



Original Research Article

Application of Plant Growth Promoting Rhizobacteria (PGPR) Isolated from the Rhizosphere of *Sesbania bispinosa* on the Growth of Chickpea (*Cicer arietinum* L.)

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ABSTRACT

Keywords

PGPR;
Escherichia coli DACG2;
Pseudomonas fluorescens DACG3;
Burkholderia sp DACG1.;
Sesbania bispinosa;
Cicer arietinum,
Bio-fertilizer

Plant Growth Promoting Rhizobacteria (PGPR) are beneficial bacteria that colonize plant roots and enhance plant growth by wide variety of mechanism. Initially twelve isolates of bacteria designated as DD1 to DD12, isolated from the rhizospheric soil of Dhaincha (*Sesbania bispinosa*) were screened on the basis of their efficiency in PGPR characteristics and finally three most efficient PGPR strains, i.e. - DD3, DD4 and DD6, were selected and further identified as *Escherichia coli* DACG2 (GenBank Accession number JN858966) *Pseudomonas fluorescens* strain DACG3 (GenBank Accession No. KP641168) *Burkholderia sp.* DACG1 (GenBank Accession No. JN639877) using 16 S rRNA sequencing technology. Subsequently, to investigate the effect of PGPR isolates on the growth of *Cicer arietinum* L., a pot culture experiment and a field experiment were conducted using a randomized complete block design with 3 replications and 9 treatments. The treatments consisted of uninoculated control and all cases of single, dual and triple inoculants of these 3 bacteria. The maximum rates of plant height, number of leaves/plant, pod bearing branches, pods/plant, nodules/plant and 100 seed weight were recorded by applying the combined inoculation of *Escherichia coli* DACG2+ *Pseudomonas fluorescens* DACG3+ *Burkholderia sp* DACG1 as compared with other inoculation treatments and uninoculated control. Therefore, present study suggests that PGPR isolates viz. *Escherichia coli*, *Pseudomonas fluorescens*, and *Burkholderia sp.* may be used as biofertilizers to enhance the growth and productivity of chickpea.

Introduction

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. The interaction between plant and microbes mainly plant growth promoting rhizobacteria (PGPR) are largely facilitated by the rhizospheric soil (Kloepper et al., 1980; Villacieros et al., 2003). PGPR are known to rapidly colonize the rhizosphere and suppress soil borne pathogens at the root surface (Rangajaran et al., 2003). These micro-organisms can also be beneficial to the plant by stimulating their growth and development through direct and indirect ways (Glick 1995; Gupta et al. 2000; Bloemberg and Lugtenberg, 2001; Moeinzadeh et al., 2010). Preparations of live microorganisms (bacteria, fungi) utilized for improving plant growth and crop productivity are generally referred to as biofertilizers or microbial inoculants (SubbaRao N.S., Dommergues Y.R., 1998; Vessey J.K., 2003) helps to promote growth by increasing the supply or availability of primary nutrient to the host plant (Narula N. et al., 2005).

The use of PGPR is steadily increased in agriculture and offers an attractive way to replace chemical fertilizers, pesticides, and supplements. The concept of PGPR began to gain importance and a large number of bacterial strains have been isolated, screened (Chanway C.P., Holl F.B., 1993; Cattelan A.J. et al., 1999; Bertrand H. et al., 2001) and evaluated for plant growth promotion (Lifshtiz R. et al., 1987; Chanway C.P. et al., 1989; Abbas Z., Okon Y., 1993; Glick B.R. et al., 1997; Zhang F. et al., 1997; Bashan Y., Holguin G., 1998; Mayak S. et al., 1999; Bent E. et al., 2001; Salamone I.E.G., 2000).

