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Isolation and Biochemical Characterization of Indigenous *Rhizobium* Strains from Root Nodules of Field Grown Soybean Crop

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A B S T R A C T

Total of eighteen (18) nodule samples of different 18 soybean cultivars were collected. Eighteen soybean varieties were grown in the Research field of Department of Plant Breeding and Genetics, College of Agriculture, Indira Gandhi KrishiVishwavidyalaya, Raipur (C.G.) during *kharif*2019-20. The present study was aimed to isolate the beneficial nitrogen fixing *Rhizobium* from root nodules of Soybean varieties to evaluate the effective symbiosis. The isolation, morphological and biochemical characterization were carried out in the laboratory of Department of Agricultural Microbiology, College of Agriculture, Raipur. Isolated *Rhizobium* strains were subjected to cultural and colony morphology tests on Yeast Extract Mannitol Agar (YEMA) from which top ten isolates showing good colony growth were selected for the biochemical characterization. After series of biochemical and Sugar Fermentation tests SRh-1, SRh-03, SRh-18, SRh-137 and SRh-136were identified as promising strains. This study confirms the presence of promising indigenous Rhizobia in some Soybean varieties *i.e.*, NRCSL 1, RSC 11-03, RKS 18, NRC 137 and NRC 136 over other varieties for effective symbiosis with indigenous rhizobia.

Introduction

The Golden bean, soybean is one of the legumes that have the ability to fix atmospheric nitrogen. It contains 40 % vegetable protein, 20 % oil, 21% Carbohydrates and 11.5% Iron (Purohit and Kumar, 1998).The soyprotein's high biological value, protein efficiency ratio and essential amino acid pattern make it superior to most of plant proteins. New cultivars of soybean are being developed continuously (Akhande*et al.*, 2007). It is therefore important to screen these cultivars on the basis of efficient indigenous Rhizobia to determine their performance in terms of yield and adaptation to different ecologies. The bacteria colonize within root nodules, where it converts atmospheric nitrogen to ammonia and provides organic nitrogenous compounds to the plants. It has been proven that plant productivity increases when the Rhizobia are present in rhizosphere. It provides the major biological source of fixed nitrogen in agricultural soils. A well established practice for maintaining soil fertility has been

evaluated for the cultivation of leguminous plants which replenish atmospheric nitrogen through symbiosis with rhizobia in rotation with non-leguminous plants. This study was aimed to isolate *Rhizobium* species from root nodules for better productivity.

There is an increasing demand of soybean for proteins and vegetable in tropics. To meet this requirement it is necessary to promote soybean production. Without inoculating tropical soils with *Rhizobium* many of the soybean varieties are unable to nodulate effectively. This inability to nodulate effectively with indigenous *Rhizobium* strain is one of the major factors preventing the production expansion of the soybean crop. Consequently it becomes inevitable to inoculate the crop in adequate *Rhizobium* (Deka and Azad, 2006). There is wide variation in the ability of *Rhizobium* strains to nodulate different soybean varieties. Thus, to understand responses and study the diversity and effectiveness of Rhizobia, isolation and characterization of Rhizobial population is necessary. The diversity and effectiveness of different isolates was studied on the basis of different biochemical tests *viz.* Triple sugar iron test, Starch utilization test and Catalase test (Tyagi *et al.,* 2017). The isolates with an outstanding performance are identified, in a search for efficient and competitive strains for use in commercial inoculants in Chhattisgarh. Therefore in this study, the diversity of Rhizobial isolates from soybean root nodules, collected under field condition was studied.

Materials and Methods

This study was conducted in College of Agriculture, Raipur located at 21^0 16'N latitude and 81^0 36'E longitude, at an average elevation of 298.58 meters above the mean sea level (MSL). Total of eighteen (18) Soybean varieties were collected randomly from the Soybean field. The soybean varieties were collected at 50 % flowering stage from the Research field of the Department of Plant Breeding and Genetics, College of Agriculture, Raipur and taken from the field to the laboratory for the isolation of *Rhizobium* from nodules.

