Journal of Cell and Tissue Research Vol. 18(2) 6423-6434 (2018)
(Available online at www.tcrjournals.com)
ISSN: 0973-0028; E-ISSN: 0974-0910

Original Article

ISOLATION, SCREENING AND CHARACTERIZATION OF PGPR STRAINS ISOLATED FROM THE RHIZOSPHERE OF A XEROPHYTE *EUPHORBIA VIROSA*

POPAT, T. AND VYAS, B. R. M.[?]

Department of Biosciences, Saurashtra University, Rajkot, Gujarat, India
E. mail: brmvyas@hotmail.com, Cell: 9427213279

Received: April 26, 2018; Accepted: May 25, 2018

Abstract: Application of exotic bacterial strains as biofertilizer has always posed a constraint in the success of biofertilizer technology. Only limited number of biofertilizers with applicability in wide range of soils is available in the market. Plant Growth Promoting Rhizobacteria (PGPR) with multiple plant growth promoting traits like phosphate solubilization, production of indole acetic acid, gibberellic acid, 1-aminocyclopropane 1-carboxylic acid deaminase activity and antifungal activities like siderophore, hydrogen cyanide (HCN) and extracellular enzymes production were isolated from the rhizospheric soils of Euphorbia virosa growing in semi arid region of Gujarat. Three isolates having multiple plant growth promoting activities were selected and identified on the basis of 16S rDNA sequence analysis. The application of PGPR strains in vitro has shown remarkable plant growth promotion and antifungal activity and increased in the growth of five crop plants viz. Pisum sativum (Pea), Arachis hypogaea (Ground nut), Cicer arientinum (Gram), Triticum aestivum (Wheat), Vigna radiata (Mung bean) during pot trials was observed. Our results demonstrate that microbial inoculation improved growth of crop plants and can lead to increased productivity.

Key words: PGPR strains, Biofertilizer, Phosphate solubilization

INTRODUCTION

As public opinion against the use of chemical pesticides on food crops grows, more and more pesticides have been removed from agricultural use. Hence, establishing new and effective pest control measures is a concern. One such alternative pest control strategy is the use of PGPR. This strategy has the potential to reduce or eliminate chemical pesticides on agriculturally important crops and thereby reduce the risk associated with pesticide residues in the environment. PGPR are root colonizing bacteria beneficial to various agricultural crops. Usually, PGPR promote plant growth directly by (i) nitrogen fixation, (ii) solubilization of minerals like phosphorous, potassium, iron etc., (iii) modulating plant hormone levels, (iv) ACC deaminase activity and indirectly by the production of (i) siderophores, (ii) volatile compounds, and (iii) hydrolytic enzymes to deplete pathogens and act as bio-control agents. Nevertheless, implementation of this biotechnology has been hindered by the lack of consistency and variation in responses that are obtained in field trials from site to site, year to year, or for different crops [1]. Successful establishment of the introduced bacteria depends on proper PGPR selection that must be tailored to the soil and crop combination. Other basic problems that are related to inoculum production, storage, and delivery have mostly precluded the use of non-spore forming bacteria as soil inoculants. To investigate the above mentioned problems, efforts were undertaken to isolate and screen efficient strains

exhibiting multiple plant growth promoting traits under *in vitro* conditions as well as during pot trials.

MATERIALS AND METHODS

Isolation of PGPR strains: Roots and rhizospheric soils were collected from plant *Euphorbia virosa* growing in different areas of Gujarat during winter season. Root samples were preferred from those areas of fields where plants showed more growth. Whole plant was carefully uprooted without causing any damage to the roots and also with soil attached to the roots, transferred to sterile plastic bags, packed and taken to the laboratory. Soil adhering to the roots was gently scraped and suspended in sterile DW and used to prepare various dilutions. 100 µl of diluted soil suspension was spread on Nutrient and Pikovskaya's agar plates and incubated overnight at RT. Colonies that appeared were picked and obtained in pure by sequential transfers on the same media. Colony and biochemical characteristics of isolates were observed. The bacterial cultures were preserved in 30% glycerol and stored at -10°C.

Plant Growth Promoting Activities

Qualitative phosphate solubilization: GF strains were spot inoculated on Pikovskaya agar plates containing tri-calcium phosphate as sole source of nutrient P and incubated at 30±2°C for 3 days. Phosphate solubilization index was calculated by determining ratio of (Diameter of solubilization zone / Diameter of the colony) x 100.

Quantitative phosphate solubilization: Bacterial strains were inoculated in 250 ml Erlenmeyer flask containing 50 ml Pikovskaya medium and incubated on rotary shaker (180 rpm) at 37°C. 5 ml culture was taken daily for 5 days and centrifuged (10000 rpm, 10 min). The pH and P-content of the supernatant were estimated [2].

Acid and alkaline phosphatase activities: Bacterial strains were inoculated in 100 ml Erlenmeyer flasks containing 40 ml Luria-Bertani (LB) medium and incubated on rotary shaker (180 rpm) at 37°C for 24 h. Entire content of the flasks was centrifuged (10000 rpm, 10 min) and supernatant was used as enzyme sample. Phosphatase activities were determined using reaction mixture comprising of 0.1 ml supernatant, 0.5 ml buffer (0.5 M Tris-citrate buffer, pH 8.5/0.5M Na-acetate buffer, pH 4.5), 0.1 ml $MgCl_2$ (0.1 M) and 1 ml *p*-nitrophenol phosphate (1 mg/ml). 5 ml NaOH was added after 30 min to stop the reaction and A_{405} was measured to determine the amount of *p*-nitrophenol released. 1 Unit enzyme activity is defined as the amount of activity that releases 1 mmol of *p*-nitrophenol per min in the reaction mixture [3].

Estimation of indole acetic acid: GF strains were cultivated in 100 ml Erlenmeyer flasks with 50 ml LB medium supplemented with tryptophan 100 mg/ml) and glucose (1%) and incubated at 37°C for 5 days.Cultures were centrifuged (10000 rpm, 4°C, 10 min) and 2 ml supernatant was mixed with 2 µl of o-phosphoric acid and 4 ml of Salkowski's reagent and A530 was recorded to determine IAA produced [4].

Estimation of gibberellic acid: 5 ml glucose medium [5] was inoculated with GF strains and incubated on rotary shaker (180 rpm) at 37°C for 3 days. Culture supernatant obtained by centrifugation (10000 rpm, 4°C, 10 min) was used to determine gibberellic acid produced as described by Graham et al. [6].

