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Characterization of Indigenous *Rhizobium* Strain Isolated from lentil in Rajshahi, Bangladesh

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ABSTRACT: *Rhizobium* spp. was isolated from root nodules of lentil (*Lens culinaris* L.), and its morphological, cultural, physiological and biochemical characteristics were studied. It was observed that the colonies were circular, light pink, convex, entire and opaque. The bacterium was gram negative, rod shaped, aerobic, non-spore forming and motile. Isolates were positive for Catalase, Citrate utilization test, Urea hydrolysis, Congored test, Nitrification test, Oxidase test, Triple sugar iron test, MacConkey agar test and, Motility tests. The samples were found negative for Methyl Red (MR), Voges-Proskauer(VP), Indole, Starch hydrolysis test, Hydrogen-sulfide production and Hofer's alkaline test. The isolate can ferment sucrose, glucose, fructose, mannitol and produce acid. Screening for antibiotic resistance in this study revealed that the strain were resistant to Ampicilin, Erythromycin, Gentamicin, Amoxycillin, Penicillin, Streptomycin and Nalidixic acid. The isolate grew optimally at pH 7.0 and temperature 28˚C. Moreover, the isolate was sensitive to the higher concentration of NaCl (>1%). Based on 16S rDNA sequence, the isolate was identified as *Rhizobium* sp. SOY12 strain. The results also revealed that Rhizobial inoculation induced significant changes in plant growth characteristics.

Keywords: *Rhizobium*; SOY12 strain; lentil; 16S rDNA sequence; Inoculation and Nodulation.

INTRODUCTION: Rhizobia, a unique group of the soil bacteria have a beneficial effect on the growth of legumes such as Lentil (*Lens culinaris* L.). Lentil is an important pulse crop grown widely throughout the world [1, 2]. However, the yield of lentil is very poor in Bangladesh which could be increased by using industrially produced nitrogen fertilizers. But, use of these fertilizers has led to worldwide ecological problems as well as affects the human health[3]. There is a great possibility to increase its production by exploiting better colonization of their root and rhizosphere through *Rhizobium* bacteria, which can reduce nitrogenous fertilizer use and can protect environment. The complex process by which the rhizobia produce nitrogen for the legume is called biological nitrogen fixation (BNF). The legume-rhizobium interaction is the result of specific recognition of the host legume by *Rhizobium.* Various signal molecules that are produced by both *Rhizobia* and the legume confer the specificity[4]. Exopolysaccharide (EPS) produced by *Rhizobium* is one such signal for host specificity during the early stage of root hair infection[5]. Hence, only rhizobia that are specifically compatible with a particular species of legume can stimulate the formation of root nodules which in turn increase nitrogen fixation for better production of legume. However, rhizobial inoculants which are produced commercially in have to be specific to legume species as well as to the region. Therefore, isolation and characterization of new *Rhizobium* strains are necessary for production of quality inoculant for using in cultivation of legumes in Rajshahi, Bangladesh. In this study, indigenous *Rhizobium* strains were isolated from lentil and then the isolates were characterized to uncover their suitability as a quality inoculant for lentil cultivation at Rajshahi, Bangladesh.

MATERIALS AND METHODS:

Isolation of *Rhizobium* strains: The lentil plants were collected from the Rajshahi, Bangladesh. Healthy, unbroken nodules of Lentils were used for isolation of root nodule bacteria (*Rhizobium*) with a method as described by Saha and Haque (2005)[6]. Then, the colonies of isolated *Rhizobium* were characterized with method described by Aneja, (2003)[7]. The isolates were also tested for gram staining[8].

Identification of *Rhizobium* strains by 16S rDNA gene sequence: Genomic DNA was extracted from the bacterial cells using TIANamp Bacteria DNA kit (Tiangen, China) and purified according to the manufacture′s instruction. The amplification products were separated by electrophoresis of 10 µl. (7 µl PCR product 3 µl loading dye, Bromothymol blue) of the reaction product in 1.0% agarose gel (wv^{-1}) in Tris-

