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Morphological and Biochemical Characterization of Rhizobia Isolated from Root Nodule of *Crotolaria junceae* L. Grown in Assam

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Abstract: ***In the present study 14 bacterial strains were isolated from root nodule of Crotolaria junceae L. from 4 different sites of Assam. The isolates were investigated for their morphological characteristics. Three different confirmatory tests were performed with the isolates. The isolates were also investigated for their biochemical characteristics and compared with biochemical characteristics of reference strains Rhizobium leguminoserum MTCC-99, Bradyrhizobium japonicum MTCC-120 and Mesorhizobium thiogangeticum MTCC-7001 obtained from IMTECH, Chandigarh. The isolates showed close similarity in their morphological features but showed variation in confirmatory test results. The study revealed that most of the isolates are closely related to Rhizobium leguminoserum MTCC-99 and Mesorhizobium thiogangeticum MTCC-7001 based on their morphological and biochemical characteristic.***

Keywords: *Crotolaria junceae* L., *Rhizobium leguminoserum* MTCC-99, *Bradyrhizobium japonicum* MTCC-120 and *Mesorhizobium thiogangeticum* MTCC-7001.

1. Introduction

Nitrogen is required by all living organisms for the synthesis of proteins, nucleic acids and other nitrogen-containing compounds. The increased utilization of chemical fertilizers as a source of nitrogen for crops results in increased emissions of nitrogen oxides, soil acidification and water pollution. Rhizobia are the most well known species of a group of bacteria that acts as the primary symbiotic fixer of nitrogen. These bacteria can infect the roots of leguminous plants, leading to the formation of nodules where the nitrogen fixation takes place [1]. In last few decades, there has been a growing level of interest in environmental friendly sustainable agricultural practices, thus increasing the role of biofertilizers such as *Rhizobia* which can decrease the need for chemical fertilizers and reduce adverse environmental effects. The assessment of diversity of indigenous rhizobial strains in various regions of the world has received considerable attention and many attempts have been made to determine the composition of indigenous strains isolated from different cultivated and wild legumes [2, 3]. Rhizobia are taxonomically very diverse, so efficient classification methods are needed for determining their diversity. Usually the diversity of rhizobia was investigated by different phenotypic and biochemical methods. However, in the past few years, there have been many advances in molecular biology that have provided powerful tools mainly PCR-based which has helped in identifying the genotypic diversity of rhizobia more accurately [4].

Although most studies of rhizobia isolation were conducted on crop plants, less explored legumes like different shrubs and herbaceous plants have important roles in ecosystems. *Crotolaria junceae* L. is a member of the legume family *Fabaceae* and has great potential as an annually renewable, multi-purpose fiber crop [5]. As one of the most widely grown green manure crops throughout the tropics, it is often grown in rotation with several different crop species. Assam is one of Indian state situated in north-eastern part. Assam lies at $89^0$42' to 96^0 E longitude and 24^0 8'to $28^0$2'N latitude and has an area of 78,438 km^2. Assam is well known for its highly diverse flora and fauna. Different leguminous plants have been reported from this region which can form effective symbiosis with different strains of Rhizobia. In the present study an attempt has been made to isolate and characterize rhizobia from root nodules of *Crotolaria junceae* L. from Assam.

2. Material and Methods

2.1 Collection of sample

Root nodules of *Crotolaria junceae* L. plant were collected from four different sites namely Cachar, Karimganj, Hailakandi and N C Hill of Assam. The collected nodules were first washed with tap water and then surface sterilized with 75% ethanol for 3min and 0.1% $HgCl_2$for 5 min. Then nodules were immediately washed 5–6 times with sterile distilled water. The surface-sterilized nodules were crushed with the help of sterile glass rod and streaked on Yeast Extract Mannitol Agar (YEMA) plate. The plates were incubated at 28±2°C for 3-5 days [6]. Single colonies were picked up and were re-streaked on YEMA plates for obtaining pure cultures. Pure cultures of 3 reference strains *Rhizobium leguminoserum* MTCC-99, *Bradyrhizobium japonicum* MTCC-120 and *Mesorhizobium thiogangeticum* MTCC-7001 were obtained from Institute of Microbial Technology (IMTECH), Chandigarh and maintained as per instruction

2.2 Morphological characterization

Morphological and microscopic characteristics of all the isolates were investigated. After an incubation of 3 to 7 days at 28°C on yeast extract mannitol agar plate, individual colonies were characterized based on their size, color, shape and elevation [6]. Microscopic features of the isolates were studied by gram staining technique.