Production of ACC deaminase activity: Isolates were inoculated in 10 ml LB medium and incubated on rotary shaker (180 rpm) at 37°C for 24 h. Cultures were harvested and pellets obtained by centrifugation (10000 rpm, 4°C, 10 min) were washed twice with 0.1 M phosphate buffer (pH 7.5), resuspended in 20 ml salts medium comprising of g/l succinic acid (2), K_2HPO_4 (1), KH_2PO_4 (1), $MgSO_4.7H_2O$ (0.5), $CaCl_2$ (0.13) and $FeSO_4$ (0.0013), pH 5.4 supplemented with 3 mM ACC and incubated on rotary shaker (180 rpm) at 37°C for 24 h [7]. Biomass obtained by centrifugation (10000 rpm, 4°C, 10 min) was washed once with 0.1 M phosphate buffer (pH 7.5) and then washed thrice with 0.1 M Tris-HCl (pH 8.5). 30 µl of toluene was added and homogenized for 30 s. 200 µl of toluenized cells was mixed with 20 µl of 0.5 mM ACC, vortexed and incubated at 30°C for 15 min. 1 ml 0.56 mM HCl was added to the mixture, vortexed and centrifuged (10000 rpm, 5 min, RT). 1 ml supernatant was vortexed with 800 µl of 0.56 mM HCl. 300 µl of 2,4-DNPH was added to the mixture and incubated for 30 min at 30°C. 2 ml 2 M NaOH was added and A_{540} determined. 1 unit activity is defined as the activity that produced 1 mmol of a-ketobutyrate in minute [8].

Antifungal Activities

Siderophore production: Chrome-azurol S agar plates were spot inoculated with GF strains and incubated (37°C, 3 d). Plates were observed for growth and development of orange halo around colonies [9].

HCN production: Test tubes containing 5 ml LB medium were inoculated with GF cultures and incubated on rotary shaker (37°C, 24 h, 180 rpm). Cultures were centrifuged (4°C, 10000 rpm, 10 min) and 100 ml of *o*-phosphoric acid was added to supernatant and observed for pink color development.

Screening of Extracellular Enzyme Activities

Chitinase activity: Chitin agar plates comprising in g/l of chitin (5), yeast extract (0.5), $MgSO_4.7H_2O$ (0.2), NaCl (0.1), K_2HPO_4 (0.5) agar (15), pH 7 were inoculated with GF strains and incubated (37°C for 3 d). 1% Congo red solution was poured on the agar surface and incubated (RT, 30 min). Plates were then washed with 1 M NaCl to observe clear zones around colonies.

Pectinase activity: Pectin agar plates comprising in g/l of peptone (3), pectin (0.5), yeast extract (0.5), Na_2CO_3 (0.5), KH_2PO_4 (0.15), $CaCl_2$ (0.001) agar (15), pH 7 were inoculated with GF strains and incubated (37°C, 24 h). Iodine solution (Iodine 1 and Potassium iodide 5 g/300 ml DW) was poured on the agar surface to observe clear zones around the colonies.

CMCase activity: GF strains were spot inoculated on carboxy methyl cellulose (CMC) agar plates where CMC disodium salt was the sole carbon source; comprising in g/l of CMC (10), KH_2PO_4 (4), Na_2HPO_4 (4), $MgSO_4.7H_2O$ (0.2), $CaCl_2.2H_2O$ (0.001), $FeSO_4.7H_2O$ (0.004), tryptone (2.0), agar (15), pH 7 and incubated (37°C, 3 d). 1% Congo red solution was poured on the agar surface and incubated (RT, 30 min). 1 M NaCl was used to flush the Congo red from agar plates.

Protease activity: Skim milk agar plates (nutrient agar with 1% skim milk powder) were spot inoculated with GF strains and incubated (37°C, 24 h) and observed for growth.

Amylase activity: Starch agar plates (nutrient agar with 5% starch solution) were spot inoculated with GF strains and incubated (37°C, 24 h). Iodine solution was poured on the agar surface to observe clear zone around the colonies [10]

Lipase activity: The media (nutrient agar with 1% tween 80) was inoculated with GF strains incubated (37°C, 3 d) and observed for growth [11].

Identiūcation with BIOLOG system: The three bacterial strains with potential *in vitro* plant growth promoting and antifungal activities were tested for C-source utilization pattern and identiūed using Biolog system. The Biolog GENIII micro plate analyzes a microorganism in 96 phenotypic tests including carbon source utilization assays and chemical sensitivity assays. The test panel provides a phenotypic ūnger-print of the microorganism which can be used to identify it at the species level. Tetrazolium redox dye is used to colorimetrically indicate utilization of the carbon sources or resistance to inhibitory chemicals. Brieūy, pure cultures were raised on Biolog Universal Growth (BUG) medium and 24 h old cultures was used to inoculate into inoculation fluid by using sterile cotton swab. Turbidity of the inoculants was adjusted to 85% for Inoculation Fluid A (IFA) and 65% for Inoculation Fluid B (IFB) by using turbidity meter. The microbial suspensions of 100 µl were inoculated into each well of GENIII microplate using 8-channel repeating pipette. Plates were incubated at 30°C and observed for color development at intervals of 12 h for 24–48 h. Color development was recorded using a microplate reader (Model EL311, BioTek Instruments, USA). Finally readings of the color development (A_{590}) of these plates were compared with BIOLOG database (Microlog System™, Release 4.0) and isolates were identifed at species level.

PCR Amplification and phylogenetic analysis: The selected PGPR bacterial strains were characterized by 16S rDNA sequence analysis. Genomic DNA was extracted [12]. PCR amplifcations were performed using primer 8f (5'-AGA GTT TGA TCC TGG CC AG-3') and 1492r (5'-GGT TAC CTT GTT ACG ACT T-3') using BDT v3.1 cycle sequencing kit on ABI 3730x1 genetic analyzer. The PCR products were resolved using 1% agarose gel. Contigs of approximately 1300 bp 16S rDNA were generated from forward and reverse sequence data using aligner software. 16S rDNA sequence of the isolate was compared with 16S rDNA sequences available by the BLASTN search in the NCBI, GenBank database (http://www.ncbi.nlm.nih.gov). Multiple sequence alignment

was performed using Clustal W2. Distance matrix was generated using RDP database and the phylogenetic tree was constructed using MEGA 4 [13].

Pot Trial Study

Preparation of Bacterial Inocula: Bacterial strains were grown in 100 ml LB medium in 250 ml Erlenmeyer flask on shaker (125 rpm) at 30°C for 120 h. Cells were concentrated by centrifugation (5000 X *g*, 10 min) and resuspended in 10 ml sterile DW. 5 ml suspension having 10^8 cells/ml was mixed with 5 ml jaggery (2%).

Seed priming: Seeds of *Pisum sativum* (Pea), *Arachis hypogaea* (Ground nut), *Cicer arientinum* (Gram), and *Triticum aestivum* (Wheat) were treated with 0.2% sodium hypochlorite solution for 30 sec, washed thrice with sterile DW, dried overnight, mixed with bacterial inocula and dried overnight in the laminar air flow.