Now, Dhaincha (*Sesbania bispinosa*) is a crop generally cultivated for its nutritive value to soil. It is cultivated in monsoon season almost throughout India and grows well in loamy, clayey, black and sandy soils. It is an ideal green manure crop as it is fast growing, succulent, and easily decomposable with low moisture requirements. It forms both root and stem nodules in association with *Rhizobium sp.*, fixes more atmospheric N₂ and produces maximum amount of organic matter in the soil. It enriches concentration of Ca, P, S & micronutrients (Khan et al. 2010). It is a very important intercrop relayed with rice and other cereals. Nutrients derived from Dhaincha are very cheap as compared to those of chemical fertilizers. It increases physical and biological properties of soil, add organic matter to it and it gives long term residual effect to the following crops.

However, till date, very few studies have been found on PGPR association of Dhaincha plants. Moreover, a better knowledge of PGPR habiting rhizospheric soil of Dhaincha and their implications on growth and yield of other crop might change traditional crop management practices. The plant growth promoting bacterial strains must be competent, able to survive and colonize in the rhizospheric soil (Cattelan et al., 1999). The good results obtained in vitro cannot always be dependably reproduced under field conditions (Chanway and Holl, 1993; Zhender et al., 1999). The variability in the performance of PGPR may be due to various environmental factors that may affect their growth and exert their effects on plant. The environmental factors include climate, weather conditions, soil characteristics or the composition or activity of the indigenous microbial flora of the soil.

Chickpea (*Cicer arietinum*) is one of the major pulse crops not only in India but

throughout the world and is the most important staple food in several developing countries. It ranks 5th among grain crops (Smithson *et al.*, 1985), and is imperative due to its high nutritive contribution in Indian diet. It has considerable importance in fodder also. But the efficiency in India is far behind as compared to world productivity. So there is a tremendous scope for enrichment of Gram seed production in our region. Chemical fertilizers are 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 to use PGPR that could contribute to the improvement of chickpea growth. To achieve the maximum growth promoting interaction between PGPR and nursery seedlings it is important to discover how the rhizobacteria exerting their effects on plant and whether the effects are altered by various environmental factors, including the presence of other microorganisms (Bent *et al.*, 2001). Therefore, it is necessary to develop efficient strains for chickpea production. One possible approach is to explore soil microbial diversity for PGPR having combination of plant growth promoting activities. So keeping in view the above constrains, the present study was designed to determine the effect of plant growth promoting rhizobacteria (PGPR) inhabiting the rhizosphere of Dhaincha (*S. Bispinosa*) for improvement of germination and plant Growth of chickpea (*Cicer arietinum* L.).

Material and Methods

Bacterial culture and Identification

The rhizospheric soil of *S. bispinosa* was collected from four different spots both from local farmer's field (around Kalyani area of Nadia district) and research plots of Horticultural faculty of Bidhan Chandra

Krishi Viswavidyalay, State Agricultural University). The bacterial strains from collected soil samples were isolated by soil dilution plate count technique. Isolated bacterial strains was tested for their PGPR activities and based on higher PGPR activities three isolates were selected for further experiments. Bacterial cultures were also molecularly characterized by 16S rDNA partial gene sequencing. The three Bacterial cultures were maintained on the respective slants media and store at 4°C further use.

Chickpea seed germination: Germination tests were carried out to determine the effect of inoculation of PGPR strains on the rates of seed germination. For this, Chickpea (*Cicer arietinum* L.) seeds were used as plant materials. Healthy seeds were surface sterilized with 0.1% HgCl₂ for 2 min and rinsed six times with sterile distilled water. Three PGPR strains were grown in respective broth on shaking incubator (180 rpm) at 28 ± 2°C for 24 h.

The surface sterilized seeds of chickpea were inoculated in broth culture of the PGPR strain cultures for 30 min including normal water (C) as control. Six inoculated seeds of each treatment were placed in separate petri-plate containing soaked (with distilled water) filter papers the petri-plates were incubated at 25±2 °C for 6 days. Seed germination was recorded regularly starting from the 2nd day on the basis of number of the germinated seed out of total germination. Each treatment was replicated three times. Percentages of germination were calculated.

Effect of single and co-inoculation on growth, yield and nodulation of chickpea

Both pot and field experiments were conducted to test the potential of selected PGPR isolates alone as well as in

combination for promoting growth, nodulation and yield of chickpea under natural conditions.

