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CHARACTERIZATION OF *PAENIBACILLUS DURUS* (PNF_{16}) A NEW ISOLATE AND ITS SYNERGISTIC INTERACTION WITH OTHER ISOLATED RHIZOBACTERIA IN PROMOTING GROWTH AND YIELD OF CHICKPEA

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ABSTRACT

Application of PGPR in crop production and protection is well known and can also contribute in reducing use of agrochemicals. However, the performance of PGPR is influenced by various biotic and abiotic factors. Isolating new efficient PGPR strain well adapted to local soil agroclimatic conditions is expected to perform with more consistency. In this study five selected rhizobacteria isolated previously were first tested *in vitro* for plant growth promoting (PGP) characteristics. One of the isolate PNF_{16} was identified as *Paenibacillus durus* by 16S rRNA gene sequence analysis. PNF_{16} alone and in combination with other rhizobacteria (*Azotobacter* (AZT_3), *Achromobacter* (PNF_{11}), *Bacillus* (Bc_1), *Pseudomonas* (Ps_5) and *Mesorhizobium* (IARI) were tested for plant growth promoting effect under pot conditions in two consecutive years. PNF_{16} was found to produce 21.7 µg ml^{-1} of indole acetic acid like substances, hydroxymate type of siderophores (Salicylate type 11 µg ml^{-1} and benzoate type 6.5 µg ml^{-1}) and solubilized phosphate (405.33 µg ml^{-1}). PNF_{16} -*Mesorhizobium* combination was found significantly better compared to other combinations for growth parameters, nodulation and yield of chickpea over control. Similar study was also performed for other tested strain. Significant increase in plant growth (32%), nodulation (43%) compared to untreated control was recorded. Co-inoculation also showed synergy and increased the number of pods per plant, 1000-grain weight, dry matter yield, grain yield and protein content by 23%, 22%, 21%, 18% and 4.4% respectively, compared to control. The results indicated the potential usefulness of PNF_{16} alone and in combinations in enhancement of nodulation and stimulation of plant growth in chickpea and adapted to soil condition of the region.

Keywords: *Paenibacillus*; *Mesorhizobium*; PGPR, plant-microbe interaction, nodulation, chickpea

INTRODUCTION

Interactions between plants and micro-organisms in the rhizosphere can clearly affect crop yields. Rhizobacteria that benefit plant growth and development are called 'PGPR'. The term 'PGPR' was introduced in 1978 by Kloepper and colleagues. Since then a large number of bacteria have been identified and reported as PGPR (*Acetobacter, Achromobacter, Arthrobacter, Azoarcus, Azospirillum, Azotobacter, Bacillus, Burkholderia, Clostridium, Enterobacter, Flavobacterium, Frankia, Hydrogenophaga, Kluyvera, Microcoleus, Phyllobacterium, Pseudomonas, Rhizobium, Serratia, Staphylococcus, Streptomyces, and Vibrio*) **(Bashan *et al.*, 2005)**. However commonly used PGPR in field application is limited to only few microorganisms. The plant growth promoting rhizobacteria may enhance plant growth either directly or indirectly. Direct mechanisms include (i) the ability to produce the plant growth regulators (indoleacetic acid, gibberellins, cytokinins and ethylene) **(Glick., 2012),** (ii) Asymbiotic N_2 fixation **(Ahmad *et al.*, 2008),** (iii) Solubilization of mineral nutrient like phosphates **(Taurian *et al.*, 2010),** Indirect mechanisms involve (i) antagonism against phytopathogens **(Gururani *et al.*, 2013),** (ii) Production of siderophores **(Haas and Défago, 2005),** (iii) Production of extra cellular cell wall degrading enzymes for phytopathogens β,1-3 glucanase **(Ribeiro and Cardoso, 2012),** Chitinase **(Ribeiro and Cardoso, 2012),** (iv) Antibiotic production **(Mazurier *et al.*, 2009)** and (v) cyanide production (**Ribeiro and Cardoso, 2012**). By modifying the microbial balance in the rhizosphere, PGPR can stimulate plant growth indirectly by inhibiting other deleterious microbes or root pathogens **(Berendsen *et al.*, 2012).** On the other hand, diazotrophs are able to decrease or prevent the deleterious effects of plant pathogens mostly through the synthesis of antibiotic and fungicidal compounds **(Mavingui and Heulin 1994; Dobbelaere *et al.*, 2003),** competition for nutrients (siderophore production) or by the induction of induced systemic resistance (ISR) against pathogens **(Timmusk and Wagner 1999; Dobbelaere *et al.*, 2003; Gururani *et al.*, 2013).**

