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Molecular Characterization and diversity of Rhizobium sp. from Centrosema viriginianum

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Abstract

The study was conducted to find out the diversity of the rhizobia that nodulate Centrosema viriginianum from different location of Kottayam district. Ten isolates were isolated from five different locations of Kottayam district, Kerala. All strains were gram negative, and produce white gummy colonies on CRYEMA medium. The YEMA medium was enriched with BTB (25 μg /ml) also helped to selectively identify Rhizobium sp. The rhizobia that nodulated the collected plants were diverse in colony morphology, growth at different pH ranges as well as in their ability to utilize different carbon sources. About 60% were found to be fast growers with the rest (40 %) alkalizing the medium are grouped under very slow growers. Various biochemical and molecular characterization tests were conducted and the results confirmed that isolated strains were Bradyrhizobium and Rhizobium sp.

Keywords: Rhizobium sp., Bradyrhizobium , Centrosema viriginianum, Bromothymol blue, Molecular characterization.

Introduction

Nitrogen is one of the most important elements for growth and productivity of plants. Because of high solubility of nitrogen resources, it is easily removed from the soil profile and becomes out of reach for crop plants (Flint, Harrison et al. 2008). Hence there is a constant need to renew nitrogen content of soil by appropriate procedures. Due to destructive effects of exogenous nitrogen supplies on the environment, livestock and human health (Cockburn et al., 2013) legumes could play a crucial role as natural providers of soil nitrogen via the establishment of a symbiotic relationship with Rhizobia (Ivanov et al. 2012). Soil microorganisms specifically bacteria called Rhizobia are able to colonize the rhizosphere, infect legume roots and biologically fix nitrogen in the soil through symbiotic process. Biological Nitrogen fixation is a process of converting elemental nitrogen into the form of ammonia (4 NH^+) and nitrate (3 NO^-) available to plants (Gothwal, et al. 2008). Rhizobia can live on plant residues (saprophytes) or entirely within plants (endophytes) or (rhizobacteria) or in close association with the plant (Anwer, 2013). Based on ability to fix nitrogen, Rhizobia are classified into slow (Bradyrhizobium) and fast growing Rhizobia. The process in which the Rhizobia colonize the rhizosphere, infect the roots and fix nitrogen leads to plant development and grain yield improvement (Deshwal et al. 2013).Legumes are one of the most diverse plants on earth widespread in tropics and temperate zones. They belong to one of superfamily of angiosperms (Leguminosae

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/Fabaceae) the order Fabales, in eurosid clade (Forest and Chase, 2009). Legumes can grow in much degraded soils because they have the ability to fix nitrogen in association with Rhizobia (Freitas et al., 2004). Besides its major role in the traditional diets throughout the world, legumes provide a multiple benefits to both soil and other crops through intercropping (Stajkovic et al., 2011). Despite vast and potential uses of grain legumes like soybean, cowpea, common bean and peas as human food, animal feed and soil fertility enhancer, they can be grown in different agro-ecological zones (Hendawey and Yones, 2013). In most cases, native nitrogen fixers are competitive to inoculants but not efficient strain and possibly incompatible to the host plant (Wilkinson et al., 1996). Therefore, relying on native nitrogen fixers without prior information on its efficiency and compatibility with host legume leads to crop production failure. The interaction between plants and soil microorganisms occurs much in the rhizosphere (Marschner et al., 2011). The legume-rhizobial symbiosis has a large impact on success of legumes hence the atmospheric nitrogen the organisms fix can be more than the fertilizer nitrogen an average farmer can afford to buy and apply .Therefore, legume-Rhizobia symbiosis can provide easy and inexpensive way to enhance soil fertility and improve crop production. The root nodulating Rhizobia approximately reduce 20 million tons of atmospheric nitrogen to ammonia which is 50% - 70% of the world biological nitrogen fixation. The higher fixed nitrogen in hosts determines the success of symbiotic relationship between legumes and Rhizobia (Crews, 1999). However, host range expansion may be limited by the symbiont distribution while hosts can potentially acquire different Rhizobia when invading new habitats (Parker et al.,2006)

Materials and methods

Collection of Samples

Root nodules were collected from the young and healthy seedling of Centrosema viriginianum plant from different location of Kottayam district, Kerala state. Centrosema viriginianum plants were uprooted carefully so as to get nodule intact.