Layout, Design and Treatments: In these experiments, three PGPR bacteria, either alone or in combination, were applied along with two controls (water and medium) and the total nine treatment combinations were laid out in a Randomized Block Design with three replications.

Pot experiments

Pot trials were conducted in the net house of the Department of Botany, University of Kalyani, West Bengal. Chickpea seeds were sown into sterilized pots. Five seeds were sown in each pot containing 12 kg soil per pot which were thinned to one plant after 15 days of germination. Pots were placed in the net house under ambient light and temperature. The inocula for the pot trials were prepared by culturing the selected bacterial strains on nutrient agar. A single colony was transferred to 250 ml flasks containing nutrient broth, and grown aerobically in flasks on a rotating shaker (95 rpm) for 24 h at 27°C. The bacterial suspension was then diluted in sterile distilled water to a final concentration of 10^8 CFU ml⁻¹. 1ml of log culture (10^8 cells) of each bacterial isolates was transferred as inoculum in the corresponding treatments. Treated and non treated pots were irrigated with sterilized water daily. After every 7 days interval 2ml of microorganism inoculum was inoculated in the corresponding pot as booster dose. At maturity stage and data regarding yield and yield contributing parameters were collected and analyzed statistically.

Field Experiment

Field experiments were conducted at two different sites of the Iswaripur Village(35°

43' N and 47° 8' E with an altitude of 2100 m) of Nadia District, West Bengal during the two successive rabi seasons of 2012-13 and 2013-14 to study the effect of PGPR on growth and yield of chickpea. The soil was sandy having pH 6.2. The selected PGPR isolates were tested alone as well as in combination under field conditions. All the agronomic practices were same as used in pot experiments. Each plot size was 3x4 m. inoculated and uninoculated seeds were sown in the well-prepared plots on November 9 and 26 in the year 2012 and 2013 respectively with 30cm x 20 cm spacing after proper land preparation by cultivating 2 times followed by planking. Treatments were replicated thrice, using randomized complete block design (RCBD). Canal water was used for irrigation when needed. . The inoculums were applied as basal dose in all the plots then after every 7 days interval 2ml of microorganism inoculum was sprayed in the surrounding rhizosphere of the corresponding plant as booster dose. At maturity, the crop was harvested and data about growth and yield parameters were recorded.

Statistical analysis. The data were analyzed with Statistical Package for Social Sciences (SPSS, ver. 21), and Sigma Plot (ver. 11) Software.

Results and Discussion

Molecular identification and the treatment combinations of the PGPR strains

The three PGPR isolates were identified by 16 S rDNA sequencing. 16 S rDNA gene sequences compared with the available sequences in the databank with help of BLAST homology search. DD3 was identified as *Escherichia coli* DACG2 and deposited it in the GenBank database with accession number JN858966 while DD4 was

identified as *Pseudomonas fluorescens* strain DACG3 (GenBank Accession No. KP641168) and DD6 as *Burkholderia sp.* DACG1 (Gene Bank Accession No. JN639877). These three PGPR strains, either alone or in combination, along with two controls (water and medium) were used as treatments. The combinations are presented in the Table 1.

Effect of isolated PGPR strains on germination of Chickpea seeds

Our experiments showed that PGPR Inoculation significantly enhanced seed germination and seedling vigour of Chickpea. However, the rate of enhancement varied with bacterial strains. The experimental results of different seed treatments in chickpea revealed significant different responses against seed germination. (Table-2). All the three treatment sets were found to be significantly superior and effective in increasing 15 per cent more germination of chickpea in comparison to control sets. The results also indicated that the PGPR strains initiate the germination of *C. arietinum* seed at the 2nd day of treatment connecting to active participation in germination system though on day 4 it was abruptly reduced whereas in control set initiation of germination occurred on 3rd day. 100 percent seed germination occurred in PGPR inoculated seeds on 3rd (in treatment 1 and treatment 2) and on 4th (in treatment 3). The observation on first count at germination can also considered as seed vigour which was very much supportive to seedling establishment in field.

