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EFFECT OF PLANT GROWTH PROMOTING RHIZOBACTERIA (PGPR) IN SEED GERMINATION AND ROOT-SHOOT DEVELOPMENT OF CHICKPEA (*Cicer arietinum* L.) UNDER DIFFERENT SALINITY CONDITION

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ABSTRACT

Plant growth promoting rhizobacteria (PGPR) are beneficial bacteria that colonize plant roots and enhance plant growth by a wide variety of mechanisms. Ten isolates of bacteria designated as SS01, SS02, SS03, SS04, SS05, SS06, SS07, SS08, SS09 and SS10 were successfully isolated and morphologically and biochemically characterized. Subsequently to investigate the effect of PGPR isolates on the growth of chickpea, a pot culture experiment was conducted in 2013 at National Institute Biotechnology, Bangladesh net house. Prior to seeds grown in plastic pots, seeds were treated with PGPR isolates and seedlings were harvested after 21 days of inoculation. All the isolates were gram negative in reaction, catalase positive, produced indole acetic acid (IAA) as well as performed phosphate solubilization, able to degrade cellulose and have the adaptability in wide range of temperature and showed positive growth pattern in medium. Most of isolates resulted in a significant increasing of shoot length, root length and dry matter production of shoot and root of chickpea seedlings. Application of PGPR isolates significantly improves the percentage of seed germination under saline conditions. The present study, therefore suggested that the use of PGPR isolates SS04, SS10 and SS08 as inoculants biofertilizers might be beneficial for chickpea cultivation in saline condition.

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INTRODUCTION

Salinity, an abiotic stress is a severe problem for temperate and tropical agriculture system, which is increasing day by day and affecting 20% of global agriculture (Mayak et al., 2004). The harmful effects of presence of salts in soil result in increased level of ethylene in root, ionic imbalance and hyper-osmotic condition in plants (Niu et al., 1995; Mayak et al., 2004). Many efforts have been made to minimize the severe effects of salt stress on the crop growth and productivity. Biological approaches such as inoculation of seeds/plants with plant growth promoting bacteria (PGPR) and application of growth regulators to induce resistance against stress have already been attempt (Hayat et al., 2010). The most suitable solution or method/approach in this regard is to use the salt tolerant bacterial isolates that may induce salt tolerance thus being useful in facilitating plant growth and yield under salt stress (Bacilio et al., 2004). Rhizosphere is the hotspots of microorganisms and plant growth promoting rhizobacteria (PGPR) are a heterogeneous group of bacteria that can be found in the rhizosphere, at root surfaces and in association with roots, which can improve the extent or quality of plant growth directly and/or indirectly (Gray and Smith, 2005). In last few decades, a large array of bacteria including the species of *Pseudomonas, Burkholderia, Agrobacterium, Erwinia, Xanthomonas, Azospirillum, Bacillus, Enterobacter, Rhizobium, Alcaligenes, Arthrobacter, Acetobacter*, *Acinetobacter, Achromobacter, Aerobacter, Artrobacter, Azotobacter, Clostridium, Klebsiellla, Micrococcus, Rhodobacter, Rhodospirrilum, Flavobacterium* and *Serratia* have been reported to enhance plant growth (Kloepper *et al*., 1989; Kim and Kim, 2008; Joshi and Bhatt, 2011). PGPR have been demonstrated to increase growth and productivity of many commercial crops including rice, wheat, maize, barley, pea, groundnut, fababean, cucumber, tomato, sorghum, cotton, black pepper, and banana (Ashrafuzzaman *et al*., 2009; Khalid *et al*., 2004; Maleki *et al*., 2010; Mehnaz et al., 2001). The mechanism by which PGPR promote plant growth are not fully understood, but are thought to includes the ability to produce phytohormones, asymbiotic N_2 fixation against phytopathogenic microorganisms by production of siderophores, the synthesis of antibiotics, enzymes and fungicidal compounds (Ahmed *et al*., 2006). Significant increases in growth and yield attributing agronomical important crops in response to inoculation with PGPR have been reported (Biswas et al., 2000). Another major benefit of PGPR is to produce antibacterial compounds that are effective against certain plant pathogen and pests. Under salt stress, PGPR have positive effect in plants on such parameters as germination rate, tolerance of drought, yield and plant growth (Kokelis-Burelle et al., 2006). Plant growth promoting rhizobacteria (PGPR) induced plants salt stress tolerance has been well studied and is considered to be the cost-effective solution to the problem. PGPR isolated from saline soils improve the plant growth at high salt (Mayak *et al*., 2004; Barassi *et al*., 2006). These PGPR tolerance wide range of salt stress and enable plants to withstand salinity by hydraulic conductance, osmotic accumulation, sequestering toxic Na^+ ions, maintaining the higher osmotic conductance and photosynthetic activities (Dodd and Alfocea, 2012). The bacteria obtained from saline environment include, *Flavobacterium*, *Azospirillium*, *Alcaligenes*, *Actinobacterium*, *Pseudomonas* (Rodriguez *et al*., 1985; Moral *et al*., 1988; Ilyas *et al*., 2012), *Sporosarcina*, *Planococcus* (Ventosa *et al*., 1983), *Bacillus* (Upadhyay *et al*., 2009), *Thalassobacillus*, *Halomonas*, *Brevibacterium*, *Oceanobacillius*, *Terribacillus*, *Enterobacter*, *Halobacillus*, *Staphylococcus* and *Virgi bacillus* (Roohi *et al*., 2012) can tolerate in high salinity in soil. Yachana and Subramanian (2013) found that seeds treated with *Pseudomonas pseudoalcaligenes* and *Bacillus pumilus* under saline conditions, as well as non-saline conditions, showed higher germination and survival percentages as compared to non-treated seeds. Kumar *et al*., (2009) stated that plants inoculated at 1% salinity with the mixture of both PGPRs showed a marginal decrease in germination, survival and plant height, while dry weight showed a marginal increase.