Seed sowing and plant growth: The primed seeds were sown in pots filled with *ca.* 1 kg of garden soil. 3 seeds were planted in each pot and watered daily with 5 ml water. Plants were harvested after 3 weeks and their morphological parameters were analyzed. The soil was air dried, sieved and analyzed for microbial count. The seeds treated with LB medium containing 2% jaggery served as control. Each treatment was replicated 4 times. All the seeds were allowed to germinate in natural condition at ambient temperature. Plants were harvested after 20 days, vigor index [14], fresh weight, dry weight (weight after drying the tissues for 48 h at 60°C) and available phosphorous conc. were determined.

Statistical Analysis: All the data were statistically analyzed using WASP-Web Agri-Stat Package (2.0). Significance of group mean values were analyzed at $P<0.05$.

RESULTS

Plant Growth Promoting Activities: GF strains solubilized phosphate by producing organic acids and acid and alkaline phosphatase enzyme activities. All the isolates produced pink colored zone on Pikovskaya's agar plate indicating production of acids. Color change was observed from yellow to pink due to reduction in pH leading to tri-calcium phosphate solubilization. The solubilization index was in the range of 67-900 (Fig. 1). Increase in the amount of soluble P and decrease in pH occurred simultaneously with incubation time (Fig. 2). All the isolates produced acid and alkaline phosphatase activities responsible for the enzymatic release of phosphate group into the medium (Fig. 3). The isolates also produced indole acetic acid for 5 days (Fig. 4 A,B). 2.48, 1.76, 2.12 and 2.52 mg/ml IAA was produced by GF1, GF3, GF7 and GF14 respectively. Other strains produced indole acetic acid in the range of 0.2-0.8 mg/ml. Gibberellic acid production in GF1 (17.8 mg/ml), GF5 (16 mg/ml), GF14 (14.6 mg/ml) and GF 19 (13.2 mg/ml) was observed to be superior to other strains (3-11 mg/ml) (Fig. 5). All isolates produce ACC deaminase activity of which GF1 GF3, GF7, GF14, GF15 and GF20 produced 1-1.2 U/ml, GF12 and GF19 0.04-0.07 U/ml while others exhibited moderate ACC deaminase activity (Fig. 6).

Antifungal Activities: Siderophore producing GF strains produced orange halo around the colonies on CAS medium. Isolates also produced HCN and enzymatic activities of lipase and protease. 18 isolates produced amylase, 15 isolates produced chitinase, 10 isolates produced pectinase and 8 isolates also produced CMCase (Table 1).

Identification of isolates: Identification of the 3 bacterial strains showing multiple PGP traits and antagonistic properties was performed using BIOLOG Micro station system: an automated identification system. Test yielded a characteristic pattern of substrate utilization for each isolate and compared to current GEN III database. Table 2 lists the carbon substrates utilized by PGPR strains GF1, GF3 and GF14.

Phylogenetic analysis: On the basis of phylogenetic analysis of 16S rDNA partial sequences, bacterial strains GF1, GF3 and GF14 were identifed as *Bacillus subtilis, B. pumilus* and *B. aerophilus* respectively. The morphological, biochemical and 16S rDNA analysis confirmed that the three potent isolates GF1, GF3 and GF14 are Gram positive and belong to genus *Bacillus*.

Pot Trial Study

Vigor index: Vigor indexes of seedlings were calculated as: VI= (Mean root length + Mean shoot length) x Germination (%) after 20 days. Seed priming with GF1, GF3 and GF14 increased VI by 8, 35 and

42% in pea seedlings; 17, 61 and 83% in gram seedlings; 78, 89 and 80% in ground nut seedlings respectively as compared to control seedlings. Vigor index of wheat seedlings were increased by 7 and 34% with GF1 and GF14 treatments while treatment with GF3 did not affect the vigor index of wheat seedlings.

Fresh and dry weight: In the case of fresh and dry weight of pea seedlings, GF3 showed higher increase than GF1 and GF14 treated seedlings. Fresh and dry weight of gram seedlings was highest in GF1 treated seedlings as compared to GF3 and GF14 treated seedlings. Highest increase (58%) in fresh and dry weight of groundnut seedlings was noted in the case of GF3. While 9 and 42% increase was observed in the case of GF1 and GF14 treated seedlings. While in case of wheat seedlings, 72% increase in GF1 and 40% increase in GF3 treated seedlings. GF14 showed 75% increase in seedling fresh and dry weight as compared to control seedlings.

Available phosphorous: Available phosphorous was found to be higher in GF strains treated seedlings as compared to control plant soils. The increased amount of available phosphorous showed that GF strains were able to solubilize phosphate present in the soil and made it available to the seedlings. All treatments caused significant increases in shoot length, root length, fresh weight, dry weight and available phosphorous as compared to control plants (Figs. 7 A,B,C,D).

DISCUSSION

The contribution of agriculture to Indian economy is steadily declining with country's broad-based economic growth. Slow agricultural growth is a prime concern today as two-thirds of India's population depend agriculture. Current agricultural practices are neither economically nor environmentally sustainable due to poorly maintained irrigation systems, improper use of chemical fertilizers and lack of knowledge about new trends and technology. Chemical fertilizers quickly improve plant growth as soil nutrients are made available to the plants immediately and are highly manipulated to produce the exact ratio of nutrients desired. But as they are made from non-renewable sources, they improve plant growth while do nothing to sustain soil health. Repeated and long term use of chemical fertilizers can change soil pH, upset beneficial microbial ecosystems, release green house gases and result in toxic buildup of chemicals such as arsenic, cadmium and uranium in the soil. The one economic and farmer-friendly solution to the constraints of chemical fertilizers is the use of live formulations of PGPR strains. A large body of evidence suggests that PGPR enhance the growth, seed emergence and crop yield and contribute to the protection of plants against certain pathogens and pests [15-19]. The mechanisms PGPR opt for plant growth promotion involves solubilization of inorganic

GF Strains	Siderophore	HCN	Extracellular Enzyme activities					
			Lipase	Protease	Amylase	Chitinase	Cellulase	Pectinase
GF1	+	+	+	+	+	+	+	+
GF2	+	+	+	+	+	+	-	-
GF3	+	+	+	+	+	+	+	+
GF4	+	+	+	+	+	-	-	-
GF5	+	+	+	-	+	+	-	+
GF6	+	+	+	+	+	+	+	-
GF7	+	+	+	+	+	+	+	-
GF8	+	+	-	+	+	-	-	-
GF9	+	+	+	+	+	+	-	-
GF10	+	+	+	+	-	+	-	-
GF11	+	+	+	+	+	-	-	-
GF12	+	+	+	+	+	+	+	+
GF13	+	+	+	+	+	+	-	+
GF14	+	+	+	+	+	+	+	-
GF15	+	+	-	+	+	-	-	-
GF16	+	+	+	-	+	+	-	+
GF17	+	+	+	+	+	+	+	+
GF18	+	+	+	+	-	+	+	+
GF19	+	+	+	+	+	-	-	+
GF20	+	+	+	+	+	+	-	+

Table 1: Antifungal activities of PGPR Strains: production of HCN, siderophore and extracellular enzymes activities