2.3 Confirmatory Tests

Three different confirmatory tests were performed to confirm the isolate as Rhizobia and to differentiate them from other contaminating microbes.

2.3.1 Growth on YEMA with Congo red

In general, Rhizobia produce white colonies, whereas many other bacteria take up the dye strongly. YEMA-Cr media was prepared and bacterial isolates were streaked on plates. The plates are then incubated at 28^{o}C for 48 hours [6].

2.3.2 Growth on Glucose Peptone Agar (GPA) medium:

Glucose peptone agar medium with Bromo-thymol blue (indicator) is widely used for isolating pure Rhizobial colonies. Rhizobia show no growth or very poor growth on glucose peptone agar medium and cause very little change in P^{H} when incubated at 25-30^{0}c. Heavy growth is indicative of contamination. Glucose peptone agar medium was prepared and bacterial isolates were streaked on plates. The plates are then incubated at 28^{o}C for 48 hours [6].

2.3.3 Keto-lactose Test

Keto-lactose test widely used to differentiate Rhizobia from other contaminating bacteria. Keto-lactose agar medium was prepared and bacterial isolates were streaked on plates. Inoculated plates were then incubated at 28^{o}c for 48 hours. Plates were then flooded with Benedict's Reagent, incubated at 25^{o}c and results were observed after 1 hour [7].

2.4 Biochemical characterization

The isolates were also investigated for seven different biochemical characteristics namely catalase test, oxidase test, nitrate reduction test, starch hydrolysis test, urease test, citrate utilization test and gelatin liquefaction test following standard procedure [7]. Biochemical characteristics of reference strains were also studied.

3. Results

In the present study a total of 14 bacterial samples were isolated from root nodule of *Crotolaria junceae* L. from four different sites of Assam. The isolates includes 4 isolates from Cachar, 4 isolates from Karimganj, 3 isolates from Hailakandi and 3 isolates from N.C.Hills. Most of the isolates showed similar colony morphology and produced white or creamy white and raised colonies when grown on YEMA plates. All the isolates were fast growers and their colonies reaches 1-3 mm in diameter after 4–5 days of incubation at 28^{0}C. The colonies were having sticky appearance showing the production of mucous. Microscopic examination revealed that the isolates were gram negative and rod in shape.

Figure 1: Pure culture of BCJ1.

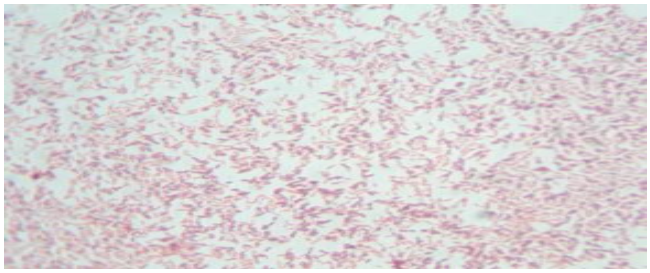

Figure 2: Gram stainig of BCJ1.

Confirmatory test was performed with all the 14 bacterial isolates. In GPA test 12 isolates showed no growth on GPA media and 2 isolates, KCJ1 and HCJ3 showed growth on GPA media. Similarly results of YEMA-Cr test revealed that 12 isolates showed growth on YEMA-CR media without absorbing the Congo red dye and 2 isolate KCJ1 and HCJ3 showed growth on YEMA-CR media by absorbing the Congo red dye. In keto-lactose test all the isolates showed no yellow color formation on keto-lactose media after adding Benedict's reagent except 1 isolate,DCJ1 that showed yellow color formation.

Table 1: Morphological characteristics of isolates.