A major problem associated with PGPBs is their inability to manifest PGP traits under natural field conditions consistently. This is mainly due to competition with native well adapted strains and specific nutrient limitation **(Vasssey, 2003).** We hypothesized that selecting a PGPB strains exhibiting multiple traits are expected to most ideal as the probability of expression of one or more PGP traits is higher. It is also expected that indigenous soil bacteria adapted to local soil and agro-climatic conditions exhibiting multiple PGP traits may be more effective under field conditions. We have screened rhizospheric soil in vicinity of Aligarh in northern India **(Ahmad *et al.*, 2006).** We found a new isolate of *Paenibacillus* sp. (PNF_{16}) which showed multiple PGP traits and characterized using 16S rRNA gene sequence analysis. The efficacy of PNF_{16} for plant growth promotion was assayed under pot experiment conditions alone and in combination with other bioinoculant such as *Bacillus* (Bc_1), *Azotobacter* (AZT_3), *Achromobacter* (PNF_{11}) and *Pseudomonas* (Ps_5) which were previously isolated in our laboratory.

MATERIALS AND METHODS

Isolation and characterization of bacterial isolates

Bacterial isolates PNF_{16} and other rhizobacteria used in this study were isolated and biochemically characterized using standard methods as described previously **(Ahmad *et al.*, 2006; 2008).**

Genetic identification of PNF_{16} by 16S rRNA partial gene sequencing

Single isolated colony PNF_{16} was inoculated in 5 ml Luria-Bertani (LB) broth and grown at 30 °C for 24 h. Cells were harvested and processed immediately for DNA isolation by standard procedure. The concentration and purity of the DNA preparation were determined by measuring optical density (OD) at 260 nm and ratio at 260/280 nm with a UV-Vis Spectrophotometer. The PCR amplification of almost full–length 16S rRNA gene was carried out with eubacterial specific

primers 16F27N (5'–CCAGAGTTTGATCMTGGCTCAG–3') and 16R1525XP (5'–TTCTGCAGTCTAGAAGGAGGTGWTCCAGGC–3') (**Pidiyar *et al.*, 2002**) in a final volume of 50µl. Briefly, the amplification reaction containing 50 ng templates DNA 25 pmole each of universal primers.0.2 mM dNTPs and 1.5 U *Taq* polymerase (Bangalore Genei, Bangalore, India) in 1X PCR buffer. The amplification reaction was carried out in Gene Amp PCR system 9700 (Applied Biosystems, USA) with the following conditions initial denaturation (2 min at 94 ^{o}C) was followed by 30 PCR cycles (94 ^{o}C for 30 s, 55 ^{o}C for 30 s, and 72 ^{o}C for 1 min) and a final extension at 72 ^{o}C for 5 min. Sequencing reactions were performed with Big Dye Terminator mix (Applied Biosystems) and samples were sequenced on 3730 DNA analyzer (Applied Biosystems). Nucleotide sequence data was deposited in the GenBank sequence database. The partial sequence were analysed with the Basic Local Alignment Search Tool (BLASTN) and compared to known bacterial sequences in NCBI GenBank using BLAST. Related sequences obtained from the database were used to construct a phylogenetic tree using MEGA4 software.

Quantification of PGP activities

Quantification of total IAA like compounds

Putative nitrogen fixer (PNF_{16}) was inoculated in nutrient medium (Yeast extract 1.5; Beef extract 1.5; Peptone 5; Sodium chloride 5, pH- 6.8) supplemented with 500 µg ml^{-1} L-tryptophan and incubated at 28 ^{o}C for 96 h. At the time interval of six hours, 5 ml of the bacterial culture was removed in sterile conditions. The cultures were centrifuged at 282 xg for 30 min. Two ml of the supernatant was mixed with 2 drops of orthophosphoric acid and 4 ml of the Salkowski's reagent. Development of pink color indicated IAA like compound production. Optical density was taken at 530 nm with the help of spectrophotometer (Spectronic 20D^{+}, Thermo Scientific, USA). This assay was carried out for ninety hours. Concentration of IAA like compounds produced by cultures was measured with the help of standard graph of IAA (Hi- media) obtained in the range of 10-100 µg ml^{-1} **(Lopper and Scroth, 1986).** Similarly quantitative estimation of IAA like compound was performed at different concentrations of tryptophan (0, 50-500 µg ml^{-1}) at 72 h of incubation.