Isolation: Healthy root nodules were washed with tap water thrice before streaking on agar plate. The nodules were sterilized externally using 95 % alcohol for 1-4 minute, followed by washing with calcium hypochlorite solution (10g/150ml distilled water) and crushing in a drop of sterile water. A loopful ground material was transferred to 5 ml of sterile water, of which 0.1 ml sample was spread onto the surface of Yeast Extract Mannitol Agar (YEMA). Plates were then incubated at 28^0C for 48 hours. Well isolated typical single colonies were re-streaked on freshly prepared YEMA plates in order to obtain pure cultures. The *Rhizobium* isolates were designated as SRh-1613, SRh-1611, SRh-3108, SRh-136, SRh-1493, SRh-03, SRh-1, SRh-137, SRh-39, SRh-130, SRh-131, SRh-147, SRh-34, SRh-992, SRh-102, SRh-11, SRh-18 and SRh-52. The eighteen soybean rhizobia isolated from 18 soybean varieties are given in Table 1 and named accordingly.

Morphological characteristics and Gram-staining of *Rhizobium* isolates: The colony characters *viz.* shape of the colony, size, elevation, margin, color, surface and motility characters were observed on YEMA medium. The *Rhizobium* isolates were gram-stained for more specified identification of the colonies. The bacterial cultures were smeared on the sterilized glass slides and fixed. Smear was stained with Crystal violet for one minute and rinsed with water. Then smear was flooded with Iodine solution for one minute. Excess of iodine solution was drained off and smear was decolorized with 95% alcohol for 30 seconds followed by washing with distilled water.

Smear was counter stained with Safranin for 30 seconds, followed by washing with distilled water and air drying. T hen, observed under oil immersion objective of research microscope (Wadhwa *et al.,* 2017).

Biochemical Tests: All the Rhizobium isolates were processed through biochemical tests *viz*. Catalase Test, Starch Hydrolysis/Amylase Test, as given in Bergey's manual of systematic bacteriology (2001). For starch utilization, inoculation by streaking of each isolate in Starch Agar Medium was done and incubated at 30^0C. After 24 hours of incubation iodine solution was flooded into the plates to determine the capability of microbes to use starch. A clear zone of inhibition around bacterial colonies indicated starch hydrolysis. The production of oxygen bubbles within a minute after addition of H_2O_2on Trypticase soy agar slants with Rhizobium culture was the indicative of catalase positive test and no bubbles signifies negative result as described by Graham and Parker (1964).

Sugar Fermentation Tests: The isolates were also examined for fermentation of the various sugars including Glucose, Lactose, Fructose, Gas and H_2S production (Triple Sugar Iron Test, TSI) as described byAneja (2007). Bacterial cultures were inoculated into the triple sugar iron agar slants by stabbing through the center of the medium to the bottom and then streaked over the surface followed by incubating at 37^0C for 2-5 days. The change in color of the slant and the formation of butt was observed.

Dehydrogenase activity in rhizosphere soil of different varieties of soybean:10 g fresh soil samples were mixed with 2.5 ml of phosphate buffer, 0.2 g $CaCO_3$, and 1 ml of 3% Triphenyl-tetrazolium chloride (TTC) and incubated at 25^0C for 24 h. After incubation, the samples were centrifuged at 3000rpm for 10 min. The supernatant liquid was discarded. The red-colored triphenyl-formazan (TPF) formed was extracted with methanol. 5 ml methanol was added to each of the tubes and vigorously shaken for a few minutes. The operation was repeated twice with 10 ml of methanol and centrifuged again the tubes. The absorbance of supernatant liquid obtained was measured for λ = 485nm as mentioned by Casida *et al.,* (1964).

Results and Discussion

Out of eighteen samples of root nodules from Soybean all were found positive for the presence of Rhizobium. Gram Negative rods with circular, Raised and smooth edges colony with musky odour were observed in all 18 strains. The data depicted in Table 2 revealed that among 18 samples tested all the isolates were found positive for the presence of *Rhizobium* on the basis of regular white mucilaginous colony growth on YEMA medium. The top ten isolates having good colony growth were preserved on YEMA medium slants for further characterization.