Pot experiment: The results of pot study showed that inoculation of Chickpea seeds with bacterial strains showed a positive effect on all the studied growth parameters (Table 2). The data presented in the Table represents that, in pot experiment, either alone or in combination, the 3 selected

PGPR strains i.e. *Escherichia coli* DACG2, *Pseudomonas fluorescens* DACG3 and *Burkholderia sp.* DACG1 showed significantly increased plant growth in all the treatments compared to both the controls. Height of the treated Chickpea plants increased up to 93% in comparison to controls. As same PGPR inoculation also had the positive effect on number of leaves per plant, which increased up to 76% in comparison to control. Enhancement of Numbers of pod bearing branches per plant is up to 70%. Number of pods per plant increased up to 45 %. Number of nodules per plant increased up to 50%.

Weight of 100 seeds increased up to 27%. The highest increase in growth parameters of Chickpea plant was recorded from Treatment 7 inoculated with all the 3 PGPR strains (combination of the 3 PGPR strains i.e. *Escherichia coli* DACG2 + *Pseudomonas fluorescens* DACG3 + *Burkholderia sp.* DACG1). It is also recorded that Treatment 4, Treatment 5, and Treatment 6 also show significant increase in comparison to Treatment 1, Treatment 2, Treatment 3 and also than controls i.e. the PGPR strains in combination performed much better than alone. The PGPRs interact with each other synergistically stimulating each other through physical or biochemical activities that may enhance some beneficial aspects of their physiology.

Field Experiment

In field experiment, all the growth parameters as influenced by the three PGPR isolate treatments have been presented in Figure 2. The maximum plant height recorded was in case of treatment 7 (combination of all the three isolates).

It is experimentally proved that PGPR have positive effect on the growth of different crops and plants (Wu S.C. *et al.*, 2005).

Table.1 Treatment combinations of the inoculums

Treatment 1 = Inoculum DD3*
Treatment 2 = Inoculum DD4**
Treatment 3 = Inoculum DD6 ***
Treatment 4 = InoculumDD3+ Inoculum DD4
Treatment 5 = Inoculum DD4+ Inoculum DD6
Treatment 6 = Inoculum DD3+ Inoculum DD6
Treatment 7 = InoculumDD3+ Inoculum DD4+ Inoculum DD6
Treatment 8 = Medium control
Treatment 9 = Water control

DD3* = *Escheritia coli* DACG2, DD4** = *Pseudomonus fluorescense* DACG3,
DD6*** = *Burkholderia* sp. DACG1

Table.2 Effect of PGPR strains on germination of Chickpea seeds

Treatments	% of germination of Chickpea seeds					
	1 st day	2 nd day	3 rd day	4 th day	5 th day	Total
Water	-	-	40	45	-	85
Treatment 1	-	35	65	-	-	100
Treatment 2	-	75	25	-	-	100
Treatment 3	-	55	40	5	-	100

Table.3 Effect of PGPR strains alone or in combination on growth parameters of Chickpea
Mean and Std. Error

Treatment(s)	Plant Height (cm)	No. of Leaves /Plant	No. of pod bearing branches	No. of pods / Plant	No. of nodule / Plant	100 seeds weight (g)
Treatment 1 (X)	33.15± 3.1	42± 3.7	12± 1.3	53± 4.5	21± 1.3	22.44± 2.3
Treatment 2 (Y)	45.09± 3.2	58± 4.2	13± 1.7	59± 4.2	23± 1.2	24.7± 1.7
Treatment 3 (Z)	37.54± 2.4	47± 4.4	11± 1.2	54± 5.8	20± 1.5	22.32± 1.2
Treatment 4 (X+Y)	53.33± 4.2	67± 4.6	14± 1.4	60± 1.3	25± 1.4	25.2± 2.4
Treatment 5 (Y+Z)	51.46± 3.8	63± 5.3	16± 1.3	67± 4.2	26± 1.4	25.43± 2.3
Treatment 6 (X+Z)	50.18± 3.3	63± 6.4	13± 1.4	63± 3.4	22± 1.9	23.94± 1.4
Treatment 7 (X+Y+Z)	59.78± 4.5	69± 4.6	17± 1.2	68± 1.6	27± 1.7	25.87± 2.2
Control(Medium Control)	31.24± 2.5	39± 4.6	10± 1.2	47± 1.6	18± 1.7	20.5± 2.2
Ref. Control (Water Control)	30.32± 2.5	35± 4.6	8± 1.2	34± 1.6	12± 1.1	19.43± 1.2