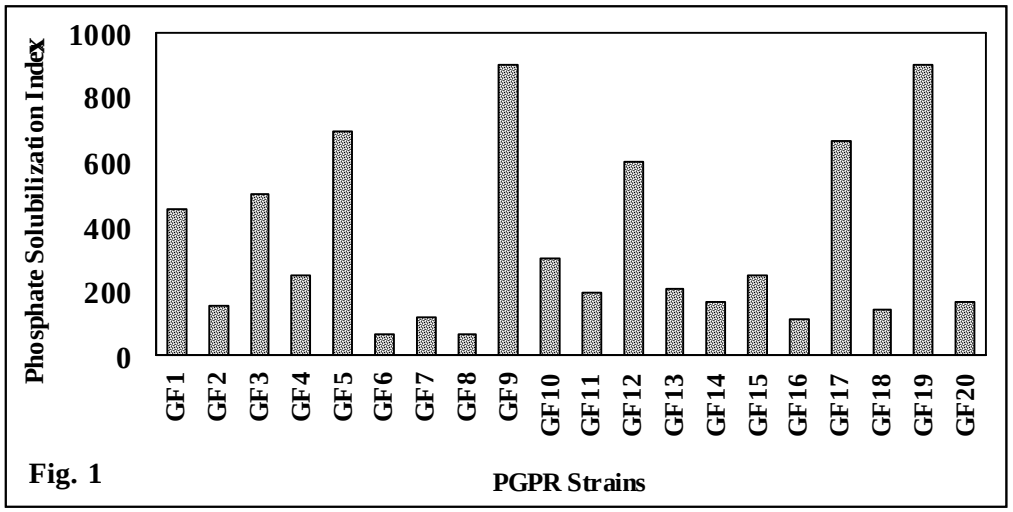

Fig. 1

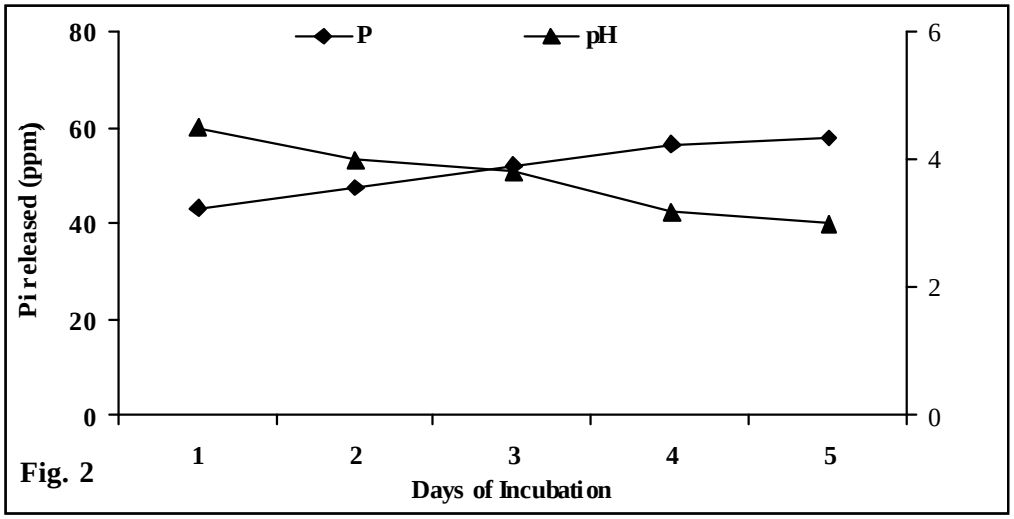

Fig. 2

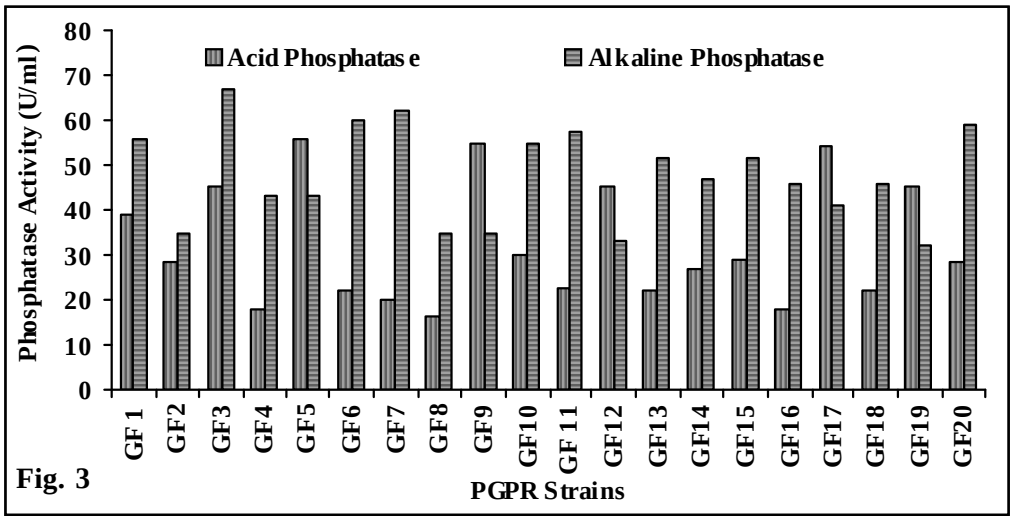

Fig. 3

Fig. 1: Phosphate solubilization index of GF strains spot inoculated on Pikovskaya's agar plate after incubation at 30±2ºC for 3 days. **Fig. 2:** Release of Pi and decrease in pH during phosphate solubilization by shake flask cultures of GF1growing in Pikovskaya's broth incubated at 37ºC for 5 days. **Fig. 3:** Acid and alkaline phosphatase activities produced by shake flask cultures of GF strains growing on LB medium at 37ºC