Quantification of phosphate solubilization

The phosphate solubilization was quantified by measuring the soluble phosphate released in the culture medium, using standard method as described earlier (**Ahmad *et al*., 2008**). Briefly a 100 ml of sterile Pikovskaya's broth was separately inoculated with fully grown culture (10^{7} CFU ml^{-1}) of the selected isolates. The uninoculated medium served as a control. At different time intervals during growth, 10 ml of culture was removed from each flask and centrifuged at 3136 xg for 30 min and pH of the supernatant was also checked. Supernatant was transferred into tubes. To each sample, 10 ml chloromolybdic acid and 5 drops of chlorostannous acid was added and volume was adjusted to 50 ml with distilled water. The blue colour developed was read at 600 nm. Amount of phosphate solubilized was calculated using the calibration curve of KH_2PO_4.

Detection of siderophore

Siderophore production was detected by universal assay as described by **Schwyn and Nielands (1987)**. Briefly, first the glasswares were deferrated with 6N HCl overnight. Solution A, the dark blue solution (dye) (Chrom azurol S 60.5; Hexadecyltrimethylammonium bromide 72.9 in 100 ml water) was autoclaved. MM9 medium (Sucrose 342.3; Calcium chloride 147.0; Magnesium sulphate 246.4; Dipotassium hydrogen orthophosphate 20; Sodium chloride 0.2; Sodium molybdate 0.005) was deferrated separately in 3% 8-hydroxyquinoline for the removal of iron. The medium was separately washed with chloroform and traces of 8-hydoxyquinoline and chloroform were removed. The pH of Pipes (Piperazine-N, N'- bis[2-ethanesulfonic acid] buffer (30.24g) was adjusted at pH 6.8 by adding 50% (w/v) NaOH solution. All the three solutions A, B and C were autoclaved separately. After cooling the solution up to 50^{o} C, all the three solutions were added aseptically. The dye solution was finally added along the glassware with enough agitation to achieve mixing without generation of foam. Chrome azurol S agar plates were prepared and divided into equal sectors and spot inoculated with 10 µL (10^{7} CFU ml^{-1}) of test organism and incubated at 28 ^{o}C for 48-72 h. Development of yellow-orange halo around the growth was considered as positive for siderophore production. The yellow-orange halo was measured in mm around the colonies of bacterial isolates which were positive for the siderophore production.

Quantification of siderophore

Bacterial isolate PNF_{16} was further examined for the quantification of hydroxymate type of siderophores using the method of **Reeves *et al*., (1983)**. Briefly, the glasswares and the medium were deferrated by 6N HCl and 3% of 8-hydroxyquinoline in $CHCl_3$. The single colony of bacterial isolate was inoculated in 5 ml deferrated nutrient broth to raise the inoculum. One ml (10^{7} CFU ml^{-1}) of bacterial culture was inoculated in 30 ml nutrient medium and incubated at 28 ^{o}C for three days. The fully grown cultures were centrifuged at 3136 xg for 20 min. The supernatant was adjusted at pH 2.0 with diluted HCl. Equal quantity of ethyl acetate was added twice in a separating funnel and separated, the resultant 60 ml ethyl acetate fraction was taken. Five ml of ethyl acetate fraction was mixed with 5 ml of Hathway's reagent. The absorbance was read at 700 nm for benzoate and at 560 nm for salicylates. The concentrations of benzoates and salicylates were calculated with the help of standard 2-3 dihydoxy benzoic acid (1-10 µg ml^{-1}) and salicylic acid (1-30 µg ml^{-1}) respectively.

Experimental design to assess inoculation response of test isolates on plant growth and yield of chickpea

The pot experiments on chickpea (*Cicer arietinum* L.) var. Avrodhi (purchased from Chola Beez Bhandar, Aligarh, India) were carried out in two consecutive years. Ten seeds of chickpea were sown in each earthen pots of 3 kg capacity (25 x 22 cm) in sandy clay loam soil (sand 667 g kg^{-1}, silt 190 g kg^{-1}, clay 143 g kg^{-1}, organic carbon 0.4%, Kjeldahl N 0.75 g kg^{-1}, Olsen P 16mg g^{-1}, Cation exchange capacity 11.7 cmol kg^{-1} and anion exchange capacity 5.1 cmol kg^{-1}, Calcium 30.45 mg l^{-1}, Magnesium 19.67 mg l^{-1}, Sodium 11.38 mg l^{-1}, Carbonate 18.45 mg l^{-1}, Bicarbonate 85.38 mg l^{-1}, Sulfate 16.18 mg l^{-1}, Chloride 26.69 mg l^{-1}, pH 7.4). Plants were thinned to three plants per pot ten days after emergence (DAE). The experiment was conducted in a complete randomized design and the pots were maintained in an open field conditions. There were eight treatments (i) Control, (ii) basal dose fertilizer, (iii) *Mesorhizobium* sp. , (iv) *Mesorhizobium* + *Azotobacter* (AZT_3), (v) *Mesorhizobium*+ *Achromobacter* (PNF_{11}), (vi) *Mesorhizobium*+ Fluorescent *Pseudomonas*(Ps_5), (vii) *Mesorhizobium* + *Bacillus* (Bc_1), (vii) *Mesorhizobium* + *Paenibacillus* (PNF_{16}) with each treatment was replicated three times and each pot contained three plants were used. Plants were watered with tap water. At each sampling time three plants were removed. The minimum dose of inorganic fertilizers was applied (Diammonium phosphate (DAP) @ 6.5x 10^{-2} g kg^{-1} to 4.5x 10^{-2} g kg^{-1} of soil).