Borate buffer (0.089M Tris, O.O89M boric acid, and 0.002M EDTA, pH 8), stained with ethidium bromide (1.6 mg/ml). The gel electrophoresis was carried out at 70 V at room temperature for ~ 1.0 hour in electrophoresis unit (Bio-Rad, USA) and DNA bands were visualized using UV transilluminator in gel documentation system. A 1 kb DNA ladder was used as molecular weight markers. The PCR products were purified using TIANquick Midi purification kit (Tiangen, China) according to the manufacture′s protocol. The total DNA yield and quality were determined spectrophotometrically by Nano Drop 2000 (Thermo Scientific, USA). Sanger sequencing work flow using dye terminator technology was followed for the present study sequencing analysis was performed on a~ 800 bp PCR product. The sequence analysis was performed using the ABI 3130 genetic analyzer and Big Dye Terminator version 3.1 cycle sequencing kit. The 16S rRNA genes in the Gene Bank by using the NCBI Basic Local Alignment Search Tool (BLASTn) (http://www.ncbi.nih.gov/BLAST). A distance matrix was generated using the Jukes-cantor corrected distance model. The phylogenetic trees were formed using Weighbor (Weighted Neighbor Joining: A likelihood-Based Approach to Distance-Based Phylogeny Reconstruction) with alphabet size 4 and length size 1000.The 16S rRNA gene sequences were deposited to Genbank using BankIt submission[9].

Determination of optimum growth conditions: To determine the optimum pH of bacterial growth, culture medium was adjusted to pH 5.0, 7.0 and 9.0. For determination of optimum temperatures, inoculated media were incubated at 20°C, 28°C and 37°C. For determination of effect of salinity, inoculated media were incubated at 1%, 2%, 3% and 4% of NaCl. The growths of bacteria at different condition were determined at different time intervals by measuring optical density at 660 nm with photoelectric colorimeter.

Seed and soil inoculation with *Rhizobium* and its effect on growth parameters in Lentil: Seed inoculation was done by slurry method using adhesive[6]. Then, inoculated seeds of Lentils were sown in in pots. For soil inoculation, liquid culture of *Rhizobium* was sprayed thoroughly in inner part (1-1.5 inches below the surface) of soil in pots. Then, fresh and dry seeds of Lentil were sown in pots. During the experiment the soils in pots were kept moistened.

Statistical analysis: Unless indicated otherwise, all experiments were independently conducted three times and data were pooled for presentation as mean±SEM. All data were analyzed with Prism software (GraphPad, La Jolla, CA, USA) using two-tailed unpaired Student's t-tests. P-values <0.05 were considered significant.

RESULTS AND DISCUSSION: In this study, it was found that the colonies were circular, light pink, convex, entire and opaque. It was also observed that the isolates were gram negative, rod shaped, aerobic and non-spore forming bacterium. The isolates were showed hazy appearance in the motility media and also were positive for Catalase, Citrate Utilization Test, Urea Hydrolysis, Congored test, Nitrification test, Oxidase Test, Triple Sugar Iron Test, MacConkey Agar Test and motility Tests. This result is supported by the finding of Lupwayi and Haque (1994)[10]. The isolates were found negative for Methyl Red (MR) and Voges-Proskauer (VP) reaction. These findings are in close agreement with Elsheikh and wood (1986)[11].

Utilization of different carbon sources is an effective tool to characterize the isolates[12, 13]. In the present study sucrose, fructose, galactose, maltose and mannitol (25 mg Hi-media, India) and 20% solution of glucose, lactose, arabinose and xylose were utilized for this test. Isolates could utilize all the nine sugar. Similar results have been reported by some other paper (Stowers, 1983; Sadowsky *et al.* 1983)[14, 15].

Resistance patterns of the isolates to thirteen antibiotics were studied. Screening for antibiotic resistance in our study revealed that most of the isolates were resistance to Ampicillin, Erythromycin, Gentamicin, Amoxycillin, Penicillin, Streptomycin and Nalidixic acid. But, the isolates were sensitive to Mecillinam Ciprofloxacin Cotrimoxazole Pefloxacin Ceftazidime and Tetracycline which is agreed with the results of Jordan(1984)[16] for the genus *Rhizobium* .