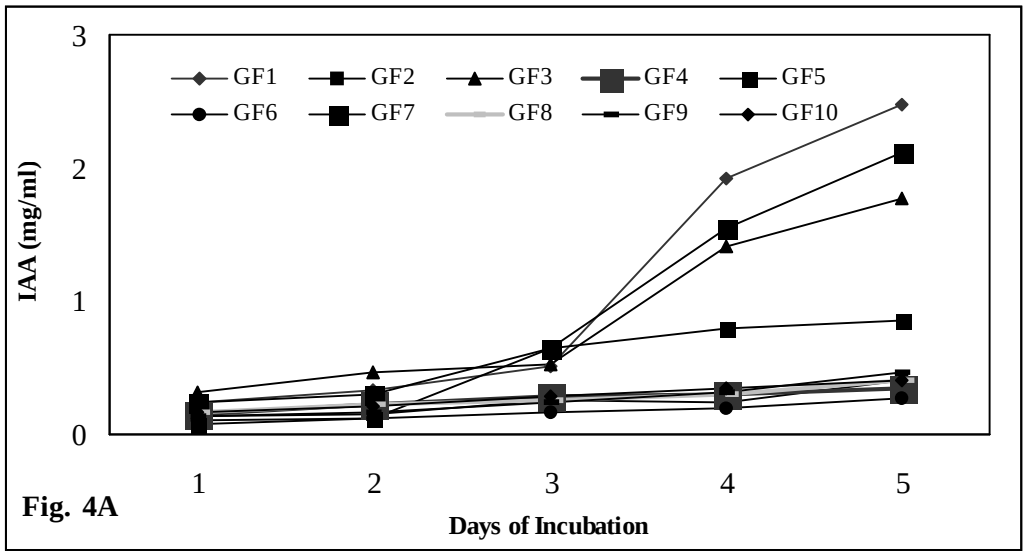

Fig. 4A

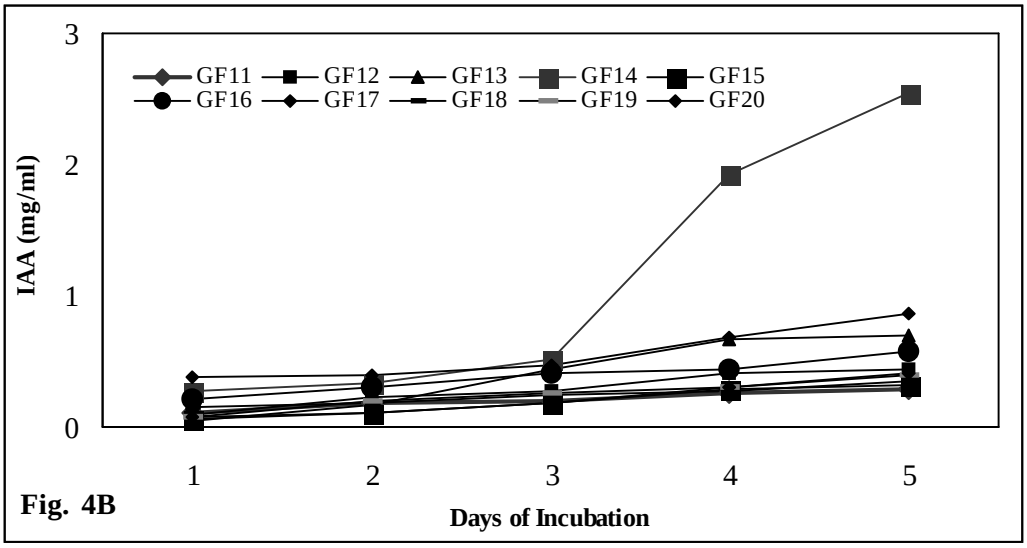

Fig. 4B

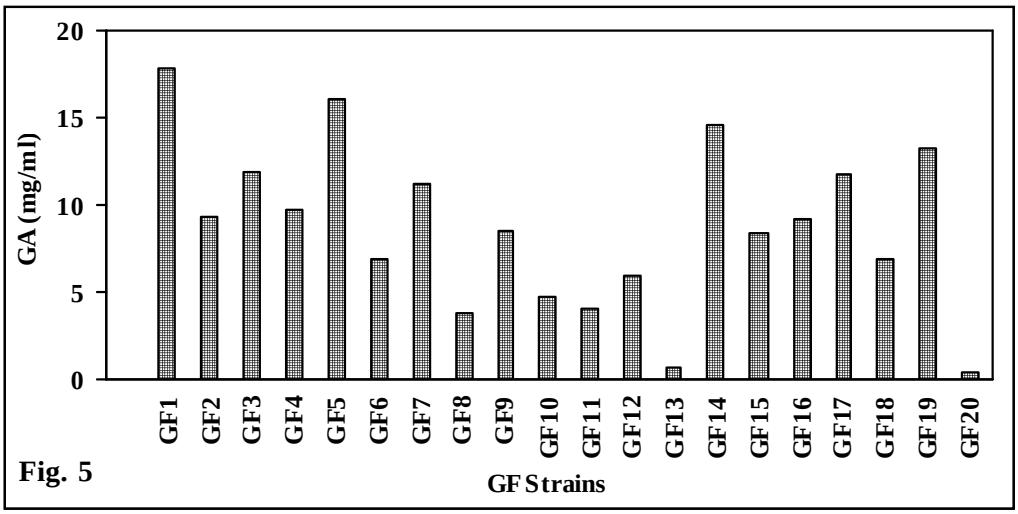

Fig. 5

Fig. 4 (A and B): Production of indole acetic acid (IAA) by shake flask cultures of GF strains growing in LB medium amended with tryptophan (100 µg/ml) and glucose (1%) at 37°C for 5 days. **Fig. 5:** Production of gibberellic acid (GA) by shake flask cultures of GF strains growing in glucose medium at 37°C for 3 days

Table 2: Substrates utilized as carbon sources by selected PGPR strains GF1, GF3 and GF14 as determined by GEN III Biolog Micro Plate Assays (- negative reaction; + positive reaction; ± moderate; +- mismatched positive; -+ mismatched negative).

Substrate	GF1	GF3	GF14	Substrate	GF1	GF3	GF14
Water	-	-	-	D-Tagatose	-	-	-
α-Cyclodextrin	-	-	-	D-Trehalose	-	-	±
β -Cyclodextrin	+	-	-	Turanose	-	-+	-
Dextrin	-	-	-	Xylitol	-	-	-
Glycogen	-	-	-	D-Xylose	-	-	-
Inulin	-	-	-	Acetic Acid	-	-	-
Mannan	-	-	-	α -Hydroxybutyric Acid	-	-	-
Tween 40	+	+	+-	β -Hrdroxybutyric Acid	+	-	-
Tween 80	+	+	+	α -Hydroxybutyric Acid	-	+	-
N-acetyl-D-Glucosamine	-	+	+	p-Hydroxy-phenyl acetic Acid	-	-	-
N-acetyl- α -D-Mannosamine	-	+	+-	α -Ketoglutaric Acid	-	-	-
Amygdalin	-	-	-	α -Ketovaleric Acid	+	-	±
L-Arabinose	-	-	-	Lactamide	-	-	-
D-Arabitol	-	-	-	D-Lactic Acid Methyl Ester	-	-	-
Arbutin	-	+	+	Propionic Acid	+	-	-
D-Cellobiose	-	-	-	Pyruvic Acid	-+	-+	-
D-Fructose	±	+-	+	Succinaic Acid	-	±	±
L-Fucose	-	-	-	Succinic Acid	-	-	-
D-Galactose	-	-	-	N-Acetyl-L-Glutamic Acid	+-	-	-
D-Galacturonic Acid	-	-	-	L-Lactic Acid	-+	-	-
Gentiobiose	-	-+	-	D-Malic Acid	-	-	-
D-Gluconic Acid	-	-	-	L-Malic Acid	`	+	+
α-D-Glucose	-+	-+	-+	Pyruvic Acid Methyl Ester	+	-	-
m-Inositol	-	-	-	Succinic Acid Mono-Methyl Ester	-+	-	-+
α-D-Lactose	-	-	-	L-Alaninamide	-	-	-
Lactulose	-	-	-	D-Alanine	-	-	-
Maltose	-	-	+	L-Alanine	-	-	-+
Maltotriose	-	±	-	L-Alanyl-Glycine	-	-	-
D-Mannitol	-	+-	+	L-Asparagine	-	-	-+
D-Mannose	-+	+	-	L-Glutamic Acid	-	-	-
D-Melezitose	-	-	-	Glycyl-L-Glutamic Acid	-	-	-
D-Melibiose	-	-	-	L-Pyroglutamic Acid	-	-	-
α-Methyl-D-Galactoside	-	-	-	L-Serine	-	-	+
β -Methyl D-Galactoside	-	-	-	Putrescine	-	-	-
β -Methyl-D-Glucose	-	-	-+	2,3-Butanediol	-	-	-
α-Methyl-D-Glucoside	-	-	-	Glycerol	-	+	+
β -Methyl-D-Glucoside	-	+	+	Adenosine	-	-+	-
α-Methyl-D-Mannoside	-	-	-	2'-Deoxy Adenosine	+-	-	-
Palatinose	+-	-	-	Inosine	-	-	+
D-Psicose	-+	-	+	Thymidine	-	+	+
D-Raffinose	-	-	-	Uridine	-	±	+
L-Rhamnose	-	-	-	Adenosine-5'-Monophosphate	-	-	-
D-Ribose	-	-	-	Thymidine-5'-Monophosphate	-	-	-
Salicin	+	+	+	Uridine-5'-Monophosphate	-	-	-
Sedoheptulosan	-	-	-	D-Fructose-6-Phosphate	-	-	-
D-Sorbitol	-	+	+	α -D-Glucose-1-Phosphate	-	-	-
Stachyose	-	-	-	D-Glucose-6-Phosphate	-	-	-
Sucrose	+-	+	+	D-L- α -Glycerol Phosphate	+-	±	±