Isolation of Rhizobium species from Centrosema viriginianum roots

Root nodules of Centrosema viriginianum was washed with sterile water followed by surface treatment with 95% alcohol then surface sterilized with 0.1% mercuric chloride for 2-3 minutes and again washed for at least 10 times with sterile water as to remove the traces of mercuric chloride. The nodules were transferred in culture tube half filled with sterile water and crushed with a sterile glass rod to obtain a milky bacterial suspension. After serial dilution suspension was streaked on YEMA (Yeast Extract Mannitol Agar) media plates and incubated at 28°C. After 10 days of incubation at 28° C, Rhizobium colonies were obtained. Pure isolates were used for further analysis.

Colony Morphology:

The colony morphology of isolates was examined on YEMA plates. After an incubation of 3-5 days at 28° C, individual colonies were characterized based on their colour, shape, appearance, colony diameter, transparency and margin (Dubey and Maheshwari, 2007).

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Biochemical charecterization of Rhizobium

The isolates were checked for contamination and particularly to distinguish the Rhizobium from Agrobacterium using the following biochemical tests:Gram stining, IMViC, catalase and oxidase tests

Congo red Yeast Extract Mannitol Agar medium

Incorporated medium (2.5 ml of 1% aqueous solution of the dye per one liter of YEMA) colonies of Rhizobia in Congo red are of white, translucent, glistening, elevated in nature and comparatively smaller with entire margin in contrast to the stained Agrobacterium colonies. The composition of the medium was Mannitol (10 g), K_2HPO_4 (0.5 g), $MgSO_4$.7H2O (0.2 g), NaCl (0.1 g), Yeast Extract (1 g), $CaCO_3$ (1 g), Agar – Agar (20 g), and distilled water (1000 ml) and a final pH - 7.0. The medium was sterilized at 15 lbs at 121^0C for 15-20 min in an autoclave. Three plates were streaked for isolation from each nodule. Upon incubation up to 10 days, the colonies of bacteria emerging were picked up and transferred to YEMA slants.

Bromothymol blue test (Santamaria, 2006)

The YEMA medium was enriched with bromothymol blue (25 µg /ml) to selectively identify Rhizobium.All the samples were subjected to grow on BTB added medium. The positive samples showed moist and gummy colonies after incubation for 48 hrs at 28°C and surrounding medium plate were yellow due to acid production.

Phylogenetic analysis

Genomic DNA was isolated using NucleoSpin® Tissue Kit (Macherey-Nagel) following manufacturer's instructions. PCR amplification reactions were carried out in a 20 µl reaction volume which contained 1X PCR buffer (100mM Tris HCl , pH-8.3; 500mM KCl), 0.2mM each dNTPs (dATP, dGTP, dCTP and dTTP), 2.5mM $MgCl_2$, 1 unit of AmpliTaq Gold DNA polymerase enzyme, 0.1 mg/ml BSA, 4% DMSO, 5pM of forward 16S-RS-F CAGGCCTAACACATGCAAGTC and reverse primers 16S-RS-R GGGCGGWGTGTACAAGGC. The PCR amplification was carried out in a PCR thermal cycler (GeneAmp PCR System 9700, Applied Biosystems).

ExoSAP-IT Treatment

ExoSAP-IT (USB) consists of two hydrolytic enzymes, Exonuclease I and Shrimp Alkaline Phosphatase (SAP), in a specially formulated buffer for the removal of unwanted primers and dNTPs from a PCR product mixture with no interference in downstream applications. Five micro litres of PCR product is mixed with 2 µl of ExoSAP-IT and incubated at 37°C for 15 minutes followed by enzyme inactivation at 80°C for 15 minutes.

Sequencing using Big Dye Terminator v3.1

Sequencing reaction was done in a PCR thermal cycler (Gene Amp PCR System 9700, Applied Biosystems) using the Big Dye Terminator v3.1 Cycle sequencing Kit (Applied Biosystems, USA) following manufactures protocol. The sequencing PCR temperature profile consisted of a 1st

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cycle at 96°C for 2 minutes followed by 30 cycles at 96°C for 30 sec, 50°C for 40 sec and 60°C for 4 minutes.

Sequence Analysis

The sequence quality was checked using Sequence Scanner Software v1 (Applied Biosystems). Sequence alignment and required editing of the obtained sequences were carried out using Geneious Pro v5.6

