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Original Article

Characterization of Some *Rhizobium* Isolates and Their Effectiveness on Pea

M Solaiman Talukder[1], ARM Solaiman[1*], Delowara Khanam[2] and M Golam Rabbani[1]

[1]*Department of Soil Science, Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU), Salna, Gazipur 1706, Bangladesh,* [2]*Division of Soil Science, Bangladesh Agricultural Research Institute (BARI), Joydebpur, Gazipur 1701, Bangladesh*

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Six isolates of *Rhizobium* were isolated from pea (*Pisum sativum*) to characterize their cultural properties and observe their effectiveness on host legume. In a laboratory study *Rhizobium* isolates showed characteristic pattern of reactions in respect of growth rate, colony characteristics and acid/alkali production on different growth media. The effect of inoculations of *Rhizobium* isolates on nodulation, growth and nitrogen fixation of pea were assessed by a pot experiment on a clay loam soil of Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur. Inoculation treatments comprised of *Rhizobium* isolates R_1, R_2, R_3, R_4, R_5 and R_6. Among the *Rhizobium* isolates R_3 and R_5 of pea at 50% flowering stage had significant positive effective on nodulation, growth and nitrogen fixation of pea. There were high positive correlations among the number and dry weight of nodules, N content and uptake by shoot of pea.

Keywords: *Rhizobium*, Pea, Nodulation, Growth, Nitrogen fixation

Introduction

Pea (*Pisum sativum*) is one of the main grain legume crops in Bangladesh. It is a high protein content food crop. As pea is a short durable crop. Its cultivation is highly profitable and preferable to the farmers. In Bangladesh, only 17,192 ha of land is under pea cultivation where its production is 13,735 MT, which is lower than other vegetables[1]. Inclusion of peas in crop rotation helps improvement of soil fertility and yield of the succeeding crops[2]. Pea like other legumes is capable of fixing and utilizing atmospheric nitrogen through symbiotic relationship with *Rhizobium* bacteria at the root of the crop.

Rhizobium inoculants significantly improves yield in many leguminous crops and can minimize the use of synthetic nitrogenous fertilizer, which is rather expensive and causes injury to soil properties[3]. The crop thus improves soil and economizes crop production not only for itself but also for the next cereals or non-legume crops grown in the relation and there by reducing the requirement of added nitrogen fertilizers. Many researchers[4-10] have reported the beneficial effects of inoculation of grain legumes. Seed inoculation with *Rhizobium* strains is known to influence nodulation and growth of pea[11]. It was observed that the maximum green pod yield of 30.78 g/plant (115% increase over uninoculated control) and mature seed yield of 5.10 g/plant (86% increase over uninoculated control) were obtained when seeds of pea were inoculated with *Rhizobium* inoculant[12]. Most soils of Bangladesh contain appreciable number of ineffective *Rhizobium* strain. But the number of *Rhizobium* strains effective on pea is very scarce in soils of Bangladesh.

Keeping these facts in mind the present investigation was, therefore, carried out to characterize some *Rhizobium* strain isolated from root nodules and to assess their effectiveness in respect of nitrogen fixation in pea.

Materials and Methods

A laboratory experiment was conducted to characterize six *Rhizobium* isolates recovered from nodules of pea collected from Jessore, Ishourdi (agro-ecological zone, AEZ 11), Faridpur, Rajbari (AEZ 12) and Barisal (AEZ 13) districts. Plants of pea were selected randomly from farmers' field of the respected AEZs. Nodules separated from roots of pea were washed in fresh water and preserved in vial containing silica gel. Silica gel was used to soak the water of the nodules that might cause rotting of the nodules. The collected nodules were surface sterilized by exposing them to 95% alcohol for 5-10 sec. Then the nodules were immersed in 0.1% acidified mercuric chloride for 3-4 min. These nodules were then washed with six changes of sterile water. The nodules were then crushed and streaked on YEM agar medium.

YEM agar medium contained the following constituents: K_2HPO_4 (0.5 g), $MgSO_4.7H_2O$ (0.2 g), NaCl (0.2 g), $CaCO_3$ (0.2 g), $FeCl_3.6H_2O$ (0.01 g), mannitol (10 g), yeast extract (0.4 g), agar (Difco, USA) (15 g), deionized water to 1 litre was used. The pH of the medium was adjusted to 7.0 with HCl solution. The medium was inoculated with the *Rhizobium* strains and incubated for one week. Colonies of the strains developed on the medium observed for their morphology and appearance. The YEM agar medium containing bromothymol blue indicator[13] was used for identification of strains. The reaction of the rhizobial strains on this medium was

**Corresponding author:*
Dr. ARM Solaiman, Professor, Department of Soil Science, Bangabandhu Sheikh Mujibur Rahman Agricultural University (BSMRAU), Salna, Gazipur 1706, Bangladesh
Tel (Office): (02) 9205310-14, Ext 2040; Tel (Home): (02) 9342649; Cell: 01552 495381; Fax: +880 (02) 9205310; E-mail: arm_solaiman@yahoo.com

noted every week. Fast-growing rhizobial strains produce acid in this medium, turning the medium yellow and slow growing rhizobia produce alkali, which turns the medium blue[13].