phosphorous that supports viability and growth of soil microflora enabling them to colonize plant rhizosphere and promote plant growth and health. Another prime mechanism of inducing plant growth is production of plant growth hormones like indole acetic acid (IAA) and gibberellic acid (GA). Gradual increase in IAA production by GF strains is noted where GF1 (2.48 mg/ml), GF3 (1.76 mg/ml), and GF14 (2.54 mg/ml) showed higher IAA production. These results are in an agreement with those reported by others 0.195

mg/ml by *Azospirillum brasilense* [20], 1.1 to 12.1 mg/ml by 30 isolates from different locations and plant hosts [21] and 0.1 mg/ml produced by *Bacillus megaterium* [22]. IAA loosens plant cell walls leading to increased amount of root exudation that provides additional nutrients to support the growth of rhizobacteria [23]. They attract more rhizosphere bacteria by increasing more amount of root exudation. Bacterial IAA stimulates development of root systems, overproduction of root hairs in plants and release of plant cell walls during elongation [24]. PGPR species possess ability to produce significant amount of IAA and thereby accomplish another very important PGPR trait. PGPR strains also possess the ability to produce significant amount of gibberellic acid. Apart from IAA and GA plant growth regulators, ethylene plays an important role in the process of fruit ripening. Increase in ethylene levels hastens the process of fruits and vegetables ripening. Early and fast ripening of fruits and vegetables causes loss of products during storage, shipping and handling. One solution to this problem is regulating the level of ethylene by ACC deaminase activity. Therefore, microbes possessing ability to secret ACC deaminase are of high interest these days. GF strains produced higher ACC deaminase activity than reported in the literature [25]. Such an increased amount of ACC deaminase activity suggests that GF strains are potential towards their role of ethylene regulation too.

Besides direct plant growth promotion, PGPR are known to induce production of antifungal compounds that suppress the growth of phytopathogenic fungi. Pesticides are widely used to control pests but they inhibit the growth of other beneficial microflora and fauna present in the soil. Chemicals present in the pesticides suppress growth of earthworms and other beneficial nematodes that increase soil fertility naturally. Therefore, PGPR that eliminate harmful effects of phytopathogens and do not adversely affect other beneficial organisms of soil are in demand. PGPR suppress growth of phytopathogenic fungi by producing siderophores [26]. Siderophores are growth inhibitors of various fungi such as *Phytophthora parasitica* [27], *Fusarium oxysporm* [28] and *Sclerotinia sclerotiorum* [29]. Another antifungal trait is the production of HCN which is well known to block respiratory metabolism. HCN is poisonous for fungal pathogen leads to the death of pathogenic fungi. One mechanism that is involved in both elimination of fungal pathogens and root colonization, is production of extracellular enzymes. Chitin, pectin and cellulose are abundantly present in exoskeleton of insect, fungi, yeast, nematodes and other invertebrates as well as cell walls of plants and are known to provide rigidity to exoskeleton of body structures. GF strains produce chitinase, pectinase and cellulase enzymatic activities to (i) break down the rigid exoskeleton of phytopathogens and supply nitrogen and carbon as a source of nutrition or precursors to plants and other beneficial microflora; (ii) play a role in prevention of pathogenesis and (iii) degrade rigidity of plant cell walls and colonize in the rhizosphere. GF strains exhibit multifactorial mechanism of antagonism towards phytopathogenic fungi. This flexibility adds one more potential trait to be considered in the development of successful biocontrol product as GF strains have their own potentiality in controlling plant diseases and pest management minimizing the use of chemical products available commercially.

The results of pot trial study showed that seed priming with GF strains increase seedling vigor index and seedling growth significantly as compared to control seedlings. Germination, seedling vigor index, fresh and dry weight of seedlings was higher in the case of treated seeds. Amongst the three impacts of PGPR i.e. neutral, deleterious and beneficial, GF strains showed exclusively beneficial effects on seed germination, seedling growth and vigor index. One of the challenges in developing PGPR for commercial application is ensuring their effects on multiple host plants. The much important observation noticed after conducting all the above assays is that the GF strains exert beneficial effects upon variety of seeds and not limited to only one or two plants irrespective of whether it is a crop, oil seed or pulse; GF strains positively affect plant growth and health. GF strains contribute to replenish adequate plant nutrition by increasing nutrient uptake and reduce negative environmental effects by multiple antifungal activities and therefore minimize the use of harmful chemical fertilizers. With a vision of versatilities such as enhancement of plant growth, ease of mass multiplication, broad spectrum of action, easy and reliable biocontrol, safe to the environment; developments in GF strains bio-formulation are to be established for better quality and quantity of crops.

Acknowledgements: We are thankful to the UGC, Govt. of India, New Delhi, for supporting this study through its Centre of Advanced Study Programme (CAS Phase-I).