Result and discussion

Isolation of samples

Ten Rhizobial isolates were obtained from different areas of kottayam districts from 5 different sites, 5 isolates grown with root nodules of Centrosema viriginianum plant and 5 isolates grown with rhizosphere soil .The Rhizobial isolates were named S(1)RN-S(5)RN and S(1)RS-S(5)RS. Root nodulating bacteria and rhizosphere bacteria were isolated from Centrosema viriginianum, and the growth period of the isolate was between 3 and 5 days which indicated that isolates were fast grower. The cells of different isolates were small to medium sized rods and were Gram negative. All the isolates grew well on yeast extract mannitol agar slants and Congo red yeast extract mannitol agar plates. Colonies of Rhizobium were observed on YEMA medium after incubation for 3-4 days at 28^0C. Raised, gummy and smooth margin colonies with creamish appearance were observed. Microscopic observation of these isolates showed them to be Gram negative rods. The YEMA medium was enriched with BTB (25 μg /ml) to selectively identify Rhizobium sp. (Vincent, 1970). The inoculated media was incubated at 28°C for 3-4 days. After incubation moist and gummy colonies were observed and surrounding medium plate was yellow due to acid production by the Rhizobium sp.

Identification of Rhizobium

Identification tests are used for the distinguishing Rhizobium from Agrobacterium. In Congo red medium, rhizobia stand out as white, translucent, glistening, elevated and comparatively smaller colonies in contrast to stained colonies of Agrobacteria. On Bromothymol Blue medium, fast growing Rhizobium showed an acid reaction, turning the medium yellow. From the above observations (table 3) we could conclude that the test organism was Rhizobium sp. slow-growing rhizobia dominate in tropical soils, in our results showed that about 80 % of isolates from nodules were fast- growing species and 60% were fast growers isolated from soil. Among the 10 isolates, 60% rhizobia are acid producers were more prominent than the alkalizers (Sanginga et al. 1989).

Biochemical characteristics of Rhizobium

The results shown in table 4.4 indicated that all the isolates of Rhizobia were positive to the Oxidase. In catalase test S (1) RN and S (4) RS were positive were as other isolates were negative. In Urease test S (1) RS, S (2) RS and S (3) RS were negative were as other isolates are positive. All the isolates not produced H_2S and S (3) RS not utilized citrate as sole source of carbon. Indole test, Starch hydrolysis and gelatin hydrolysis test were negative for all isolates .These isolates were slow-growers and

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unable to utilize starch and gelatin components. In methyl red test, the isolate S(1)RN were positive, whereas remaining Rhizobium isolates were negative. In voges-prokauer test, acetoin produced from glucose, the isolates S(2)RN, S(5)RN were positive whereas other isolates were negative. In triple sugar Iron agar test, S(5)RN and S(1)RN rhizobial isolates were fermented the glucose and sucrose with no gas in the test tubes the S(2)RN isolates were fermented the glucose not sucrose and produced gas. Genomic DNA was isolated from two Rhizobial isolates designated as S(1)RN was closely related to KF596692 which is 99% similarity ans 0.02% divergence(figure 1). Dendofram of S(4)RS (fig 2) reveals that the isolate was 100% identity to MK249882.1 belongs to Rhizobium sp.

Conclusion

Symbiotically associated Rhizobium sp from root nodule of Centrosema viriginianum was isolated and characterized as fast growers.