Rhizobium isolates collected from Faridpur were designated as R_1, R_2 and R_3, those from Rajbari as R_4 and R_5 and that from Ishsurdi as R_6. The isolates were assessed for colony characteristics, growth rate and acid/alkali production in laboratory media with a view to know their basic properties prior to more intensive study on their performance in respect of nodulation, growth, nitrogen fixation of pea.

A pot experiment was conducted at Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur to study the effect of *Rhizobium* isolates on nodulation, growth and nitrogen fixation of pea. The soil used in this experiment belongs to Salna series under Madhupur tract (AEZ 28). The collected soil samples were air-dried, crushed and passed though an mm sieve. Then the soil was autoclaved at 121°C for 1 h to destroy the indigenous organisms inhabiting there in. The soil was clay loam texture and contained 0.61% organic carbon, 0.05% total nitrogen, 0.07% available phosphorus (P), 0.84 (meq/100 g dry soil) exchangeable potassium (K), 13.75 (meq/100 g dry soil) cation exchange capacity (CEC) and had a pH 7.1. Pea seeds were collected from Bangladesh Agricultural Research Institute, Joydebpur, Gazipur. The seeds were healthy, vigorous, well matured and free from other materials.

The experiment was laid out in a randomized complete block design (RCRD) with four replications. The treatments were arranged in the experimental units randomly. Inoculation treatments included uninoculated control R_0, *Rhizobium* isolate R_1, *Rhizobium* isolate R_2, *Rhizobium* isolate R_3, *Rhizobium* isolate R_4, *Rhizobium* isolate R_5, and *Rhizobium* isolate R_6. Each pot was filled with 1 kg of soil. Basal doses of phosphorus (P), potassium (K), zypsum (S), molybdenum (Mo) at the rate of 50 kg P_2O_5/ha (in the form of triple superphosphate, TSP); 50 kg K_2O/ha (in the form of muriate of potash, MP); 20 kg S/ha (in the form of zypsum) and 1.5 kg Mo/ha (in the form of sodium molybdate) were applied to the pots during final pot media preparation. No nitrogen fertilizer was used in the experiment. Five germinated seeds were sown in each pot. Liquid rhizobial inoculants were prepared in the Soil Microbiology Laboratory of the Bangabandhu Sheikh Mujibur Rahman Agricultural University, Gazipur using YEM broth. Liquid inoculants were spread on the germinated seeds and were incorporated in soil.

Two healthy plants per pot were retained after the formation of first trifoliate leaf. All the intercultural operations like weeding, irrigation and drainage, mulching etc. were performed as and when necessary. Plant samples were collected from the pot at 50% flowering stage (50 days after sowing, DAS) of the crops. Plants were carefully uprooted with the help of 'khurpi' so that no nodules were left in the soil. The roots were washed thoroughly with water. The nodules from the roots of each plant were separately collected and counted. The shoot root and nodules materials were first air-dried and then over dried at 65°C for 72 h. Then oven-dried weight of shoot, root and nodule were recorded. The oven dried plant shoot material was ground in a grinding machine (Wiley Pulverizer, Type 1029-8, Yoshida Seisakusho Co Ltd, Japan). Total N content in the shoot material was determined by ashing the plant material using salicylic acid modified Kjeldahl method following sulphuric acid digestion and then colorimetric assay[14]. Nitrogen uptake by shoot was calculated from the data on dry matter yield and nitrogen content in shoot material of the crop. The recorded data of various characters of the crop were statistically analyzed to find out the significance of variation resulting from the experimental treatments. For this purpose, analysis of variance was worked out for each character of the crop. The difference between treatments means were compared by Duncan's Multiple Range Test (DMRT).

Results and Discussion

Growth on Congo red YEM agar

In general colonies of rhizobial isolates absorbed very little of the Congo red dye. Results presented in Table 1 show that *Rhizobium* isolates R_1 and R_6 absorbed the dye very weakly, while isolates R_2, R_3, R_4 and R_5 exhibited weak absorption of the dye. This result is consistent with that of Trinick[15] who reported that rhizobia absorbed the dye weekly compared with other bacteria.