Chickpea is the most important staple food in several developing countries and chemical fertilizers is the most important input required for chickpea cultivation. In order to make its cultivation sustainable and less dependent on chemical fertilizers, it is important to know how to use PGPR that can biologically fix nitrogen, solubilize phosphorus and induce some substances like indole acetic acid (IAA) that can be contribute to the improvement of chickpea growth. Thus the aim of this study was to determine the effect of PGPR strains that are compatible with chickpea. We also investigated the influence of PGPR with salinity on seed germination.

MATERIALS AND METHODS

Soil samples were collected from chickpea field of Tangail sadar upazila of Tangail district. After collection of soil samples, it was stored in plastic container, properly leveled and carried to the laboratory for further use. Ten grams of rhizosphere soil were taken into a 250 ml of conical flask and 90 ml of sterile distilled water was added to it. After serial dilution upto 10, an aliquot of this suspension was spread on the plates of nutrient (NA) agar medium (NH_4Cl 5.0 g; K_2HPO_4 3.0 g; Na_2SO_4 2.0 g; KH_2PO_4 1.0 g; NH_4NO_3 1.0 g; $MgSO_4$, $7H_2O$ 0.1 g; glucose 2.0 g; distilled water 1 litre and pH 7.0±0.2) in the molecular microbiology laboratory of National Institute of Biotechnology (NIB), Savar, Bangladesh. After 3 days of incubation at 28°C, bacterial colonies were streaked to other NA agar plates and incubated at 28°C for 3 more days. Typical single bacterial colonies were observed over the streak. Well isolated single colonies were picked up and different characteristics of colonies such as shape, size, elevation, surface, margin, color, odor and pigmentation etc. were observed and recorded. All the morphological and biochemical characters of the isolates were determined based on Bergey's Manual of Systematic Bacteriology (Garrity *et al*., 2001). A loopful of bacterial culture from each isolates was diluted into a test tube containing 1 ml sterile distilled water and was vortexed for 2/3 minutes. A loopful suspension was then taken on a glass slide and smeared. The slide was air dried and fixed by heating on a Bunsen flame. The slide was flooded with crystal violet solution for 3 min. The slide was washed gently in flow of tap water and air dried. The slide was observed under microscope and recorded the shape. Motility of bacteria was observed by hanging drop method. A drop of suspension was taken on a cover slip. The cover slip was hanged on a hollow slide with vaseline. The slide was then observed under microscope to test the motility of bacteria. The culture of 10 isolates were streaked on NA agar plates and incubated at 10, 20, 28, 37 and 45°C and also in the NA plate with 0, 3.0, 6.0 and 12.0 mM NaCl solutions in the medium. The bacterial isolates were designated as SS01, SS02, SS03, SS04, SS05, SS06, SS07, SS08, SS09 and SS10. A single colony of bacterial culture was grown on nutrient broth medium. A loopful of the respective culture was transferred to the 100 ml of conical flask then incubated for 7 days on a rotary shaker in 80 rpm. The IAA production and phosphate solubilization were then examined according to the method given by Bric *et al*., (1991) and Pikovskaya, (1984), respectively. Phosphate solubilizing capacity of the isolates was estimated using Pikovskaya's medium (Pikovskaya, 1948). The cellulose degradation test was performed by using carboxy methyl cellulose (CMC) agar plates according to the method of Kasana *et al*., (2008). Seeds of chickpea were collected from pulse research centre of Bangladesh Agricultural Research Institute (BARI), Gazipur. Prior to germination, the chickpea seeds were surface sterilized in 3% H_2O_2 and then rinsed with distilled water. The seeds were then surface sterilized with 0.024% sodium hypochlorite for 2 minutes and rinsed thoroughly in sterile distilled water. Seeds were inoculated by overnight soaking with suspensions of bacteria (approximately 10^7-10^8 cfu/ml). Seeds soaked in sterilized distilled water were used as the control. The soaked seeds with rhizobacterial isolates emerged with three different NaCl solutions which were derived from sterile distilled water by adding 0 (control), 3, 6 and 12 mM NaCl, respectively. Chickpea seeds were placed over the sterile filter paper into a petri dish and covered with tight fitting lid. Then the petri-plates were kept in an incubator maintaining the moisture and temperature of 28^0C-30^0C. Seed germination assay was laid out in Completely Randomized Design (CRD) with three replications. Germinated seeds were recorded and discarded at 24 h interval over 10 days.