Inoculum and plant culture

The standard bioinoculants, *Mesorhizobium* sp. (Indian Agriculture Research Institute, New Delhi, India) specific to chickpea were cultivated in 100 ml yeast extract mannitol broth (YEM) in 250 ml flask on rotary shaker at 125 rpm at 28 ^{o}C 2-3 days to achieve the cell density of 10^{7}-10^{8} cells ml^{-1}. All PGPR (*Azotobacter* sp., Fluorescent *Pseudomonas* sp., *Bacillus* sp., *Achromobacter* sp. and *Paenibacillus* sp.) strains were cultivated in 100 ml nutrient broth in 250 ml flasks to obtain maximum growth. The *Mesorhizobium* and all PGPR strains were mixed in the ratio of 1:1. Chickpea seeds were surface sterilized by soaking in sodium hypochlorite (5%) for 15 min and washed four times with sterile distilled water. The surface sterilized seeds were soaked in the cultures or consortium of cultures for two h at 28 ^{o}C. For control treatments as well as minimum dose of inorganic fertilizer (Di-Ammonium Phosphate) seeds were soaked in sterile distilled water. Ten seeds of each treatment were kept in 10 ml sterile NSS to take the CFU seed^{-1} of the inoculated bacteria. Inoculum density on seeds was determined by agitating ten seeds in 10 ml sterile NSS from each treatment and plated after serial dilution on the respective agar media. Plates were incubated at 28°C for 48 h, the number of colonies was counted, and the total population was expressed as CFU seed^{-1}.

Sampling and data collection

The sampling of chickpea was done at 60, 90 DAS and 145 DAS (harvest) was done. Root and shoot length and dry weight of the plants were recorded and analyzed at different stages of crop. Number of nodules and the fresh and dry weight of nodules were recorded at 90 DAS. At each sampling intervals, three pots i.e. nine plants were removed from each treatment at random. The following data were collected.

Leghaemoglobin content

Leghaemoglobin content was recorded at 90 DAS in chickpea, pink, and washed nodules from both crops were washed thoroughly and detached from the roots. Two hundred mg nodules were macerated with the help of mortar pestle in 3 ml sodium phosphate buffer, then filtered through two layers of cheese cloth. The nodule debris was discarded. The turbid reddish brown filtrate was clarified by centrifugation at 31xg for 30 min. The supernatant was diluted up to 6 ml with sodium phosphate buffer. Then equal volume of pyridine was added to extract. The solution became greenish yellow due to the formation of ferric hemochrome. The hemochrome was divided equally into two parts. To one portion few crystals of sodium dithionite was added to reduce the hemochrome. The mixture was stirred without aeration. Absorbance was read at 556 nm against the blank. Few crystals of potassium hexaferrocynate were added to other portion to oxidize the hemochrome and read at 539 nm **(Appleby and Bergersen, 1980).** Leghemoglobin was calculated by the following formula

Leghemoglobin concentration (mM) =$A_{556} - A_{539}$ x 2D / 23.4

Where D is the initial dilution, A is absorbance

Nitrogenase activity (Acetylene reduction assay) in nodules

Nitrogenase activity was recorded at 90 DAS in chickpea. The activity of nitrogenase was determined in the fresh samples by the method of **Hardy and workers (1966).** Nodules were incubated with acetylene for thirty minutes at 27 ^{o}C. The air (5cm^3) was removed with a syringe from each incubation period, and ethylene content was measured on a gas chromatograph (GC 5700, Nucon, New Delhi, India) equipped with 1.8 m Porapack N (80/100 mesh) column, a flame ionization detector and an integrator. Nitrogen was used as a carrier gas. The flow rate of nitrogen, hydrogen and oxygen were 0.5, 0.5 and 5 ml S^{-1}. The oven temperature was 100 ^{o}C and that of the detector was 150 ^{o}C. Ethylene identification was based on the retention time and was quantified by comparing with the standard curve drawn with pure ethylene. The nitrogenase activity was expressed as nM (nanomole of ethylene formed g^{-1} of nodule fresh mass h^{-1}).