Growth on peptone glucose agar

Growth in peptone glucose agar as reported by Vincent[16] indicates that most of the *Rhizobium* isolates grow either poorly or moderately in this medium. Isolate R_2 showed poor growth on the medium. Isolates R_1, R_3, R_4 and R_5 showed moderate growth on the medium. However, strain R_6 did not grow at all in this medium (Table 1).

Colony characteristics on YEM agar

Rhizobium isolates are classified as fast- and slow-growing depending on growth on YEM agar. Isolates R_1, R_3, R_4 and R_5 were found to be fast-growing on YEM agar. These isolates produced large confluent colonies with abundant gum in YEM agar after 5 to 7 days of incubation. In contrast, isolates R_2 and R_6 showed slow growth in this medium (Table 1). Colonies of these isolates on YEM agar appeared small and separate with slight gum production. The texture of the sum was usually sticky.

Acid/alkali production in YEM agar medium containing bromothymol blue indicator

The importance of production of acid or alkali by the various rhizobia as reported by Norris[13] has been emphasized when considering *Rhizobium* taxonomy. In this study, all the fast-growing *Rhizobium* isolates showed acidic reactions throughout their four weeks of growth, while all the slow-growing *Rhizobium* isolates started with an alkaline reaction after one week of growth (Table 1). It was observed that the isolates R_1, R_3, R_4 and R_5 produced acidic reaction on this medium. These isolates turned green colour of the medium to yellow. Isolates R_2 and R_6 produced alkali on this medium, which turned green colour of the medium to blue.

Table 1. *Characteristics of some Rhizobium strains isolated from nodules of pea*

Rhizobium isolate	Growth on Congo red YEM agar (Absorption of dye)	Growth on peptone glucose agar	Colony characteristics on YEM agar (Fast/slow)	Acid/alkali production in YEM agar medium containing BTB	Growth in YEM broth
R_1	Slight	Moderate	Fast	Acid	Moderate turbidity
R_2	Weak	Poor	Slow	Alkali	Moderate turbidity
R_3	Weak	Moderate	Fast	Acid	Moderate turbidity
R_4	Weak	Moderate	Fast	Acid	Moderate turbidity
R_5	Weak	Moderate	Fast	Acid	Moderate turbidity
R_6	Slight	Nil	Slow	Alkali	Moderate turbidity

YEM = Yeast extract mannitol; BTB = Bromothymol blue indicator.

Growth in YEM broth

Results reported in Table 1 indicate that *Rhizobium* isolates showed visible turbidity in YEM broth after 5 to 7 days of incubation. All the isolates produced moderate turbidity in this medium. The differences in texture of extracellular polysaccharide of the fast- and slow-growing rhizobia have been shown to be due to differences in monosaccharide comparison[17].

Number of nodules per plant

The effect of *Rhizobium* inoculation on number of nodules per plant of pea was significant (Table 2). Plant inoculated with *Rhizobium* strains produced significantly higher number of total and effective nodule as compared to that of uninoculated control. Solaiman and Rabbani[9] observed that pea inoculated with *Rhizobium* inoculant produced the highest number of nodules at pre-flowering and pod filling stages. The highest number of effective and total (70.50 and 92.75) nodule was obtained with isolate R_3. The second highest number of total and effective nodule was obtained with isolate R_5, which was statistically similar to the effect of isolates R_6 and R_4. The lowest number of nodule was obtained with the isolate R_2, which was statistically similar to the effect of isolate R_1.

Table 2. *Effect of Rhizobium inoculation on number and dry weight of nodules of pea*

Inoculation treatment	No. of nodules/ plant		Dry weight of nodules (mg/plant)	
	Effective	Total	Effective	Total
Control	00.00^{d}	00.00^{e}	00.00^{c}	00.00^{c}
R_1	26.00^{c}	39.00^{cd}	80.80^{b}	102.50^{b}
R_2	19.00^{c}	28.00^{d}	92.99^{b}	114.39^{b}
R_3	70.50^{a}	92.75^{a}	140.34^{a}	176.72^{a}
R_4	35.75^{bc}	55.75^{bc}	75.95^{b}	98.35^{b}
R_5	48.75^{b}	71.75^{b}	130.20^{a}	167.39^{a}
R_6	35.25^{bc}	59.00^{bc}	150.00^{a}	182.43^{a}
CV (%)	13.69	15.71	12.78	14.33

Means in a column followed by same letter(s) are not significantly different at 5% level by DMRT.

Dry weight of nodule

There was a significant variation in dry weight of nodule per plant of pea with different *Rhizobium* isolates (Table 2). Plants inoculated with isolate R_6 produced the highest dry weight of effective and total (150.00 and 182.43 mg/plant) nodule, which was statistically similar to the effect of the isolates R_3 and R_5. The lowest dry weight of total nodule was obtained with the isolate R_4 whose effect was statistically similar to R_1 and R_2. This result was in agreement with Solaiman and Rabbani[8] who reported that *Rhizobium* inoculant significantly increased dry weight of nodules per plant in edible-podded pea. There was a positive correlation between the number and dry weight of nodules (r = 0.907).