Germination percentage was calculated by the following formula (Li, 2008):

$$\text{Germination percentage (GP)} = \frac{\text{Total number of seeds germinated}}{\text{Total number of seeds taken for germination}} \times 100$$

An amount of 0.3 kg sand was placed into a pot. Ten PGPR inoculated seeds were sown at 4 to 5 cm depth of sand in each plastic pot and laid out in a completely randomized block design (RCBD) with three replications. The chickpea plants were harvested after 21 days of seed sowing through separating of plants from soil. Shoot length (cm $plant^{-1}$) and root length (cm $plant^{-1}$) and dry weight of shoots and roots of each plant were recorded after drying in an oven for 1 day at 70°C. The data was analyzed statistically by MS-STATC statistical program. The significance of differences between mean values was evaluated by DMRT according to the methods of Gomez and Gomez (1984).

RESULTS AND DISCUSSION

Isolation and Characterization of PGPR

Ten bacterial isolates were successfully isolated from the rhizosphere of chickpea. They were designated as SS01, SS02, SS03, SS04, SS05, SS06, SS07, SS08, SS09 and SS10 as shown in Table 1 and the morphological characteristics of PGPR isolates widely varied. The isolates were found to be first growers. All the isolates produced round shape and raised colonies having smooth shiny surface with smooth margin (Table 1). They differed in color but all were odorless and no pigmentation was observed in the colonies of NA agar plates (Table 1). Diameters of the colonies of isolates varied from 0.2 to 2.0 mm (Table 1). PGPR colonize plant roots and exert beneficial effects on plant growth and development by a wide variety of mechanisms.

