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***Rhizobium* sp.CCNWYC119: a single strain highly effective as biofertilizer for three different peas (Pigeon pea, Sweet pea and Chick pea)**

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ABSTRACT

Rhizobium spp. was isolated from root nodules of Pigeon pea (*Cajanus cajan* L.), Sweet pea (*Lathyrus sativus* L.), Chick pea (*Cicer arietinum* L.). The isolates were rod shaped, aerobic, gram negative, motile and non-spore forming. Isolates were positive to Catalase, Citrate utilization, Urea hydrolysis, Congored, Nitrification, Oxidase, Triple sugar iron and MacConkey agar test. The isolates can ferment all nine sugars. Then, the isolates identified as *Rhizobium* spp. depending on above results were subjected to 16S rRNA sequencing for further confirmation and identification. Surprisingly, the isolates were same strain or member of same cluster of *Rhizobium* and identified as *Rhizobium* sp.CCNWYC119 strain based on 16S rRNA sequence (98% similarity). Then, different parameters of soil quality enrichment and plant growth viz. plant height; weight of pods and seeds; number, fresh and dry weight of nodules were studied to test the efficacy of the isolate as biofertilizer. Here, inoculant of *Rhizobium* sp. isolated from Pigeon pea was used as biofertilizer. The results showed the significant increase of nodulation, enrichment of soil of rhizosphere, plant growth and yield for all three types of inoculated peas as compared with non-inoculated control peas indicating that the isolated strain could be used as a common efficient biofertilizer for Pigeon pea, Sweet pea and Chick pea. It was also found that the isolate grew optimally at temperature 28°C and pH 7.0. Moreover, the isolate was sensitive to the higher concentration of NaCl (>1%) and to antibiotics- Mecillinam, Ciprofloxacin, Cotrimoxazole, Pefloxacin, Ceftazidime and Tetracycline.

Key words: *Rhizobium* sp.CCNWYC119, Pea, 16S rDNA sequence, biofertilizer.

INTRODUCTION

Rhizobia, a unique group of the soil bacteria have a beneficial effect on the growth of legumes such as Pigeon Pea, Sweet Pea and Chick Pea. Legumes are important crop which can grow widely throughout the world (Santos *et al.*, 2000). Peas are one of the most important legume crops in the semiarid tropics covering Asia, Africa, southern Europe, and Central and South America. The average yield of peas are low which can be attributed to lack of high yielding varieties and suitable rhizobial strains capable of fixing high atmospheric nitrogen. The soils of many countries for example Bangladesh are deficient in nitrogen fixing bacteria which causes poor yield of chickpea (Bhuiyan *et al.*, 1998). However, the yield of peas 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 (Vitousek, 1997). Hence, use of efficient *Rhizobium* strain as biofertilizer instead of chemical fertilizer could be an ecofriendly approach of production of peas. Indeed, peas are able to associate with a large diversity of indigenous rhizobia in soil with great genetic and

metabolic diversity (Fernandes *et al.*, 2012). Moreover, the legume-rhizobium association is the result of specific recognition of the host legume by specific *Rhizobium* strain. Various signal molecules produced by both *Rhizobium* and the legume control the specificity (Phillips, 1991). For example, exopolysaccharide produced by *Rhizobium* is one of such signals for host specificity during the early stage of root hair infection (Olivares *et al.*, 1984). Hence, only suitable strains of *Rhizobium* that are compatible with a particular species of legume can stimulate the development of root nodules. Thus, only compatible strain of *Rhizobium* can increase nitrogen fixation in specific type of legumes resulting in better harvesting of crops. Hence, rhizobial biofertilizer which are produced commercially have to be specific to particular legume species. Therefore, isolation, characterization and identification of new *Rhizobium* strains are necessary for production of quality biofertilizer. In this study, *Rhizobium* sp.CCNWYC119 strain was isolated from three different peas viz. Pigeon pea, Sweet pea and Chick pea. Surprisingly, inoculation of this strain was equally effective to significantly increase the different parameters

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of plant growth and yield in three studied peas indicating that this single strain of *Rhizobium* could be used as quality biofertilizer for multiple types of peas.