Out of 18 strains, 10rhizobia strains showing good colony growth were screened through a series of various biochemical and Sugar fermentative tests depicted in Table 3. All the *Rhizobium* isolates were able to show starch hydrolysis. Two of the *Rhizobium* isolates *i.e.*SRh-1 and SRh-136 showed good clear zone.

All the top 10 *Rhizobium* isolates showed positive for catalase test. This finding is in close agreement with Saldana *et al.,* (2003); Shahzad *et al.,* (2012) and Gauri *et al.,* (2017). All the top ten *Rhizobium* isolates showed positive test for glucose, lactose and sucrose fermentation. Similar results were reported by Sharma *et al.,* (2009) and Tyagi *et al.* (2017).

Table.1 Isolation of Rhizobium strains from root nodules of different Soybean varieties

S. No.	Soybean *Rhizobium* Isolates	Different Soybean varieties from which Rhizobium isolated
1.	SRh-1613	PS 1613
2.	SRh-1611	PS 1611
3.	SRh-3108	DS 3108
4.	SRh-136	NRC 136
5.	SRh-1493	MACS 1493
6.	SRh-03	RSC 11-03
7.	SRh-1	NRCSL 1
8.	SRh-137	NRC 137
9.	SRh-39	AMS 100-39
10.	SRh-130	NRC 130
11.	SRh-131	NRC 131
12.	SRh-147	NRC 147
13.	SRh-34	DSb34
14.	SRh-992	KDS 992
15.	SRh-102	BAUS 102
16.	SRh-11	SKF-SP-11
17.	SRh-18	RKS 18
18.	SRh-52	JS 97-52

Table.2 Colony morphology and Gram's reaction of indigenous *Rhizobium* Isolates of Soybean varieties

S. No.	Rhizobium Isolates	Morphological characters		Colony characters				
		Shape	Gram's Reaction	Colony growth	Margin	Elevation	Shape	Colour
1.	SRh-1613	Rod	Negative	+	Regular	Convex	Circular	Whitish pink and Glistening
2.	SRh-1611	Rod	Negative	+ + +	Regular	Convex	Circular	Whitish pink and Glistening
3.	SRh-3108	Rod	Negative	+ + +	Regular	Convex	Circular	Whitish pink and Glistening
4.	SRh-136	Rod	Negative	+ + +	Regular	Convex	Circular	Whitish pink and

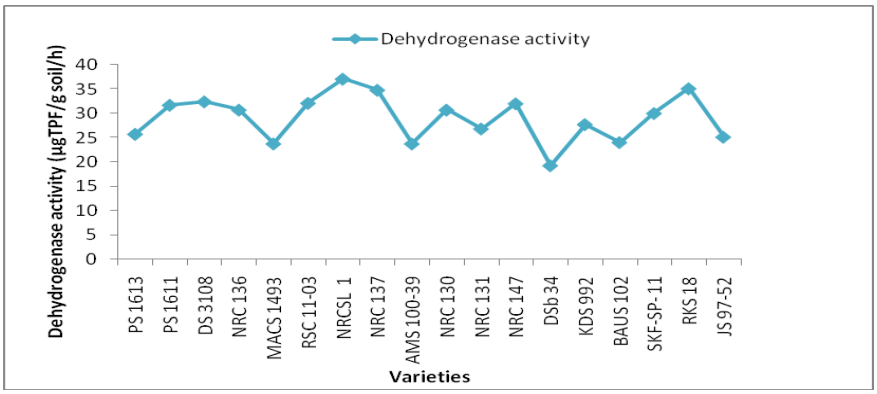
								Glistening
5.	SRh-1493	Rod	Negative	+	Regular	Convex	Circular	Whitish pink and Glistening
6.	SRh-03	Rod	Negative	+ + +	Regular	Convex	Circular	Whitish pink and Glistening
7.	SRh-1	Rod	Negative	+ + +	Regular	Convex	Circular	Whitish pink and Glistening
8.	SRh-137	Rod	Negative	+ + +	Regular	Convex	Circular	Whitish pink and Glistening
9.	SRh-39	Rod	Negative	+	Regular	Convex	Circular	Whitish pink and Glistening
10.	SRh-130	Rod	Negative	+ + +	Regular	Convex	Circular	Whitish pink and Glistening
11.	SRh-131	Rod	Negative	+ + +	Regular	Convex	Circular	Whitish pink and Glistening
12.	SRh-147	Rod	Negative	+ + +	Regular	Convex	Circular	Whitish pink and Glistening
13.	SRh-34	Rod	Negative	+	Regular	Convex	Circular	Whitish pink and Glistening
14.	SRh-992	Rod	Negative	+	Regular	Convex	Circular	Whitish pink and Glistening
15.	SRh-102	Rod	Negative	+	Regular	Convex	Circular	Whitish pink and Glistening
16.	SRh-11	Rod	Negative	+	Regular	Convex	Circular	Whitish pink and Glistening
17.	SRh-18	Rod	Negative	+ ++	Regular	Convex	Circular	Whitish pink and Glistening
18.	SRh-52	Rod	Negative	+	Regular	Convex	Circular	Whitish pink and Glistening