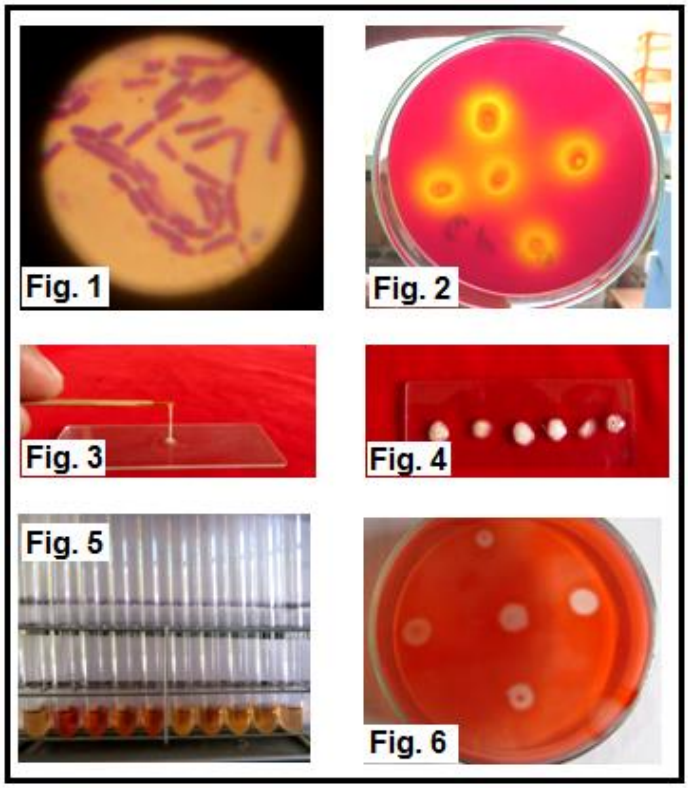

Figure 1. Gram staining of PGPR isolates; **Figure 2.** Phosphate solubilization plate assay test of PGPR isolates; **Figure 3.** KOH test of PGPR isolates; **Figure 4.** Catalase test of PGPR isolates; **Figure 5.** Indole acetic acid (IAA) production by PGPR isolates; **Figure 6.** Cellulose degradation plate assay test of PGPR isolates

Table 1. Morphological characteristics of 3-day old colony of PGPR isolates

Isolates	Shape	Size (mm)	Elevation	Surface	Margin	Colour
SS01	Round	0.9-1.1	Raised	Smooth shiny	Smooth	Off whitish
SS02	Round	0.9-1.1	Raised	Smooth shiny	Smooth	Pinkish
SS03	Round	1.0-1.5	Raised	Smooth shiny	Smooth	Brownish
SS04	Round	1.0-1.5	Raised	Smooth shiny	Smooth	Yolk brown
SS05	Round	1.9-2.0	Raised	Smooth shiny	Smooth	Yellowish
SS06	Round	1.0-1.5	Raised	Smooth shiny	Smooth	Yolk yellowish
SS07	Round	1.5-2.0	Raised	Smooth shiny	Smooth	Whitish
SS08	Round	0.2-0.5	Raised	Smooth shiny	Smooth	Yellowish
SS09	Round	0.9-1.1	Raised	Smooth shiny	Smooth	Whitish
SS10	Round	0.5-1.0	Raised	Smooth shiny	Smooth	Off whitish

Table 2. Cell shape, motility, odor, pigmentation, gram staining, catalase test, KOH test and cellulose degradation test of isolated PGPR of chickpea rhizosphere

Isolates	Cell shape	Motility	Odour	Pigment	Gram staining	KOH test	Catalase test	Cellulose degradation
SS01	Rod	Motile	Odourless	None	-	-	+	+
SS02	Rod	Motile	Odourless	None	-	-	+	+
SS03	Rod	Motile	Odourless	None	-	-	+	+
SS04	Rod	Motile	Odourless	None	-	-	+	+
SS05	Rod	Motile	Odourless	None	-	-	+	+
SS06	Rod	Motile	Odourless	None	-	-	+	+
SS07	Rod	Motile	Odourless	None	-	-	+	+
SS08	Rod	Motile	Odourless	None	-	-	+	+
SS09	Rod	Motile	Odourless	None	-	-	+	+
SS10	Rod	Motile	Odourless	None	-	-	+	+

Table 3. Growth of PGPR isolates at different temperature (10^0C-45^0C) conditions

Isolates	Temperature				
	10^0C	20^0C	28^0C	37^0C	45^0C
SS01	+	++	++	+	-
SS02	+	++	++	+	-
SS03	+	++	++	++	+
SS04	++	++	++	++	++
SS05	+	++	++	+	-
SS06	+	++	++	+	-
SS07	+	++	++	+	-
SS08	+	++	++	+	-
SS09	+	++	++	+	-
SS10	+	++	++	++	-

(- = No growth, + = weak growth and ++ = good growth)

Table 4. Growth of PGPR isolates at different NaCl (0-12.0 mM) concentrations

Isolates	NaCl concentration (mM)			
	0 mM	3.0 mM	6.0 mM	12.0 mM
SS01	++	++	++	+
SS02	++	++	++	+
SS03	++	++	++	++
SS04	++	++	++	++
SS05	++	++	++	+
SS06	++	++	++	++
SS07	++	++	++	+
SS08	++	++	++	+
SS09	++	++	++	++
SS10	++	++	++	++

(+ = weak growth and ++ = good growth)