MATERIALS AND METHODS

Isolation and characterization of *Rhizobium* strains: The Pigeon Pea, Sweet Pea and Chick Pea plants were collected from the Rajshahi, Bangladesh. Healthy, unbroken nodules of peas were used for isolation of *Rhizobium* with a method as described by Saha and Haque (2005). Briefly, nodule was surface sterilized by immersing in 0.1% w/v mercuric chloride for 2 minutes and then 95% alcohol for 2-3 minutes. Each nodule was then crushed in a small aliquot of sterile physiological saline and then milky fluid was streaked on sterile yeast extract mannitol agar (YEMA) containing congo red. The plates were incubated at 28°C for five days. The typical well-isolated white, raised colonies were picked up to streak on YEMA plates for purification. Then, the purified *Rhizobium* was characterized with Catalase test, Citrate Utilization test, Urea Hydrolysis, Congored test, Nitrification test, Oxidase test, Triple Sugar Iron test, MacConkey Agar test, Methyl Red, Voges-Proskauer reaction, Starch hydrolysis, Hydrogen-sulfide production and Hofer's alkaline test. All these tests were accomplished according to the methods described by Aneja, (2003). The isolates were also tested for gram staining (Vincent, 1970).

Identification of *Rhizobium* strains by 16S rDNA gene sequence: Genomic DNA was extracted from the bacterial cells using TIANamp Bacteria DNA kit (Tiagen, 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, 0.089M 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 (Tiagen, China) according to the manufacture's protocol. The total DNA yield and quality were determined spectrophotometrically by NanoDrop 2000 (Thermo Scientific, USA). Sanger sequencing work flow using dye terminator technology was followed for the present study sequencing analysis was performed on a 4 800 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. (Saitou and Nei 1987).

Antibiotic sensitivity test: Sensitivity of antibiotic to the isolated bacteria was performed as described by Saha and their colleagues (Saha *et. al.* 2009). Briefly, 1ml of fresh broth culture of *Rhizobium* was spread uniformly on a nutrient agar plate with a sterile glass spreader. The plate was air-dried for few minutes and then antibiotic discs were placed on inoculated nutrient agar plates which were incubated at 37°C for 24 hours. After incubation, clear zones indicated inhibition of growth of the *Rhizobium*.

Seed and soil inoculation with *Rhizobium* and its effect on growth parameters in peas: Seed inoculation was done by slurry method (Saha and Haque, 2005). Briefly, legume seeds were disinfected with 0.2% HgCl₂ (2-3 min.) followed by 6-7 washings with sterile water. Disinfected seeds (about 50) were suspended in 20-40ml thick cell suspension of *Rhizobium* (10¹² cells/ml) or in sterile saline for 30 minutes. Then, the seeds were air dried for 30 minutes in sterile petriplates. Soil sample (2 kg) was taken in polycarbonate bags and sterilized in an autoclave (121°C, 1 hour) for three consecutive days. Sterilized and non-sterilized natural soil samples were transferred separately into sterile plastic pots. Then, sterilized soil was inoculated by spraying liquid culture of *Rhizobium* (10¹² cells/ml) thoroughly in inner part (1-1.5 inches below the surface) of soil in pots. Then, inoculated and non-inoculated dry seeds were sown separately in pots with inoculated soil and natural soil respectively. During the experiment the soils in pots were kept moistened. After growth of pea plants, different parameters of plant growth, yield and nodulation were measured at different time intervals. Soils of rhizospheres were analyzed with standard protocols from Regional Laboratory, Soil Resource Development Institute, Rajshahi, Bangladesh.

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.

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, the bacteria isolated from Pigeon pea, Sweet pea and Chick pea showed typical characteristics of *Rhizobium*. These were motile and positive for Catalase, Citrate Utilization Test, Urea Hydrolysis, Congored test, Nitrification test, Oxidase Test, Triple Sugar Iron Test and MacConkey Agar Test. This result is supported by the finding of Lupwayi and Haque (1994). The isolates were found negative for Methyl Red (MR), Voges-Proskauer (VP) reaction, Starch hydrolysis, Hydrogen-sulfide production and Hofer's alkaline test. Similar findings for *Rhizobium* were reported by Elsheikh and Wood (1986). Utilization of different carbon sources is an effective tool to characterize the isolates (Erum and Bano, 2008). All of the three isolates could utilize all the studied sugars viz. sucrose, fructose, galactose, maltose, mannitol, glucose, lactose, arabinose and xylose indicating that all the isolates could be belonging to same taxa. Comparable characteristics of *Rhizobium* have been reported by some other papers (Stowers, 1983; Sadowsky *et al.* 1983).

The use of 16S rRNA gene sequences to identify bacteria has been certainly the most common method (Janda and Abbott, 2007). Hence, the isolated bacteria were subjected to 16S rRNA sequence based identification. Interestingly, the sequence analysis and subsequent BLASTn analysis indicated that the 16S rRNA sequence of three isolates collected from Pigeon pea, Sweet pea and Chick pea had 98% similarity to that of *Rhizobium* sp.CCNWYC119 (Fig. 1). It was reported that an unknown isolate and the reference strain have to be same strain or belonging to same cluster of strains when 16S rRNA sequence of these two strains is >97% similar (Janda and Abbott, 2007). Thus, all three isolates were identified as *Rhizobium* sp.CCNWYC119 which can efficiently colonize the root nodules of the three different types of peas. Hence, only the isolate collected from Pigeon pea was selected for further characterization as well as used as a biofertilizer for Pigeon pea, Sweet pea and Chick pea.