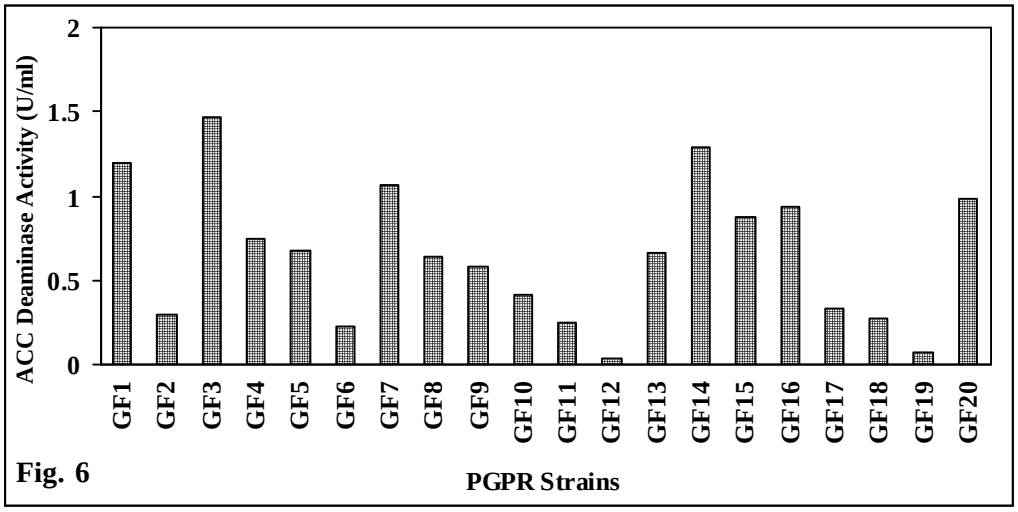

Fig. 6

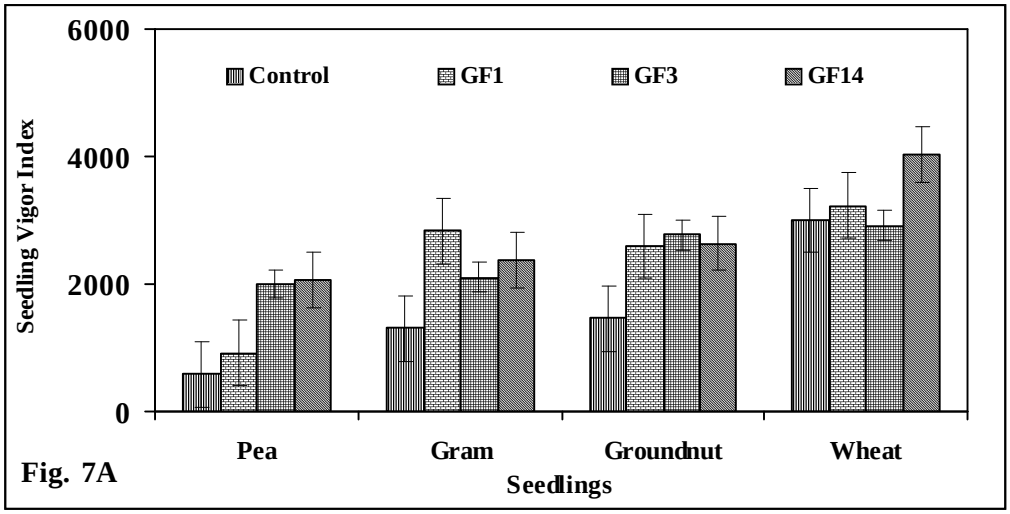

Fig. 7A

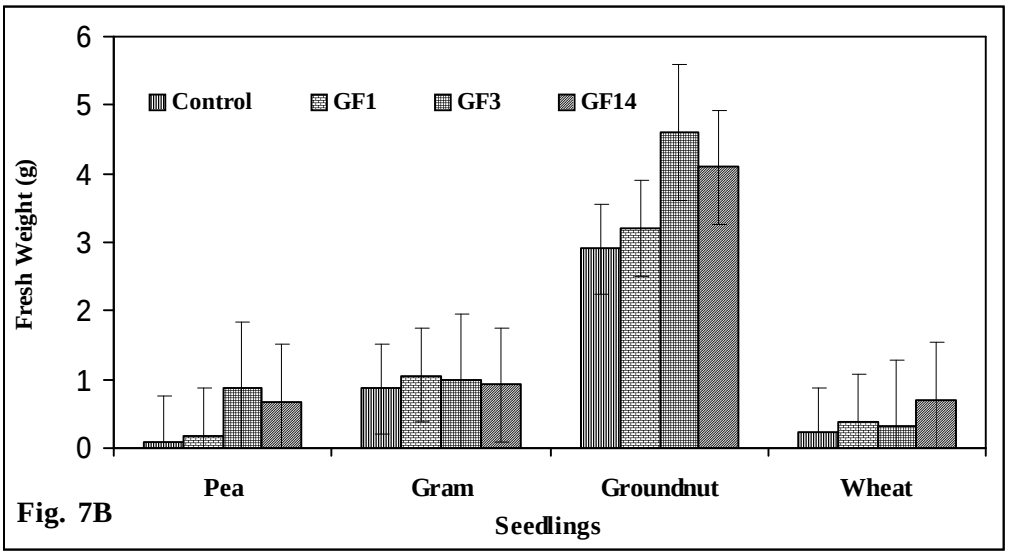

Fig. 7B

Fig. 6: Production of ACC deaminase activity by shake flask cultures of GF strains in salts medium amended with 3 mM ACC at 37°C for 24 h. **Fig. 7A** and **B:** Seedling growth parameters of pea, gram, groundnut and wheat seeds primed with *Bacillus subtilis* GF1, *Bacillus pumilus* GF3 and *Bacillus aerophilus* GF14 after 20 days of growth in pot trials **(A)** Seedling vigor index; **(B)** Fresh weight (g).