Microscopic Observation of PGPR Isolates

Microscopic observations were performed to examine some characteristics of PGPR isolates such as shape, gram reaction and motility (Table 2). On the one hand, all the isolates were found in rod shaped, motile and gram negative (Figure 1) in reaction. On the other hand, all the isolates were found to be positive in response to catalase test (Figure 4) and in cellulose degradation test (Figure 6). Catalase activity in the bacterial isolates may potentially be very advantageous. Bacterial isolates with catalase activity positive are highly resistant to environmental, mechanical and chemical stress (Glick *et al*., 1998). In case of KOH test, all the isolates were tested as negative (Figure 5) and also the isolates were positive in phosphate solubilization NA plate assay test (Figure 2). It was also found to be that growth of isolates on NA agar plates varied in temperature and NaCl concentration (Table 3 and 4). The growth of all isolates was good in the temperature range of 20 to 28°C. In addition, SS03 and SS04 isolates were found to grow at 45°C and isolates SS03, SS04, SS06, SS09 and SS10 were able to grow in 12.0 mM NaCl solutions.

Production of IAA and Solubilization of Phosphorus

As shown in Figure 7, all the isolates produced indole acetic acid (IAA) in various ranges. On the contrary, SS01 was found to be a weak IAA producer in comparison to the minimum IAA producer isolates viz. SS02, SS03, SS04, SS05 and SS09. SS06 and SS07 produced the medium quantity IAA producer. On the other hand, only SS10 produced the highest IAA among the isolates. In phosphate solubilization, SS01 solubilized the lowest amount of phosphate and isolates SS04, SS06, SS07 and SS09 were found to be the medium phosphate solubilizers. Moreover, it was noticed that all the isolates solubilize phosphate in the nutrient agar medium plate assay (Fig. 2). Furthermore, SS05 solubilized the highest amount of phosphate followed by SS02, SS03 and SS08 (Fig.7). It has been reported that IAA production by PGPR can vary among different species and it is also influenced by culture condition, growth stage and substrate ability (Mirza *et al*., 2001). PGPR have been shown to solubilize precipitated phosphates and enhance phosphate availability to chickpea that represent a possible mechanism of plant growth promotion under field condition (Verma *et al*., 2001).

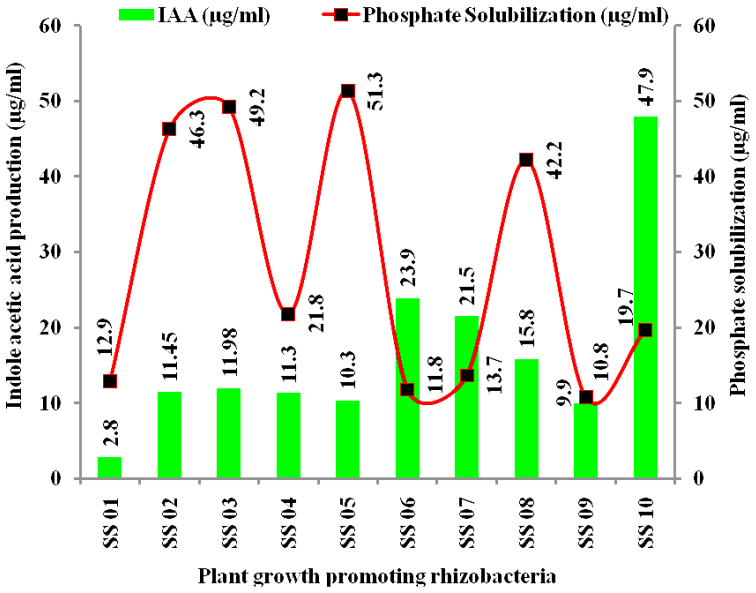

Figure 7. Indole acetic acid (IAA) production and phosphate solubilization by the isolates

Seed Germination

The first observation of this study was that increasing NaCl concentration decreased the germination percentage in chickpea seeds (Fig. 10). When NaCl treatments compared to each other it was seen that the effect of PGPR on germination percentage varies with bacterial isolates. The effect of PGPR on germination rate of seeds under saline conditions was statistically significant ($p<0.05$). Shannon and Grieve, (1999) reported that salinity showed the germination rate and at low concentration the only was on germination rate and not total percentage of seeds.

The results of our study clearly showed that PGPR improved germination percentage and rate according to the control in spite of the use of high concentrations of NaCl. Nelson, (2004) noted that PGPR were able to exert a beneficial effect upon plant growth such as increasing the germination rate.