Protein estimation

The protein content of seeds was estimated by the method as described by **Lowry *et al*. (1951).**

Yield parameters

At the time of harvesting, yield parameters including number of pods plant^{-1}, weight of seeds plant^{-1} and weight of thousand seeds were determined.

Statistical analysis

The data is pooled data of two years. The measured parameters were subjected to statistical calculation using one way analysis of variance (ANOVA) by SPSS ver.11.00. The difference among means was calculated at 5% probability level. When analysis of variance showed significant treatment effects, the least significant difference (LSD) ($P \leq 0.05$) was applied to make comparisons among means by Tukey's test.

RESULTS

The bacterial isolates used in this study were isolated previously from rhizospheric soil and characterized biochemically using standard methods as listed in Table 1.

Table 1 Origin, morphological characteristics, PGP activity and presumptive identification of selected bacterial isolates

Strain	Morphology	Spores/ cysts	Gram staining	Origin	PGPR traits	Most significant alignment	Identification method	References
Mesorhizobium sp.	Rods	–	Negative	Commercial biofertilizer for chickpea IARI, New Delhi	Symbiotic nitrogen fixer	-		
AZT_3	Oval	+	Negative	Rhizosphere *Trifolium alexandrium*	IAA, P solubilization, Siderophore, Antifungal activity, ammonia	*Azotobacter chroococcum*	Morphological, Biochemical (Bergey' manual)	**Ahmad *et al*. (2008)**
Ps_5	Rods	–	Negative	Rhizosphere *Triticum aestivum*	IAA, P solubilization, Siderophore, Antifungal activity, ammonia, HCN	*Pseudomonas fluorescens*	Morphological, Biochemical (Bergey' manual)	**Ahmad *et al*. (2008)**
Bc_1	Rods	+	Positive	Rhizosphere *Vigna mungo*	IAA, P solubilization, Siderophore, Antifungal activity, ammonia, HCN	*Bacillus* sp.	Morphological, Biochemical (Bergey' manual)	**Ahmad *et al*. (2008)**
PNF_{11}	Rods	–	Negative	Rhizosphere *Cicer arietinum*	IAA, P solubilization, Siderophore, Antifungal activity, ammonia	*Achromobacter* sp.	16S rRNA gene analysis	**Ahmad *et al*. (2006)**
PNF_{16}	Rods	–	Positive	Rhizosphere *Cicer arietinum*	IAA, P solubilization, Siderophore, Antifungal activity, ammonia	*Paenibacillus* sp.	16S rRNA gene analysis	This study

The bacterium PNF_{16} was found gram-positive, rod shaped, motile, without spores and cyst. Colonies on glucose broth (GB) medium are circular, convex, white and translucent. The nucleotide sequence of PNF_{16} was submitted to GenBank (accession number: KC477263). The analysis of 16S rRNA sequence indicated that PNF_{16} shared a maximum 99% homology with *Paenibacillus durus* (accession number: JQ436899). Further a phylogenetic tree was constructed from multiple sequence alignment of 16S rRNA gene sequence (Fig. 1).

The internal numbers indicates bootstrap values.

Assays for plant growth promotion traits

Isolate PNF_{16} belonging to putative nitrogen fixers was subjected to quantitative estimation of PGP traits. Indole-3-acetic acid (IAA) like molecules production in the presence of different concentration of tryptophan was analyzed. The isolate was found to produce maximum amount (21.7 µg ml^{-1}) of indole-3-acetic acid like molecules at 500 µg ml^{-1} of tryptophan concentration and at 72 h of incubation (Fig. 2 and 3). IAA like molecules production was increased significantly by increasing the concentration of tryptophan from 100-500 µg ml^{-1}. Isolate PNF_{16} solubilized phosphate (405.33 µg ml^{-1}) at 12th day of incubation (Fig. 4).The isolate also produced hydroxymate type of siderophores (salicylate type 11.0 µg ml^{-1} and benzoate type 6.5 µg ml^{-1}).

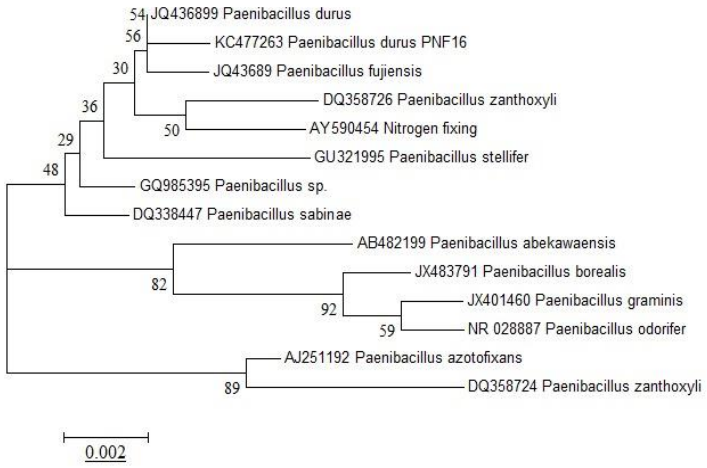

Figure 1 Phylogentic tree of PNF_{16} (GenBank accession number: KC477263) constructed using neighbor- joining method based on 16S rRNA gene sequences of strain PNF_{16} and related species.