Plant height

The plant height of pea was significantly influenced by different treatments (Table 3). The highest plant height (44.75 cm) was recorded with the isolate PR_3, which was statistically similar to the isolates R_4, R_1 and R_2. The lowest plant height was obtained in uninoculated control. This result resembles with that of Hossain and Solaiman[18] who stated that plant height of mungbean increased significantly due to inoculation seeds with *Rhizobium* isolates.

Table 3. *Effect of Rhizobium inoculation on plant height, root length, dry weight of shoot and root of pea*

Inoculation treatment	Plant height (cm)	Root length (cm)	Dry weight of shoot (mg)	Dry weight of root (mg)
Control	24.50^{c}	16.25^{b}	1293.00^{d}	165.00^{b}
R_1	39.50^{ab}	21.50^{a}	1592.00^{bcd}	507.50^{a}
R_2	39.00^{ab}	24.50^{a}	1950.00^{abc}	497.50^{a}
R_3	44.75^{a}	22.75^{a}	2003.00^{ab}	625.00^{a}
R_4	41.25^{a}	24.25^{a}	2210.00^{a}	437.50^{a}
R_5	33.75^{b}	23.50^{a}	1538.00^{cd}	540.00^{a}
R_6	32.75^{b}	25.50^{a}	1645.00^{bcd}	550.00^{a}
CV (%)	10.93	14.48	13.64	15.49

Means in a column followed by same letter(s) are not significantly different at 5% level by DMRT

Root length

There was no significant difference among the strains in recording root length of pea (Table 3). However, all the strains produced higher root length compared to uninoculated control. The highest root length (25.50 cm) was obtained with the strain R_6 and the lowest root length was obtained with uninoculated control. This result was in agreement with Hossain and Solaiman[18].

Dry weight of shoot

It was noted that dry weight of shoot was influenced by *Rhizobium* inoculation (Table 3). The highest dry weight of shoot (2210 mg/plant) was recorded with PR_4, which was statistically similar with the isolates R_3 and R_2. Dry weight of shoot recorded with isolate R_4 was 70% higher than that of uninoculated control. The lowest dry weight of shoot was recorded with uninoculated control. This result supports the findings of Solaiman and Rabbani[6] who reported that *Rhizobium* inoculant significantly increased dry weight of shoot per plant in pea.

Dry weight of root

It is evident (Table 3) that there was no significant difference among the isolates in recording dry weight of root of pea. However, all the isolates produced higher dry wt of root compared to uninoculated control. The highest dry weight of root (625 mg/plant) was recorded with the isolate R_3 and the lowest was obtained from uninoculated control. Solaiman and Rabbani[6] reported that *Rhizobium* inoculant significantly increased dry weight of root per plant of pea.

Nitrogen (N) content in shoot

The performance of *Rhizobium* strains in recording N content in shoot was statistically different (Table 4). The highest N content (3.77%) in shoot was obtained with the isolate PR_6, which was statistically similar to that obtained with the isolates R_5 and R_3. Solaiman and Rabbani[6] reported that *Rhizobium* inoculant significantly increased N content in shoot per plant of pea. The lowest N content in shoot was obtained from uninoculated control.

Table 4. *Effect of Rhizobium inoculation on nitrogen (N) content in shoot and nitrogen (N) uptake by shoot of pea*

Inoculation treatment	N content in shoot (%)	N uptake by shoot (mg/plant)
Control	1.70^c	22.00^d
R_1	2.27^b	36.26^c
R_2	2.60^b	50.77^b
R_3	3.21^a	64.34^a
R_4	1.25^c	27.57^c
R_5	3.25^a	49.98^b
R_6	3.77^a	62.08^a
CV (%)	11.52	12.43

Means in a column followed by same letter(s) are not significantly different at 5% level by DMRT

Nitrogen uptake by shoot

The highest N uptake (62.08 mg/plant) was recorded with the strain PR_3, which was statistically similar to that of PR_6 (Table 4). All the isolates performed better than uninoculated control in respect of N uptake by shoot. This result was in agreement with that of Solaiman and Rabbani[6] who reported that *Rhizobium* inoculant significantly increased N uptake by shoot in pea. The lowest N uptake by shoot was recorded from uninoculated control.

The present study demonstrates that two *Rhizobium* isolates, namely R_3 and R_5, had significant positive effective on nodule formation and nitrogen fixation in pea. High positive correlations also observed with respect to the number and dry weight of nodules, nitrogen content and uptake by shoot of pea.

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