The highest growth was observed at 28ºC (Figure 1b). The organisms were found to be temperature sensitive as at higher and lower temperatures, a low growth was observed that might be due to a hindrance in the metabolic activity. Similarly, the best growth of *Rhizobium* was found at pH 7 (Figure 1a). The experiments also showed that the cells were able to grow at 1% NaCl but unable to grow at higher concentration of NaCl, showing that the isolate was sensitive to the salt concentration (Figure 1c). Similar findings have been reported by Kucuk *et al*., in 2006[17]. In addition, Hashem and their colleagues in 1998[18] had proposed that salt stress may decrease the efficiency of the *Rhizobium*-legume symbiosis by reducing plant growth and photosynthesis.

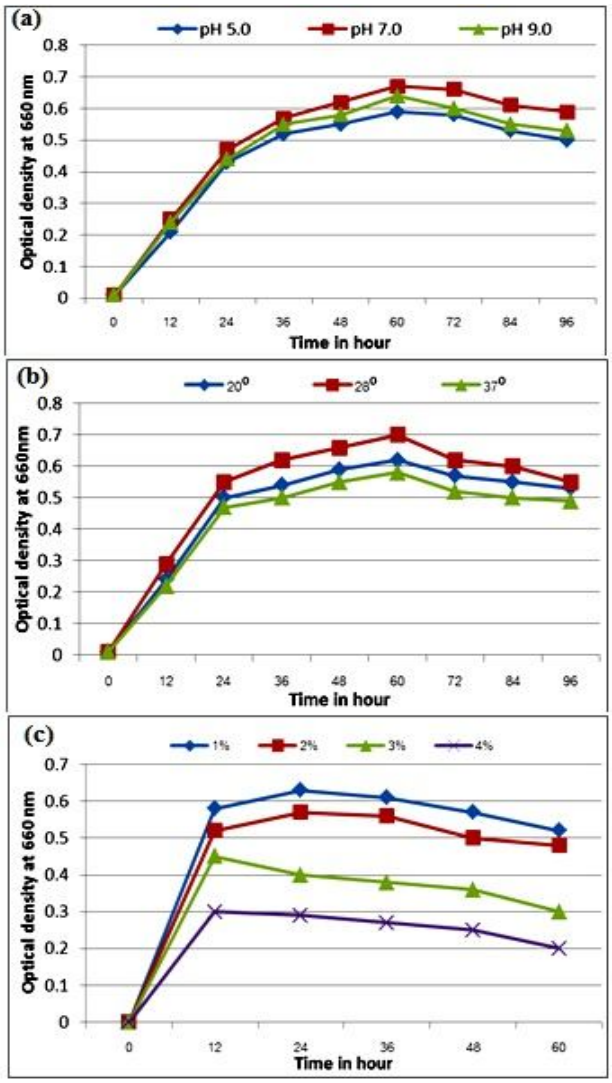

Figure 1: Growth characteristics of the isolates Lentil at different pH (a), temperature (b) and salinity (c).

In this study, the isolated bacteria were identified through 16S rDNA gene sequencing. The 16S rDNA sequence revealed that the isolated strain was homologous to bacterial strain *Rhizobium* sp. SOY12. The phylogenetic distances shown in Figure 2 indicate that the relationships between this group and the *Agrobacterium* species. Moreover, this phylogenetic tree clearly showed that the isolates were belonged to the genus *Rhizobium.*

The results obtained in this study show some interesting aspects on the growth effects of *Rhizobium* inoculation on Lentil, which was grown in pots under controlled environment. For all studied parameter of lentil growth viz. number, fresh weight and dry weight of nodules, plant height, pod weight and seed weight, the result showed significant differences between inoculation and control plants (Table 1). These findings can be supported by other studies as reported that Rhizobial inoculation induced significant changes in plant growth characteristics [19, 20, 21].