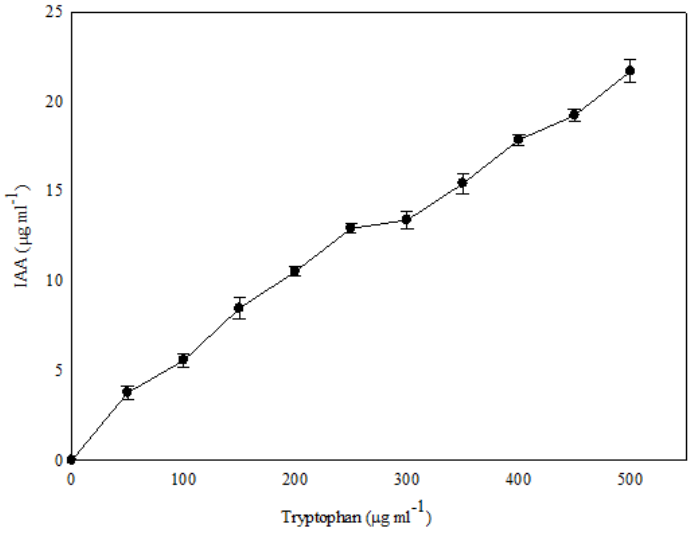

Figure 2 Tryptophan dependent IAA like substances production by PNF16 at

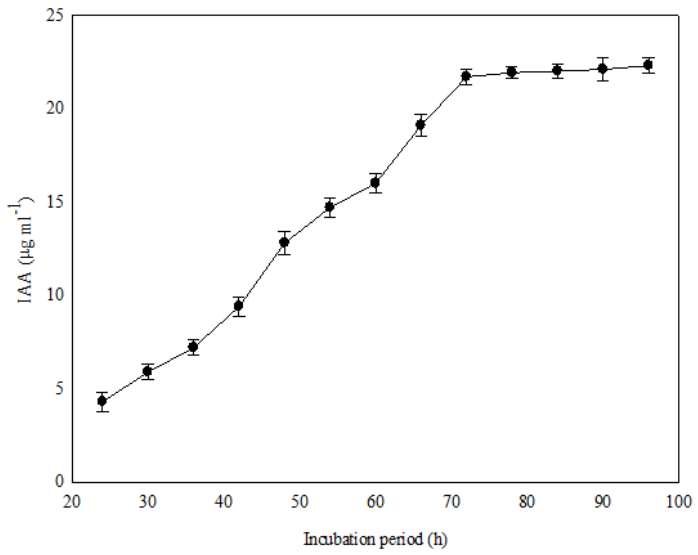

Figure 3 Time dependent production of IAA like substances by PNF_{16} at 500 72 h of incubation µg ml^{-1} tryptophan concentration.