Table.3 Biochemical characterization of Rhizobial isolates of Soybean

S. No.	Soybean varieties	Rhizobium Isolates	Biochemical Characterization						
			Amylase test	Catalase test	Utilization of Carbon source (TSI)				
					Glucose fermentation	Lactose fermentation	Sucrose fermentation	Gas production	H_2S production
1.	NRCSL 1	SRh-1	++ +	+ + +	+	+	+	+	+
2.	RSC 11-03	SRh-03	+ + +	+ + +	+	+	+	+	+
3.	RKS 18	SRh-18	+ + +	+ + +	+	+	+	+	+
4.	NRC 137	SRh-137	++ +	+ + +	+	+	+	-	-
5.	NRC 136	SRh-136	+ + +	+ + +	+	+	+	-	-
6.	NRC 147	SRh-147	+ +	+ +	+	+	+	-	-
7.	NRC 131	SRh-131	+ +	+ +	+	+	+	-	-
8.	DS 3108	SRh-3108	+ +	+ +	+	+	+	-	-
9.	PS 1611	SRh-1611	+ +	+ +	+	+	+	-	-
10.	NRC 130	SRh-130	+ +	+ +	+	+	+	-	-

***+:** Positive result, -: no results (Negative result)

Fig.1 Dehydrogenase activity in rhizosphere soil of different Soybean varieties



These findings corroborate with the results of Michael (2006); Singh (2008) and Erum (2008) who also reported these sugar tests positive during isolation and characterization of *Rhizobium*. The three isolates (SRh-1, SRh-03 and SRh-18) were shown positive for the gas and H_2S production, while other seven isolates were shown negative for the same. After screening through various biochemical and Sugar fermentative tests the top five isolates were SRh-1, SRh-03, SRh-18, SRh-137 and SRh-136. The dehydrogenase activity of rhizosphere soils from all target soybean varieties has been depicted in Fig 1.The DHA was observed between 19.2 to 37.1µgTPF/g soil/h. The maximum dehydrogenase activity was shown by soil of soybean variety NRCSL 1 (37.1µgTPF/g soil/h) followed by RKS 18 (35.1µgTPF/g soil/h).

In conclusion from biochemical studies of indigenous soybean rhizobia isolates and microbial activities in Soybean rhizosphere soils, it is seen that SRh-1, SRh-03, SRh-18, SRh-137 and SRh-136 were promising indigenous *Rhizobium* isolates. Screening of indigenous strains as per local Chhattisgarh agro-climatic condition is being significant

and can be used as bio-inoculants in Soybean crop cultivation for enhancing the productivity.

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