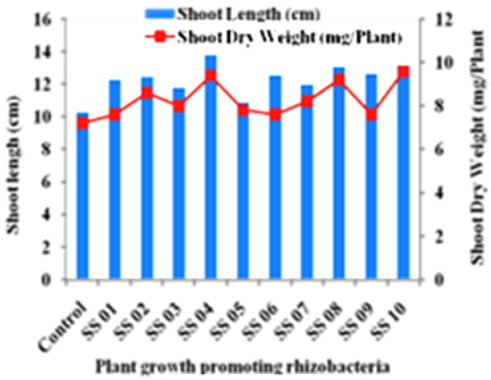

Fig. 8a

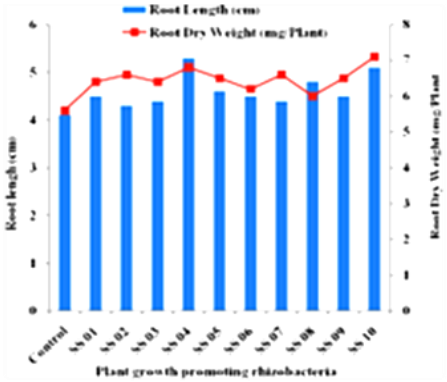

Fig. 8b

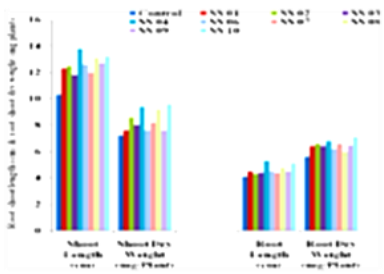

Fig. 9

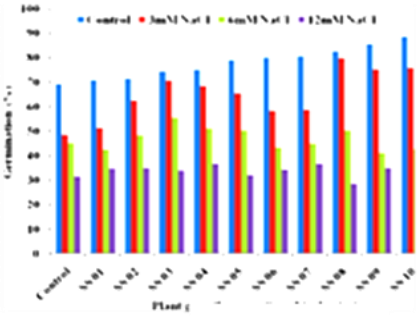

Fig. 10

Figure 8a. Effect of PGPR on shoot length (cm) and shoot dry weight (mg/plant) of chickpea; **Figure 8b.** Effect of PGPR on root length (cm) and root dry weight (mg/plant) of chickpea; **Figure 9.** Effect of PGPR on shoot length (cm), shoot dry weight (mg/plant), root length (cm) and root dry weight (mg/plant) of chickpea; **Figure 10.** The effect of PGPR on seed germination of chickpea under salinity condition

Length and Dry Weight of Shoot and Root

The PGPR isolates significantly affected the length of shoots and roots of chickpea seedlings. Results reveal that the shoot length increased in PGPR treated plants over un-inoculated control (Figure 8a, 8b and 9). The highest shoot length 13.80 cm was recorded in SS04 isolate which was statistically similar to isolates SS08 (13.10 cm plant^{-1}) and SS10 (13.20 cm plant^{-1}). A significant increase in shoot dry matter of chickpea seedling was observed in response to PGPR isolates. The highest shoot dry matter was recorded in isolate SS10 (9.60 mg plant^{-1}) followed by SS04 (9.40 mg plant^{-1}) and SS08 (9.20 mg plant^{-1}). Root length ranged from 4.10 to 5.30 cm plant^{-1}. The isolate SS04 produced the highest root length (5.30 cm plant^{-1}), in comparison to other isolates SS05 and SS08 also showed superior root length, respectively (Figure 8b and 9). A significant variation in root dry weight was observed in response to different PGPR isolates. In this study, the effectiveness of PGPR isolates on shoot length, root length and dry weight of shoot and root were investigated. Most of the isolates significantly increased shoot length, root length and dry matter production of shoot and root of seedlings (Figure 8a, 8b and 9). Bacteria produce IAA to promote root growth by stimulating cell division or elongation (Patten and Glick, 2002).

Our results suggested that PGPR are able to enhance the production of IAA, solubilization of phosphorus and resistance to pathogen and pests, thereby improving growth of chickpea plant. The use of PGPR as inoculants biofertilizers is an efficient approach to replace chemical fertilizers and pesticides for sustainable chickpea cultivation in Bangladesh and other developing countries. Further investigations, including efficiency test under green house and field conditions needed to clarify the role of PGPR as biofertilizers that exert beneficial effects on plant growth and development.

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