Isolates	Sample Collection site	Colony size (mm)	Colony color	Colony shape	Gram staining
ICJ1	Cachar	2-3	White	Round	-
ICJ2	Cachar	2-3	White	Round	-
DCJ1	Cachar	1-3	Yellow	Round	-
DCJ3	Cachar	2-3	White	Round	-
KCJ1	Karimganj	1-3	White	Round	-
KCJ3	Karimganj	2-3	White	Round	-
BCJ1	Karimganj	1-2	White	Round	-
BCJ2	Karimganj	1-3	Yellow	Round	-
HCJ1	Hailakandi	1-2	White	Round	-
HCJ3	Hailakandi	2-3	White	Round	-
HCJ 4	Hailakandi	1-3	White	Round	-
NCCJ1	N. C. Hills	2-3	White	Round	-
NCCJ2	N. C. Hills	1-3	White	Round	-
NCCJ4	N. C. Hills	2-3	White	Round	-
R. leuminoserum MTCC-99	-	1-3	White	Round	-
B. japonicum MTCC-120	-	1-3	White	Round	-
B. cepacia MTCC-4684	-	1-3	White	Round	-

Table 2: Confirmatory test of isolates

Isolates	GPA Test	YEMA-CR Test	KLA Test
ICJ1	-	+	-
UCJI/ICJ2	-	+	-
DCJ1	-	+	+
ZCJIII/DCJ3	-	+	-
KCJ1	+	-	-
UCJII/KCJ3	-	+	-
BCJ1	-	+	-
JCJ/BCJ2	-	+	-
ZCJI/HCJ1	-	+	-
ZCJII/HCJ3	+	-	-
HCJ 4	-	+	-
NCCJ1	-	+	-

NCCJ2	-	+	-
NCCJ4	-	+	-
R. leuminoserum MTCC-99	-	+	-
B. japonicum MTCC-120	-	+	-
B. cepacia MTCC-4684	-	+	-

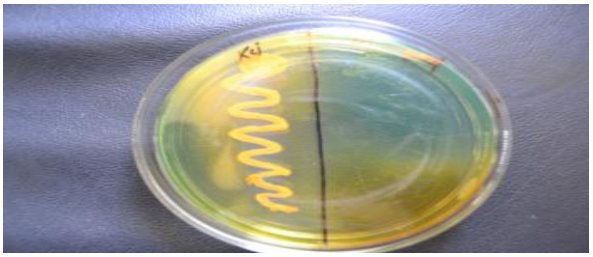

Figure 3: GPA test results of KCJ1 and DCJ1.

Figure 4: YEMA-Cr test results of KCJ1 and ICJ1.

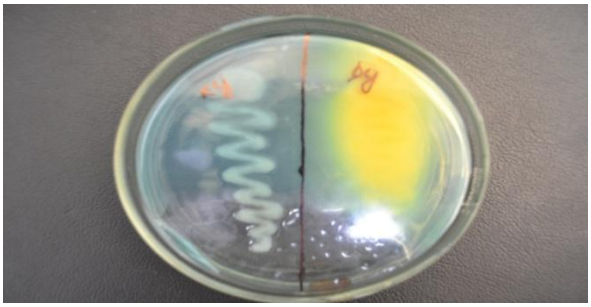

Figure 5: Ketolactose test results of KCJ1 and DCJ1.

After performing all the confirmatory tests, the isolates were investigated for their biochemical characteristics along with the reference strains. Most of the isolates showed positive result for catalase test, oxidase test, nitrate reduction test, urease test and negative result for starch hydrolysis test, citrate utilization test and gelatin liquefaction test. Except isolate DCJ3 which showed negative result for nitrate reduction test and HCJ3 which showed negative results for urease test.

Table 3: Biochemical characteristics of isolates

Isolates	Catalase test	Oxidase test	Nitrate test	Starch test	Urease test	Citrate test	Gelatin test
ICJ1	+	+	+	-	+	-	-
ICJ2	+	+	+	-	+	-	-
DCJ1	+	+	+	-	+	-	-
DCJ3	+	+	-	-	+	-	-
KCJ1	+	+	+	-	+	-	-
KCJ3	+	+	+	-	+	-	-
BCJ1	+	+	+	-	+	-	-
BCJ2	+	+	+	-	+	-	-
HCJ1	+	+	+	-	+	-	-
HCJ3	+	+	+	-	-	-	-
HCJ 4	+	+	+	-	+	-	-
NCCJ1	+	+	+	-	+	-	-
NCCJ2	+	+	+	-	+	-	-
NCCJ4	+	+	+	-	+	-	-
R. leuminoserum MTCC-99	+	+	+	-	+	-	-
B. japonicum MTCC-120	+	+	+	-	+	+	-
B. cepacia MTCC-4684	+	+	+	-	+	-	-