Figure.1 A bar graph showing the germination percentage of chickpea seeds after 4th day of inoculation with the three PGPR strains along with control. DD3 = Escherichia coli DACG2, DD4 = Pseudomonas fluorescence DACG3, DD6 = Burkholderia sp. DACG1

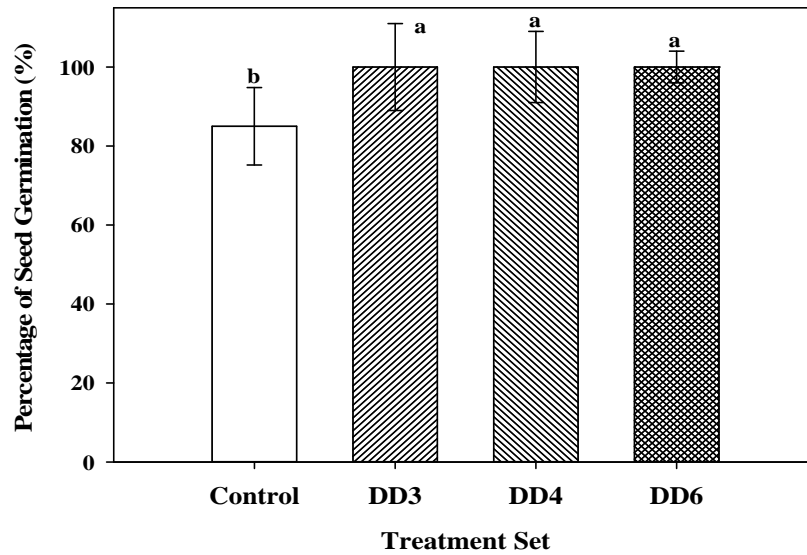
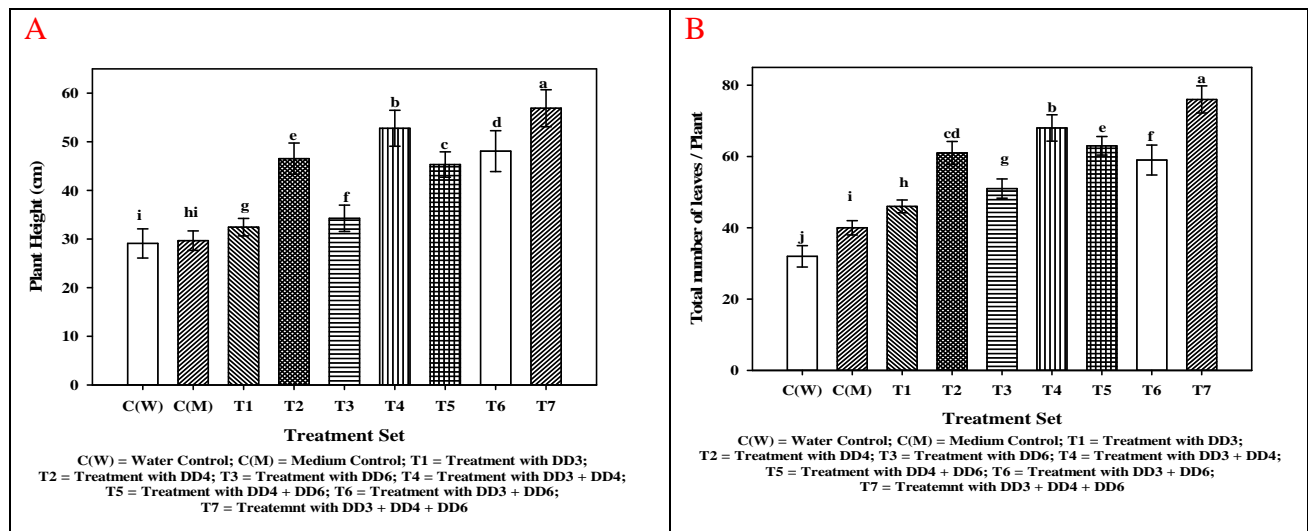
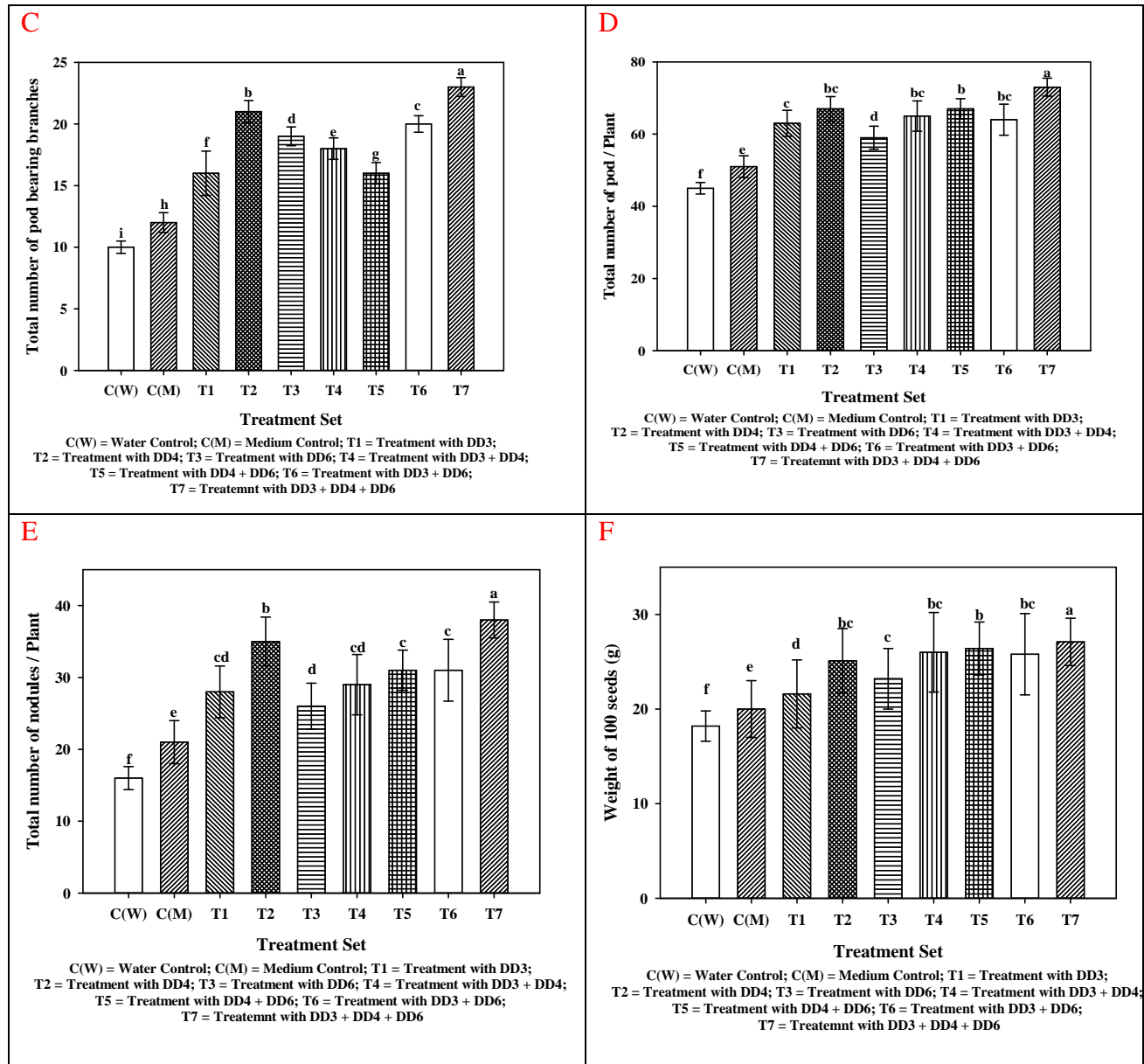


