of three replicates.
*Nitrogen content was significant at 5% between different plant parts (Fc = 8.02, Ft = 3.00)

Identification of *Rhizobium* strains

From the characterization tests it is evident that all the isolates form five *Indigofera* species are gram negative rods; acid producers; non 3-ketolactose producers and showed no growth in Hofer's medium and Glucose–peptone agar and are finally confirmed as *Rhizobia* by the nodulation test.

Enzymatic studies

The ability to produce different enzymes to utilize various organic substrates is an important biochemical characteristic feature of *Rhizobium* strains. Among the enzymatic activities studied, all the five isolates showed tryptophanase, nitrate reductase, citrase and catalase activities and do not exhibit phenyl alanine deaminase and DNase activities (Table 3). Variation was observed among the isolates for the enzymatic activities like protease, urease, gelatinase, caseinase, lipase, amylase and lysine decarboxylase. The variation in enzymatic activities of rhizobial isolates was reported by various workers. Graham and Parker (1964) reported that out of 79 strains of rhizobia studied, 58 are positive to nitrate reduction while 21 are negative. In the present study 4 out of 5 isolates were positive to nitrate reductase activity. Salve and Gangawanae (1992) reported that out of the 13 isolates studied by them some are positive and some are negative. In the present study only the isolate from *I. trita* showed amylase activity. Nitrate reductase activity, production of ammonia and catalase activities play an important role in nitrogen fixation metabolism. Enzymes like urease, protease, amylase, gelatinase play an important role during nodule formation. Thus, the *Rhizobium* isolates which produce these enzymes are considered as best for nodulation and nitrogen fixation.

Table 3: Enzymatic/Biochemical activities of *Rhizobium* isolates of *Indigofera* species

Enzymatic activities tested	Name of the isolates				
	I. trita	*I. linnaei*	*I. astragalina*	*I. parviflora*	*I. viscosa*
Tryptophanase	+	+	+	+	+
Nitrate reductase	+	-	+	+	+
Lysine decarboxylase	+	+	-	-	+
Amylase	+	-	-	-	-
Urease	+	+	-	-	-
Catalase	+	+	+	+	+
Citrase	+	+	+	+	+
Gelatinase	-	+	+	-	+
Caseinase	-	-	+	-	-
Lipase	-	+	-	-	+
Protease	+	+	+	-	+
Phenylalanine deaminase	-	-	-	-	-
DNase	-	-	-	-	-
Litmus milk reaction	Acid	Alkaline	Proteolysis	Proteolysis	Litmus reduction
Phosphate solubilization (Pikovskaya's medium)	-	-	-	-	+

Much variation was observed among the isolates with respect to litmus milk reaction. The isolate from *I. trita* produced acid and the isolate from *I. linnaei* produced alkaline. The two isolates from *I. astragalina* and *I. parviflora* showed proteolysis and the isolate from *I. viscosa* showed litmus reduction with serum zone formation. Growth reactions of *Rhizobium* in litmus milk, one of the characterization test to differentiate different isolates, have been studied by various workers (Graham and Parker, 1964; Muthusamy *et al*., 1973; Rangaswami and Oblisami, 1962; Oblisami, 1974) and these studies revealed that, the *Rhizobium* isolates from clover, pea and *Vicia* group produce alkalinity with the production of serum zone. While the rhizobia from cowpea, soyabean and lupin, produce alkalinity without the production of serum zone. However, Basak and Goyal (1980) reported that 4 isolates from *Acacia* and *Albizia* produce acidity without serum zone production. In the present study, the isolate from *I. viscosa* produced serum zone with acid reaction. This indicates that the present *Rhizobium* isolates from *Indigofera* species appears to be quite different from most of the *Rhizobia*, with respect to litmus milk reaction.

Among the five isolates studied the isolate from *I. viscosa* only showed clear zone around the colony in Pikovskaya's medium, indicating the ability to solubilize tri calcium phosphate. Thus, phosphate solubilization, an important criterion for plant growth promoting rhizobacteria, was exhibited by only *I. viscosa* isolate.

Protein profiles

In the present study, electrophoretic banding pattern of whole cell protein of five *Rhizobium* isolates was performed to study the similarity between them. Maximum of five bands were observed in the isolate from *I. astragalina* and four in the isolate from *I. viscosa*, while rest of the isolates produce only 3 bands (Figure 1). SDS-PAGE of whole-cell proteins of rhizobial strains from wild legumes, exhibited protein profiles with peptide bands ranging from 5–19 bands per profile was reported by Zahran *et al.* (2003). In the present study, all the five isolates showed two common bands at mol. wt. of 66,000 Da and 43,000 Da and shared rest of the three bands between different isolates. Sridevi and Mallaiah (2008) studied the SDS PAGE analysis of whole cell–protein and reported that *Rhizobium* isolate from *Sesbania sesban* produced a band at 70,000 Da for the outer membrane receptor protein involved in siderophore transport. Similarly in the present study also a common band for all the isolates was observed just above 66,000 Da, indicating that these isolates are also potential siderophore producers. The isolate from *I. viscosa* showed one additional band with mol. wt. of 29,000 Da, which was not shared by any other isolates. The isolates from *I. linnae, I*. *astragalina* and *I. viscosa* shared a common band with mol. wt. of 14,300 Da. In *I. astragalina* among the five bands, one band with approx. mobility at 23,000 Da was shared with that from *I trita* isolate while another band at approx mobility at 18,000 Da was shared with that from *I. parviflora* isolate. The SDS-PAGE analysis of whole cell proteins not only helps in identifying of the rhizobial strains (Roberts *et al.*, 1980; Fabiano and Arias, 1990) but also useful in the differentiation among the isolates within the same serogroup (Broughton *et al.*, 1987). By conducting the SDA-PAGE analysis of four *Azorhizobium* strains isolated from stem nodules of *Sesbania rostrata* Dreyfus *et al.* (1985) concluded that all the four strains have identical protein gel electropherograms and are closely related. In the present study the similarity index studies based on the number of bands, revealed that the isolate from *I. astragalina* showed 75% similarity with *I. trita*, *I. linnaei*, and *I. parviflora*, while the isolate from *I. viscosa* was different from other isolates by having an additional band. This clearly shows that the isolates were related with reference to number of bands but differ with reference to the quality

and quantity of the proteins. Zahran *et al.* (1994) reported a change in SDS PAGE protein banding pattern among the salt tolarent (that can grow up to 3% NaCl) and halophylic strains (that grow in the range of 3 – 10% NaCl) of *Rhizobia*. Similarly, the difference in banding pattern observed in the present study by the presence of an additional band in the isolate from *I. viscosa* can be correlated to the fact that this was the only halophylic isolate among the five isolates that can grow at 6% NaCl concentration (data not presented but studied). Thus, the isolate from *I. viscosa* with its halophylic nature and phosphate solubilizing capacity, can be exploited as an effective bioinoclulant.

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