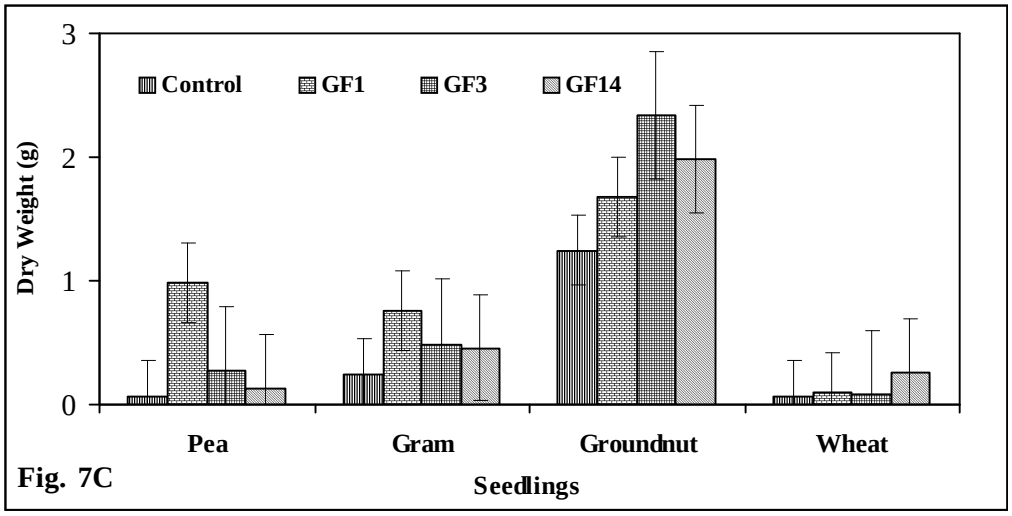

Fig. 7C

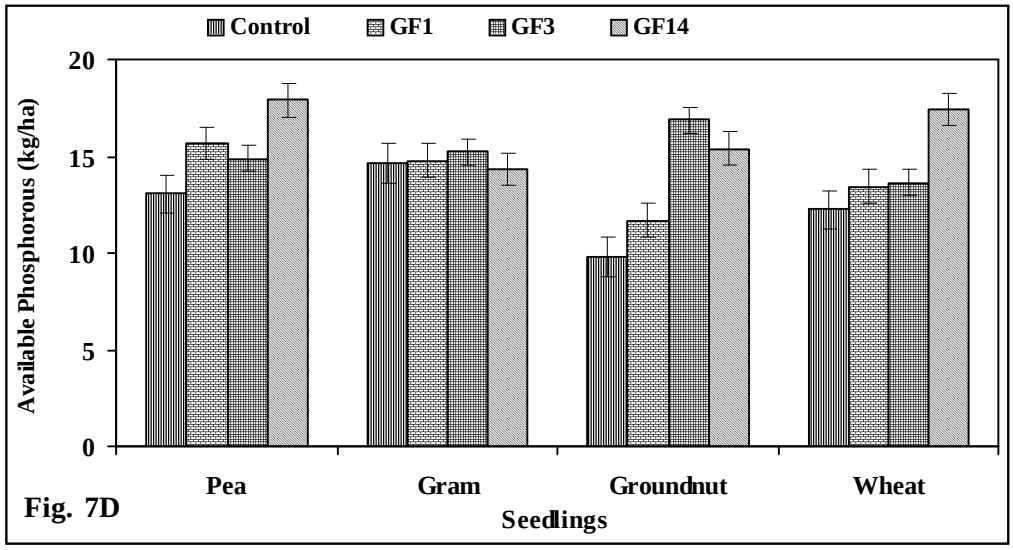

Fig. 7D

Fig. 7C and D: Seedling growth parameters of pea, gram, groundnut and wheat seeds primed with *Bacillus subtilis* GF1, *Bacillus pumilus* GF3 and *Bacillus aerophilus* GF14 after 20 days of growth in pot trials (C) Dry weight (g); (D) Available phosphorous (ppm).

REFERENCES

[1] Lambert, B. and Joos, H.: Trends Biotchnol., 7: 215-219 (1989).

[2] Olsen, S.R., Cole, C.V., Watanable, F.S., and Dean, L.A.: *Estimation of available phosphorus in soil by extraction with $NaHCO_3$*. USDA circular No. 939 (1954).

[3] Tabatabai, M.A. and Bremner, J.M.: Soil. Biol. Biochem., 1: 301-307 (1969).

[4] Brick, J.M., Bostock, R.M. and Silverstone, S.E.: Appl. Environ. Microbiol., 57: 535-538 (1991).

[5] Sanchez, M.: Appl. Microbiol., 11(6): 523-528 (1963).

[6] Graham, H.D. and Thomas, L.B.: J. Pharmacetuical Sci., 50(1): 44-48 (1961).

[7] Honma, M. and Shimomura, T.: Agri. Biol. Chem., 42: 1825-1831 (1978).

[8] Penrose, D.M. and Glick, B.R.: Can. J. Microbiol., 47: 368-372 (2001).

[9] Schwyn, B. and Neilands, J.B.: Anal. Biochem., 160: 47-56 (1987).

[10] Bragger, J.M., Daniel, R.M., Coolbear, T. and Morgan, H.W.: Appl. Microbiol. Biotechnol., 31: 556-561 (1989).

[11] Haba, E., Espuny, M.J., Busquets, M. and Manres, A.: J. Appl. Microbiol., 88: 379-387 (2000).

[12] Sambrook, J., and Russell, R.W.: *Mol. Coning: A Lab. Manual*. Cold Spring Harbor Lab.Press, NY (2001).

[13] Tamura, K., Dudley, J., Nei, M. and Kumar, S.: *MEGA4: Molecular Evolutionary Genetics Analysis software version 4.0*. Mol. Biol. Evol. 24:1596-1599 (2007).

[14] Abdul Baki, A.A. and Anderson, J.D.: Crop Sci. 13: 630-633 (1973).

[15] Dey, R., Pal, K.K., Bhatt, D.M. and Chauhan, S.M.: Microbiol. Res. 159(4): 371-394 (2004).

[16] Kloepper, J.W., Ryu, C. and Zhang, S.: Phytopathology, 94: 1259-1266 (2004).

[17] Kokalis-Burelle, N., Kloepper, J.W. and Reddy, M.S.: Appl. Soil Ecol., 31: 91-100 (2006).

[18] Herman, M.A.B., Nault, B.A. and Smart, C.D.: New York Crop Protect., 27: 996-1002 (2008).
[19] Minorsky, P.V.: Plant Physiol., 146: 323-324 (2008).
[20] Karthikeyan, J.K., Cheruth, A.J. and Azooz, M.M.: Acad. J. Plant Sci., 2: 69-73 (2009).
[21] Khalid, A., Arshad, M. and Zahir, Z.A.: J. Appl. Microbiol., 96: 473-480 (2004).
[22] Freitas, J.R., Banerjee, M.R. and Germida, J.J.: Biol. Fertility Soil., 4: 358-364 (1997).
[23] James, E., Gyaneshwar, P., Mathan, N., Barraquio, W., Reddy, P., Iannetta, P., Olivers, F. and Ladha, J.: Mol. Plant. Microbiol. Interact., 15: 894-906 (2002).
[24] Salamone, De., Hynes, R. and Nelson, L.: Role of cytokinins in plant growth promotion by rhizosphere bacteria. In: *PGPR: Biocontrol and Biofertilization* (Siddiqui, Z., editor) Springer; The Netherlands, pp. 173-195 (2005).
[25] Duan, J.: *http://hdl.handle.nt/10012/3003* (2007).
[26] Edi Husen: Indo. J. Agric. Sci., 4(1): 27-31 (2003).
[27] Seuk, C., Paulita, T. and Baker, R.: J. Plant Pathol., 4(3): 218-225 (1988).
[28] Buysens, S., Heungens, K., Poppe, J. and Hofte, M.: Appl. Environ.Microbiol., 62 (3): 865-871 (1996).
[29] Mc Loughlin, M., Quinn, J., Bettermann, A. and Bookland, R.: Appl. Environ. Microbiol., 58(3): 1760-1763 (1992).