Figure.2 Bar graph of effects of PGPR treatments on A:Plant height, B: Number of leaves/plant, C: Total number of pod bearing branches, D: Total number of pods/plant, E: Total number of nodule/plant, F: Weight of 100 seeds (gm), DD3=Escherichia coli DACG2, DD4=Pseudomonas fluorescence DACG3, DD6=Burkholderia sp DACG1





The impact of rhizobacteria generally on plant growth and health may be classified as neutral, deleterious or beneficial (Kloepper J.W. *et al.*, 1989). Many researchers (Lifshtiz R. *et al.*, 1987; Chanway C.P. *et al.*, 1989; Abbas Z., Okon Y., 1993; Glick B.R. *et al.*, 1997; Zhang F. *et al.*, 1997; Bashan Y., Holguin G., 1998; Mayak S. *et al.*, 1999; Bent E. *et al.*, 2001) records the ability of microbial inoculants, to increase plant growth and germination rate, improve

seedling emergence, responses to external stress factors and protect plants from disease. This present investigation confirms the earlier works. It revealed that use of PGPRs with seed treatment improve seed germination, seedling emergence, seedling vigor and seedling stand over the control. Similar results have been reported in other crops such as potato, radish plants, sorghum and pearl millet (Burr T.J. *et al.*, 1978; Raju N.S. *et al.*, 1999; Niranjana S.R. *et al.*,

2004). The improvement in seed germination by PGPR was also found in work with wheat and sunflower (Shaukat K. *et al.*, 2006), where it was found that some PGPR induced increases in the seed emergence, in some cases achieving increases up to 100% greater than controls. Our results also show the higher seedling with isolated bacterial strains.

In pot experiment, it was observed that PGPR inoculation significantly increase the growth of seedlings of Chickpea. In general, inoculation resulted in early seedling growth and development. Similar findings were reported by Dobbelaere S. *et al.*, (2006) who assessed the inoculation effect of PGPR *Azospirillum brasilense* on growth of spring wheat. They observed that inoculated plants resulted in better germination, early development and flowering and also increase in dry weight of both the root system and the upper plant parts (Gravel V. *et al.*, 2007; Kozdroja J. *et al.*, 2004). Soil condition also influenced the growth promotion by bacterial strains. Martinez-Toledo M.V. *et al.*, (1988) showed that the numbers of *Azotobacter* decreased as plant growth continued in nonsterile agricultural soil, while the numbers of *Azotobacter* associated with maize roots grown in sterile agricultural soils remained similar to those of the original inoculum. This may imply rhizobacteria had a more competitive ability to survive and affect the growth of inoculated plants in the presence of indigenous micro flora (Khalid A. *et al.*, 2004). In this study, Inoculation of PGPR strains increased all parameters determined in-pot experiment as well as in field. Again, Mixed Inoculants which indicate that mixed inoculants.

The present experiment revealed that seed inoculation with all isolated bacteria resulted in an increased plant height and leaf numbers. Similar increases in plant height

and leaf area were observed in different crops such as potato, radish plants, sorghum and pearl millet inoculated with *Pseudomonas*, *Azospirillum* and *Azotobacter* strains (Burr T.J. *et al.*, 1978; Raju N.S. *et al.*, 1999; Niranjana S.R. *et al.*, 2004).

In conclusion, it was found that three Plant growth promoting rhizobacteria (viz. *Escherichia coli* DACG2, *Pseudomonas fluorescence* DACG3, *Burkholderia sp.* DACG1) isolated from the rhizosphere of *Sesbania bispinosa* significantly enhanced root and shoot length and biomass production of chickpea. Therefore it is suggested that the use of PGPR isolates of as effective biofertilizers might be beneficial for chickpea cultivation.

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