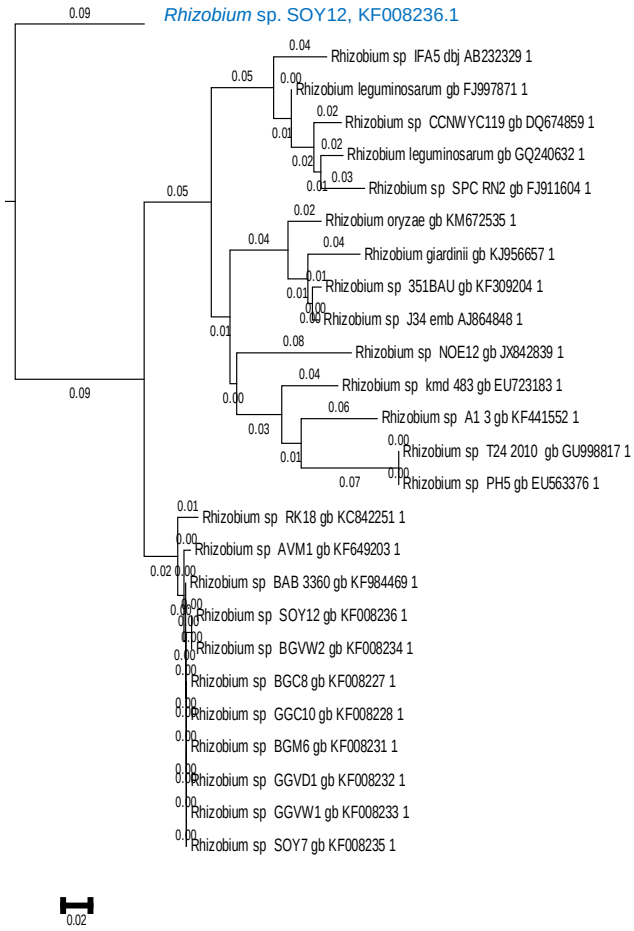

Figure 2: Unrooted Phylogenetic tree showing the genetic relationship among the cultivated bacteria Lentil and reference 16S rDNA sequences from the GenBank based on partial 16S ribosomal RNA gene sequences. Scale bar 0.02 = 2% difference among nucleotide sequences.

Table 1: Effect of *Rhizobium* inoculation on various growth parameters in Lentil.

	Length (cms)	50 pod wt. (gms)	100 seed wt. (gms)	No. of Nodules/ Plant	Fresh wt. of nodules (gms)	Dry wt. of nodules (gms)
Inoculated	28.40 ± 0.24	2.21 ± 0.04	2.07 ± 0.07	22.00 ± 1.37	0.25 ± 0.02	0.10 ± 0.01
Control	23.60 ± 0.60	1.56 ± 0.02	1.55 ± 0.04	12.40 ± 0.51	0.09 ± 0.01	0.04 ± 0.00
Degrees of freedom	08	08	08	08	08	08
Calculated value. P=0.05	<0.0001 ***	<0.0001 ***	<0.0001 ***	< 0.0002 ***	<0.0001 ***	<0.0002 ***

Plant growth and microorganism activity depends upon soil reaction and on possible condition of the soil. Hence, soil properties of pots in which the lentils were cultivated were tested. It was found that Rhizobium inoculation can increase amount of Phosphorus remarkably in soil (Table 2).

Table 2: Properties of Lentil soil types before and after inoculation with *Rhizobium* strain.

Parameters	Units	Before use	Control	Inoculated
pH	-	8.2	8.5	8.4
Organic mater	%	1.22	1.23	1.47
Potassium	Cmol/kg	0.18	0.18	0.18
Total Nitrogen	%	0.07	0.07	0.09
Phosphorous	ppm	11	11.1	22.2
Sulfur	ppm	15	11.3	7.8
Zinc	ppm	2.47	5.42	4.36

Conversely, the amount of Sulfur was decreased significantly in soil after *Rhizobium* inoculation. However, no remarkably changes were observed in other parameters of soil after *Rhizobium* inoculation (Table 2). Likewise, it was reported that *Rhizobium* inoculation in Lentil enhanced the plant soil properties than control in line with Tabatabai, (1994)[22] .

CONCLUSION: The indigenous strain *Rhizobium* sp. SOY12 which was isolated from Lentil of Rajshahi, Bangladesh possesses some unique characteristics of carbohydrate utilization, antibiotic resistance, salt tolerance and optimal growth condition. Altogether, it can be concluded from this study that the isolated indigenous *Rhizobium* sp SOY12 strain could be an efficient candidate for production of biofertilizer for using in Rajshahi, Bangladesh as it shows some positive aspects on the growth of Lentil in this region.

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REFERENCES:

1. Ford R. R. and Taylor P. W. J. (2003) Construction of an intraspecific linkage map of lentil (*Lens culinarisssp. culinaris*), *Theor. Appl. Genet*., 107, 910–916.