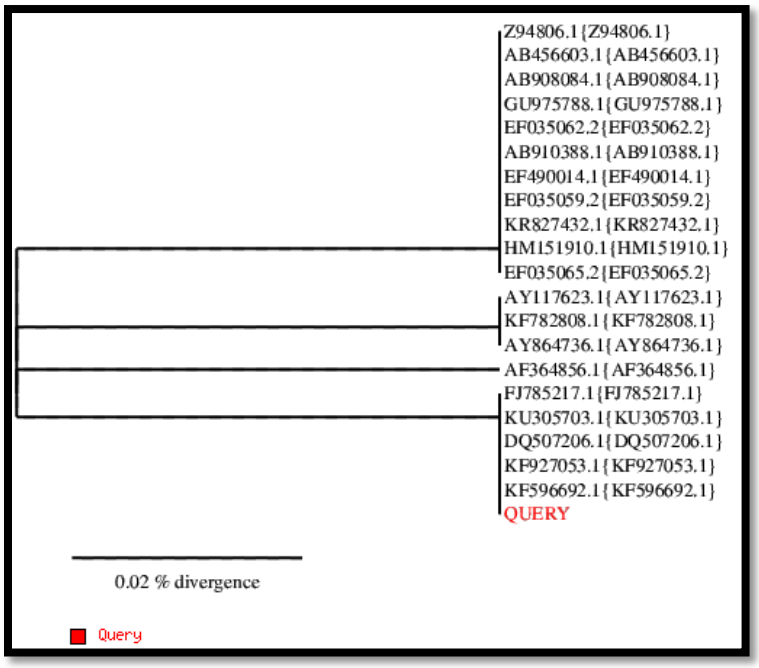

Figure 1. Phylogenetic of S1RN closely related to Rhizobium sp

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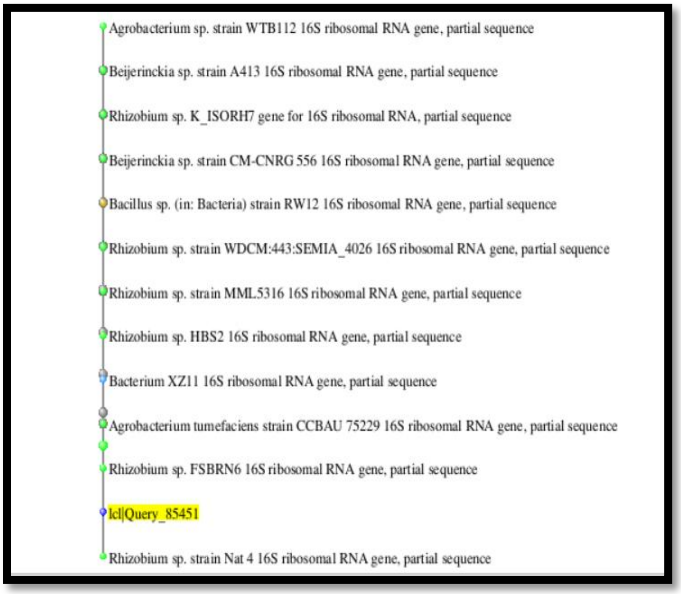

Figure 2. Phylogenetic of S4RS closely related to Rhizobium sp

Table 1. isolated colonies and acid producers

SAMPLE	ISOLATES	SITES	BTB medium
Root nodule	S(1)RN	Thrikodithanam	yellow
Soil	S(1)RS	Thrikodithanam	Blue
Root nodule	S(2)RN	Chingavanom	yellow
Soil	S(2)RS	Chingavanom	yellow
Root nodule	S(3)RN	Changanacherry	Blue
Soil	S(3)RS	Changanacherry	yellow
Root nodule	S(4)RN	Pampady	yellow
Soil	S(4)RS	Pampady	Blue
Root nodule	S(5)RN	Manimala	yellow
Soil	S(5)RS	Manimala	Blue

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Table 2: Morphological characteristics of bacteria

Isolates	Gram staining	Motility test	Colony characters
S(1)RN	Gram negative	+	Circular, convex, smooth, entire, semi translucent, gummy, creamish colonies
S(1)RS	Gram negative	+	Circular, convex, smooth, entire, semi translucent, gummy, creamish colonies
S(2)RN	Gram negative	+	Circular, convex, smooth, entire, semi translucent, gummy, creamish colonies
S(2)RS	Gram negative	+	Circular, convex, smooth, entire, semi translucent, gummy, creamish colonies
S(3)RN	Gram negative	+	Circular, convex, smooth, entire, semi translucent, gummy, creamish colonies
S(3)RS	Gram negative	+	Circular, convex, smooth, entire, semi translucent, gummy, creamish colonies
S(4)RN	Gram negative	+	Circular, convex, smooth, entire, semi translucent, gummy, creamish colonies
S(4)RS	Gram negative	+	Circular, convex, smooth, entire, semi translucent, gummy, creamish colonies
S(5)RN	Gram negative	+	Circular, convex, smooth, entire, semi translucent, gummy, creamish colonies
S(5)RS	Gram negative	+	Circular, convex, smooth, entire, semi translucent, gummy, creamish colonies

Table 3: Biochemical test

Biochemical test	S(1)RN	S(1)RS	S(2)RN	S(2)RS	S(3)RN	S(3)RS	S(4)RN	S(4)RS	S(5)RN	S(5)RS
Indole test	-	-	-	-	-	-	-	-	-	-
MR	+	-	-	-	-	-	-	-	-	-
VP	-	-	+	-	-	-	+	-	+	+
Citrate test	+	+	+	+	+	-	+	+	-	+
Nitrate test	+	+	+	+	-	+	+	+	-	+
Urease	+	-	+	-	+	-	+	+	+	+
TSI	K/K	A/A	K/K	K/A,G+	A/K	K/K	K/K	K/A	K/K	A/A
Starch	-	-	-	-	-	-	-	-	-	-
Gelatin	-	-	-	-	-	-	-	-	-	-
Catalase	-	+	-	-	-	-	+	+	-	-

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Oxidase	+	+	+	+	+	+	+	+	+	+
H_2S	-	-	-	-	-	-	-	-	-	-

*K/K-alkaline slant/alkaline butt, A/A-acid slant/acid butt, G^+-gas production

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