Fig 1: Unrooted Phylogenetic tree showing the genetic relationship between the isolate collected from Pigeon pea 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. Phylogenetic tree of bacteria isolated from Sweet pea and Chick pea (not shown) are same to that of Pigeon peas.

Study of antibiotic sensitivity pattern is vital to maintain pure culture of a bacterial isolate as well as to take a decision for using it safely in any environmental application. Screening for antibiotic resistance in our study revealed that the selected isolate was resistance to Ampicillin, Erythromycin, Gentamicin, Amoxycillin, Penicillin, Streptomycin and Nalidixic acid. But, the isolate was sensitive to Mecillinam Ciprofloxacin Cotrimoxazole Pefloxacin Ceftazidime and Tetracycline which was agreed with the results of Jordan(1984) for the genus *Rhizobium*.

Soil characteristics, such as pH, temperature and amount of salt in soil, may compromise symbiotic efficacy

and plant development. In this study, the highest growth of the isolate was found at pH 7 (Fig. 2A). Low pH values (below 5.0) are reported to be deleterious for nodulation and nitrogen fixation (Appunu and Dhar, 2006). Similarly, the best growth was observed at 28°C (Fig. 2B). The low growth was observed at low temperature that might be due to a hindrance in the metabolic activity. The experiments also showed that the isolate was able to grow at 1% NaCl but unable to grow at higher concentration of NaCl, indicating that the isolate was sensitive to the salt concentration (Fig. 2C). Similar findings have been reported by Kucuk *et al.*, in 2006. In addition, Hashem *et al.* (1998) had proposed that salt stress may decrease the efficiency of

Fig 2: Optimum pH (A), temperature (B) and salinity (C) for growth of the isolate *Rhizobium* sp.CCNWYC119. The optimum pH, temperature and salinity for bacterial growth were determined at every 12-hours interval by measuring optical density at 660 nm.

the *Rhizobium*-legume symbiosis by reducing plant growth and photosynthesis.

The results of this study showed some promising aspects of the inoculant of the selected isolate (thereafter mentioned as biofertilizer) on Pigeon pea, Sweet pea and Chick pea, which were cultivated in pots. Length of plants and weight of their pods and seeds are good indicators of growth and yield of legumes (Ramana *et al.*, 2010). Remarkably, our results showed that the length of plants and the weight of their pods and seeds were significantly increased by the use of biofertilizer as compared with these parameters of non-inoculated control

plants (Table 1,2,3). Moreover, the number and weight of root nodules per plant were enhanced considerably by the use of the biofertilizer as compared with control plant (Table 1,2,3). Similar improvement of nodulation, growth and yield of legumes by effective biofertilizer were reported by many other studies (Ramana *et al.*, 2010; Mondal *et al.*, 2013; Kumawat *et al.*, 2013).

An important feature of a rhizobial biofertilizer is to make available certain plant nutrients in the rhizospheres of inoculated plants by various actions (Gangwar and Dubey, 2012). To test efficacy of the biofertilizer, different

Table 1: Effect of *Rhizobium* inoculation on various growth parameters of Pigeon Pea.

	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	201.6 ± 2.46	12.78 ± 0.21	7.54 ± 0.15	205.4 ± 1.72	20.00 ± 0.45	13.00 ± 0.32
Control	176.6 ± 2.73	11.32 ± 0.11	6.60 ± 0.06	171.0 ± 1.61	14.80 ± 0.37	9.80 ± 0.37
P value	0.0001	0.0002	0.0004	< 0.0001	< 0.0001	0.0002

Sterilized soil was inoculated by liquid culture of *Rhizobium*. Then, inoculated and non-inoculated dry seeds were sown separately in pots with inoculated soil and natural soil respectively. Data were collected at different time intervals. The data are mean±SEM from five independent experiments. Unpaired T-test was done to calculate the P-value. P<0.05 indicates that inoculation and control (not inoculated) are significantly different.

Table 2: Effect of *Rhizobium* inoculation on various growth parameters in Sweet Pea.