2. Erskine W. (1997) Lessons for breeders from landraces of lentil, *Euphytica.*, 93, 107-112.

3. Vitousek P. M. (1997) Human alteration of the global nitrogen cycle: sources and consequences, *Ecological Applications.*, **7**, 737.

4. Phillips D. A. (1991) Flavonoids: Plant signals to soil microbes, *Rec. Adv. Phytochem.*, 2, 1-33.

5. Olivares J., Bedmar E. J. and Martinez M. E. (1984) Infectivity of *Rhizobium meliloti* as affected by extra cellular polysaccharides, *J. Appl.Bacteriol*., 56, 389-393.

6. Saha A. K. and Haque M. F. (2005) Effect of inoculation with *Rhizobium* on nodulation and growth of Bean, *Dolchos lablab*. *J. Life Earth Science*., 1(1), 71-74.

7. Aneja K. R. (2003) Experiments in microbiology plant pathology and Biotechnology, 4th edition, New age International Publishers, New Delhi, India.

8. Vincent J. M. (1970) A manual for the practical study of the root nodule Bacteria, IBP Hand Book No. 15, Blackwell Scientific publications, Oxford.

9. Saitou N. and Nei M. (1987) The Neighbor- joining method: A new method for reconstruction Phylogenetic trees, *Molecular biology and evolution*., 4(4), 406 -425.

10. Lupwayi N. and Haque I. (1994) Legume–Rhizobium technology manual Environmental sciences division International Livestock center for Africa, Addis Ababa, Ethiopia., pp1-93.

11. Elsheikh E. A. E. and wood M. (1986) Soil biology, *Biochem.*, 21, 883-887.

12. Mirza B. S., Mirza M. S., Bano A. and Malik K. A. (2007) Coinoculation of chickpea with fhizobium isolates from roots and nodules and yhytohormone producing enterobacter strains, *Aus. J. Exp. Agric*., 47(8), 1008-1015.

13. Erum, S. and Bano, A. (2008) Bariation in phytohormone production in Rhizobium strains at different altitudes of Northern areas of Pakistan, *Int. J. Agric. Biol*., 10, 536-540.

14. Stowers M. I. and Eaglesham A. R. J. (1983) A stem nodulating *Rhizobium* with physiological characterstics of both fast and slow growers, *J. Gen. Microbiol*., 129, 3651-3655.

15. Sadowsky M. J., Keyser H. H. and Bohlool B. B. (1983) Biochemical characterization of fast growing and slow growing rhizobia that nodulate soybeans, *Syst. Bacteriol*., 33,716-722.

16. Jordan D. C. (1984) Family III. Rhizobiacea Conn 1938. In: *Bergey's Manual of Systermnatic Bacteriology.* Bol I (eds krieg. N.R. and Holt.J.G) Wilhams and Wilkings Press, Baltimore, pp234-254.

17. Kucuk C., M., Kivanc M., Kinaci E. (2006) Characterization of Rhizobium Sp. Isolated from Bean, *Turk J. Biol*., 30, 127-132.

18. Hashem F. M., Swelim D. M., Kuykendall L. D., Mohamed A. I., Abdel-Wahab S. M. and Hegazi N. I. (1998) Identification and characterization of salt and thermo tolerant Leucaenamodulating *Rhizobium* strains, *Biol. Fert.Soil*., 27, 335-341.

19. Sharma D. S. and Tilak K. V. B. R. (1974) Comparative efficiency of different commercial inoculants of *Rhizobium japonicum* on field grown soybeans. ICAR, 8(4), 223-226

20. Kapur O. C., Ganguwar M. S., Tilak, K. V. B. R. (1975) Influence of zinc on symbiotic nitrogen fixation by soybean (*Glycine max* Linn.) in silt loam soil. IJAR, 9 (1), pp 51-56.

21. Dev S. P. and Tilak, K. V. B. R. (1976) Effect of organic amendments on the nodulation and nitrogen fixation by soybean, *Indian .J. Agric. Research*., 46(6), 252-256.

22. Tabatabai M. A. (1994) Soil enzymes. In: *Methods of soil Analysis Part 2. Microbiological and Biochemical properties* .Ed : Weaver RW, Angle S, Bezdicek D, Smith S, Tabatabai, M. A, Wollum, A Soil Science society of America Book Series., 5, pp775-834.