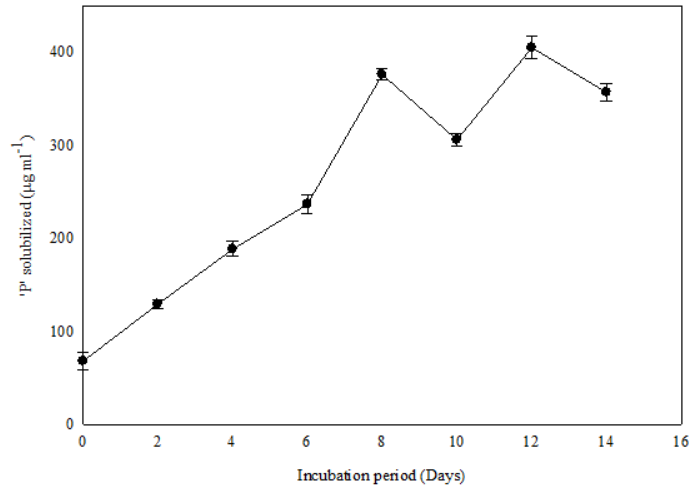

Figure 4 Phosphate solubilization by PNF_{16}

Effect of bacterial inoculation on growth, nodulation and yield of chickpea

A total of six PGPR including PNF_{16} alone and in combination were inoculated to chickpea plant by seed treatment methods under open pot conditions. Vegetative growth parameters were studied at 60, 90 days after sowing (DAS) and yield characteristics were determined at harvest (145 DAS). In general all the dual bacterial inoculation were better over control and single bacterial inoculation of *Mesorhizobium* in case of length and dry weight of plant parts i.e. root and shoot. Co-inoculation of chickpea with PNF_{16} along with *Mesorhizobium* sp. showed significant increase in root length (26%), shoot length (32%) and root and shoot dry weight (25, 27%) respectively at 90 DAS in comparison to uninoculated control (Table 2). We found that double treatment of PNF_{16} and *Mesorhizobium* was best over rest of the dual bacterial inoculation in case of vegetative parameters of chickpea.

Table 2 Effect of bacterial inoculation on growth parameters of chickpea at 90 DAS

Treatments used	Root length (cm)	Shoot length (cm)	Dry weight plant^{-1} (g)	
			Root	Shoot
Control	29.5^{c}	27^{d}	1.6^{a}	2.6^{a}
Basal dose of fertilizer	32.6^{bc}	32.6^{c}	1.7^{a}	2.8^{a}
Mesorhizobium sp.	32.7^{b}	33.2^{bc}	1.7^{a}	2.9^{a}
Mesorhizobium + *Azotobacter* (AZT_3)	32.6^{bc}	33.5^{bc}	1.7^{a}	2.9^{a}
Mesorhizobium+ *Achromobacter* (PNF_{11})	34.8^{ab}	34.6^{ab}	1.8^{a}	3.1^{a}
Mesorhizobium+ Fluorescent *Pseudomonas*(Ps_5)	33.9^{b}	33.9^{bc}	1.8^{a}	3.0^{a}
Mesorhizobium + *Bacillus* (Bc_1)	33.6^{b}	33.6^{bc}	1.7^{a}	2.9^{a}
Mesorhizobium + *Paenibacillus* (PNF_{16})	37.1^{a}	35.7^{a}	2^{a}	3.3^{a}
F value	10.982	45.152	1.27	0.728

* Column values followed by a different letters are significantly different ($P \leq 0.05$).

Nodulation parameters such as nodule number, nodule dry weight, leghaemoglobin content and nitrogenase activity was observed at 90 days after sowing (DAS). Plants treated with PNF_{16} along with *Mesorhizobium* increase the nodule number (43%) over control followed by PNF_{11} (30%), Ps_5 (27%), Bc_1 (26%) and AZT_3 (24%) (Table 3). Similarly dry weight of nodules and leghaemoglobin content showed significant increase by 42 and 63% respectively due to co-inoculation of PNF_{16} with *Mesorhizobium*. Similar trends were also observed for nitrogenase activity. *Mesorhizobium* alone inoculation showed better performance over minimum dose of chemical fertilizer as well as uninoculated control. *Mesorhizobium* and PNF_{16} were statistically significant ($P < 0.05$) over control for nodulation parameters (Table 3).

Table 3 Effect of bacterial inoculation on nodulation of chickpea at 90 DAS

Treatments used	Nodule plant^{-1}		Leghaemoglobin content[m M (g F M^{-1})]	Nitrogenase activity [nM C_2H_4 (g nodule FM.)]$^{-1}$ h^{-1}
	Number	Dry weight (g)		
Control	31.5^{d}	1.14^{d}	157.65^{e}	382.82
Basal dose of fertilizer	35^{cd}	1.185^{d}	163.05^{e}	393.09
Mesorhizobium sp.	38^{bc}	1.26^{cd}	190.35^{d}	407.9
Mesorhizobium + *Azotobacter* (AZT_3)	39.165^{bc}	1.325^{bcd}	207.1^{cd}	422.34
Mesorhizobium+ *Achromobacter* (PNF_{11})	41^{ab}	1.555^{ab}	235^{ab}	466.085
Mesorhizobium+ Fluorescent *Pseudomonas* (Ps_5)	40^{abc}	1.51^{abc}	227.1^{bc}	502.555
Mesorhizobium + *Bacillus* (Bc_1)	39.665^{abc}	1.395^{bcd}	222.05^{bc}	451.935
Mesorhizobium + *Paenibacillus* (PNF_{16})	45^{a}	1.7^{a}	256.75^{a}	436.915
F value	12.136	10.997	57.689	729.169

* Column values followed by a different letters are significantly different ($P \leq 0.05$).