4. Discussion

Crotolaria junceae L. commonly known as 'sunn hemp' is generally considered to have originated in India, where it has been cultivated since prehistoric times [8]. In the present study, a total of 14 bacterial samples were isolated from root nodules of *Crotolaria junceae* L. growing in Assam and characterized based on their morphological and biochemical features. There were several reports describing the characterization of rhizobia based on morphological and biochemical features. Gachande and Khansole (2011) isolated Rhizobia from root nodules of Soy bean (*Glycine max* L.) and characterized them as *Rhizobium joponicum* and *Bradyrhizobium japonicum* based on its morphological, cultural and biochemical characteristics [9]. The present study revealed that morphological and microscopic features of most of the isolates are very much similar with the morphological and microscopic features of reference strains. However the isolates DCJ1 and BCJ2 produce yellow color colonies on YEMA media which don't resembles with the colony color of reference strains, further rhizobia usually produces white or creamy white colonies on YEMA media. So the two isolates may not be considered as a probable rhizobial isolates. For confirming the isolates as rhizobia, 3 confirmatory tests were performed. Most of the isolates except (KCJ1 and HCJ3) showed no absorption of congo red dye on YEMA-Cr media which is consistent with the results of Trinick *et al* who reported that rhizobia don't absorbed congo red dye or absorbed very weekly compared with other bacteria [10]. Regarding the growth in glucose peptone agar Vincent *et al* reported that rhizobia showed either no growth or grow very poorly on GPA media. While further confirming these isolates, out of 14 isolates, 12 isolates showed either poor or no growth on GPA medium indicating

the features of rhizobia. Remaining 2 isolates (KCJ1 and HCJ3) invariably showed growth on the medium. Result of ketolactose test revealed that most of the isolates showed negative result for the production of 3-ketolactose from lactose. However, one isolate (DCJ1) showed positive result (showed growth on the medium) which is in contradiction to earlier work carried out by Sadowsky *et al* [11]. The reference strains showed standard confirmatory test result of Rhizobia. The isolates DCJ1, BCJ2, KCJ1 and HCJ3 that showed no resemblance of morphological characteristics and confirmatory test results with the reference strains and contradicts with the reports of earlier works may be considered as contaminating strain.

Biochemical characterization of the isolates revealed that most of the isolates were oxidase, catalase and urease, nitrate reduction positive and unable to utilize citrate, which complements with the reults of Lupwayi and Hague [12]. However, the isolate DCJ3 showed negative results of nitrate reduction test and the isolate HCJ3 showed negative results of urease test. All the isolates showed negative results for starch hydrolysis. It was also observed that the isolates did not produce gelatinase enzymes. Negative gelatinase activity of *Rhizobium* was also observed by Hunter *et al* [13]. The results also confirm that the biochemical features of all the isolates except DCJ3 and HCJ3 are similar to the biochemical features of reference strains, *Rhizobium leguminoserum* MTCC-99 and *Mesorhizobium thiogangeticum* MTCC-7001. Similar to the present study Deshwal *et al* (2014) reported the isolation and characterization of *Rhizobium leguminoserum* from root nodules of *Pisum sativum* L. based on their morphological and biochemical characteristics [14].

5. Conclusion

Thus, from the present study it can be concluded that most of the bacterial strains isolated from *Crotolaria junceae* L grown in Assam are closely related to *Rhizobium leguminoserum* and *Mesorhizobium thiogangeticum* based on their morphological, confirmatory and biochemical characteristics.

6. Future Prospects

Since, in the present study the isolates were characterized based on their morphological and biochemical features, in future suitable PCR based genotypic techniques can be employed for confirming the identity of the isolates at strain level and for predicting the phylogenetic relationship of the isolates with other known isolates. The above study can be significantly helpful to understand the diversity of rhizobia in Assam associated with the studied plant.

7. Acknowledgements

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