	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	71.00 ± 0.89	6.79 ± 0.13	7.50 ± 0.17	17.00 ± 0.71	0.27 ± 0.03	0.21 ± 0.02
Control	65.40 ± 0.93	5.780 ± 0.03	5.44 ± 0.03	11.80 ± 1.07	0.15 ± 0.03	0.104 ± 0.02
P value.	0.0025	< 0.0001	< 0.0001	0.0036	0.0112	0.0026

Sterilized soil was inoculated by liquid culture of *Rhizobium*. Then, inoculated and non-inoculated dry seeds were sown separately in pots with inoculated soil and natural soil respectively. Data were collected at different time intervals. The data are mean±SEM from five independent experiments. Unpaired T-test was done to calculate the P-value. P<0.05 indicates that inoculation and control (not inoculated) are significantly different.

Table 3: Effect of *Rhizobium* inoculation on various growth parameters in Chick Pea.

	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	42.90 ± 0.40	7.72 ± 0.09	10.38 ± 0.06	14.00 ± 0.55	0.19 ± 0.003	0.144 ± 0.002
Control	39.30 ± 0.37	6.54 ± 0.05	9.08 ± 0.07	7.00 ± 0.32	0.05 ± 0.01	0.04 ± 0.003
P value	0.0002	< 0.0001	< 0.0001	< 0.0001	< 0.0001	< 0.0001

Sterilized soil was inoculated by liquid culture of *Rhizobium*. Then, inoculated and non-inoculated dry seeds were sown separately in pots with inoculated soil and natural soil respectively. Data were collected at different time intervals. The data are mean±SEM from five independent experiments. Unpaired T-test was done to calculate the P-value. P<0.05 indicates that inoculation and control (not inoculated) are significantly different.

Table 4: Properties of initial soil and soil in rhizosphere of pea plants which were inoculated with *Rhizobium* sp.CCNWYC119 strain (Inoculated) or not inoculated with any *Rhizobium* strain (Control).

Parameters	Before use	Pigeon pea		Sweet pea		Chick pea	
		Control	Inoculated	Control	Inoculated	Control	Inoculated
pH	8.2	8.3	8.3	8.2	8.4	8.4	8.2
Organic matter (%)	1.22	1.40	1.63	1.29	1.64	1.15	1.45
Potassium (Cmol/kg)	0.18	0.28	0.23	0.24	0.28	0.32	0.27
Total Nitrogen (%)	0.07	0.08	0.11	0.07	0.10	0.08	0.10
Phosphorous (ppm)	11.5	25.5	25.9	18.7	25.4	13.5	29.3
Sulphur (ppm)	15.7	28.7	41.1	33.9	27	7.8	28.9
Zinc (ppm)	2.47	4.94	5.20	3.59	3.4	3.89	3.36

Sterilized soil was inoculated by liquid culture of *Rhizobium*. Then, inoculated and non-inoculated dry seeds were sown separately in pots with inoculated soil and natural soil respectively. After growth, soil samples were collected from rhizospheres of flowering plants. Then different factors of initial soil samples and soil samples of rhizospheres of inoculated and non-inoculated plants were measured.

parameters of soil quality in the rhizospheres of inoculated plants were compared with those of non-inoculated control plants. It was found that the biofertilizer inoculation could increase the amount of organic matter and total nitrogen content remarkably in the soil of rhizospheres of Pigeon pea, Sweet pea and Chick pea as compared with those of non-inoculated plants (Table 4). Similar findings have been conferred by other studies (Khadraji and Ghoulam, 2016; Gupta and Sahu, 2012). However, the biofertilizer could increase significantly the amount of sulphur in the soil of rhizospheres of Pigeon pea and chick pea, but not in rhizospheres of chick pea. However, no remarkable changes were observed in other parameters *viz.* pH, potassium, phosphorus and zinc of soil of rhizospheres by the use of biofertilizer (Table 4). Likewise, it was reported that *Rhizobium* inoculation in Pigeon pea, Sweet pea and Chick pea enhanced the plant soil properties than the control (Gupta and Sahu, 2012).

CONCLUSION

The three isolates of *Rhizobium* which were isolated from nodules of three different types of peas were identified

as *Rhizobium* sp.CCNWYC119 based on 16S rRNA sequence similarity (98%). The inoculant of the isolate of Pigeon pea could effectively colonize roots of Pigeon pea, Sweet pea and Chick pea as well as could promote their growth, yield and nodulation. It was also found that this biofertilizer improved the soil quality in rhizosphere especially by increasing the amount of organic matters and nitrogen content in soil. Taken together, it can be concluded that the *Rhizobium* sp.CCNWYC119 used as biofertilizer in this study could efficiently promote nitrogen fixation by increasing number and size of nodules as well as improve the soil quality of rhizospheres of Pigeon pea, Sweet pea and Chick pea which in turn enhance their growth and yield of pods and seeds.

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