The yield parameters, weight of 1000 seeds (g), number of pods plant^{-1}, number of seeds plant^{-1}, weight of seeds per plant (g) and protein content of seeds (mg g^{-1}) per plant were taken into account. The data on all yield parameters was significantly different over control. The relative inoculation responses of all four PGPR isolates are almost statistically different for yield data (Table 4). The co-inoculation increase the dry biomass by (21%), number of pods plant^{-1} (23%), number of seeds plant^{-1} (20%), weight of 100 seeds (22%), seed weight g plant^{-1} (18%) and protein content (4.4%) over control in both the year.

Table 4 Effect of bacterial inoculation on yield attributes of chickpea

Bacterial inoculations	Dry biomass (g plant^{-1})	Number plant^{-1}		Seed weight (g)		Protein content of seeds (mg g^{-1})
		Pods	Seeds	1000	(plant^{-1})	
Control	9.375 ^{d}	14.5^{c}	28.5 ^{c}	203.43	5.71	23.26 ^{e}
Basal dose of fertilizer	9.815 ^{cd}	14.665 ^{c}	30.33 ^{bc}	225.275 ^{d}	5.855 ^{e}	23.33 ^{e}
Mesorhizobium sp.	9.835 ^{cd}	15 ^{bc}	31 ^{abc}	227.58 ^{cd}	5.985 ^{de}	23.485 ^{d}
Mesorhizobium + *Azotobacter* (AZT_3)	10.41 ^{bc}	15.335 ^{abc}	31.335 ^{abc}	230.105 ^{bcd}	6.07 ^{cd}	23.58 ^{cd}
Mesorhizobium+ *Achromobacter* (PNF_{11})	10.86 ^{ab}	16.335 ^{a}	32.5 ^{ab}	239.44 ^{ab}	6.42 ^{a}	23.865 ^{a}
Mesorhizobium+ Fluorescent *Pseudomonas* (Ps_5)	10.725 ^{ab}	16.165 ^{ab}	31.5 ^{abc}	235.855 ^{bc}	6.26 ^{ab}	23.77 ^{ab}
Mesorhizobium + *Bacillus* (Bc_1)	10.595 ^{abc}	15.665 ^{ab}	31 ^{abc}	233.91^{bcd}	6.175 ^{bc}	23.66 ^{bc}
Mesorhizobium + *Paenibacillus* (PNF_{16})	11.355 ^{a}	17.83	34.165^{a}	248.495 ^{a}	6.755	24.3
F value	13.178	25.945	4.855	47.767	102.404	113.698

* Column values followed by a different letters are significantly different ($P \leq 0.05$).

DISCUSSION

Rhizobacteria can promote plant growth through phosphate solubilization, indole and siderophore production, have a potential for use as PGP inoculants to improve crops. IAA like compounds are the most common growth regulators produced by PGPR and is known to enhance plant growth. It is now common knowledge that almost all the rhizopsheric bacteria produce growth regulating substances **(Glick, 2012).** Our isolate produced significant amount of IAA like molecules with increasing concentration of tryptophan. Varying levels of IAA like molecules production was recorded in *Paenibacillus* sp., *Bacillus* sp. and *Klebsiella* sp. was also reported by **Ji *et al.* (2014**). The availability of insoluble and fixed forms of phosphorus by bacterial inoculants play an important role in increasing soil phosphorus availability and simultaneously enhancing plant growth and crop yield **(Rodriguez and Fraga, 1999; Hameeda *et al.*, 2008)**. Bacteria belonging to genera *Paenibacillus*, *Bacillus*, *Pseudomonas* etc. are reported to solubilize the insoluble phosphate compounds and aid in plant growth (**Ahmad *et al.*, 2008; Pastor *et al.*, 2014**). Siderophores can directly stimulate the biosynthesis of other antimicrobial compounds by increasing the availability of these minerals to the bacteria, which suppresses the growth of phytopathogenic fungi and function as stress factors in inducing host resistance (**Ahmad *et al.*, 2008; Zhou *et al.*, 2012**). Our results on PGP activities are in conformation with the report of **Gururani *et al.* (2013**). The isolated rhizobacteria showing multiple PGP traits are expected to perform better on inoculation because of the probability of expression of one or other traits under natural condition. It is interesting to note that PNF_{16} showed three PGP activities such as production of indole-3-acetic like molecules, phosphate solubilization and siderophore production.

Several authors have reported the beneficial effects of N_2-fixing bacteria with other PGPR on growth and yield of legumes **(Bashan *et al.*, 1993; Bashan *et al.*, 2004).** In the present study, the symbiotic N_2-fixing *Mesorhizobium* used together with the *Paenibacillus* (PNF_{16}), *Azotobacter*, *Bacillus*, *Pseudomonas* stimulated growth, nodulation, and yield of chickpea. **Wani *et al.* (2007)** reported that dual bacterial inoculation of *Mesorhizobium* and phosphate solubilizing *Pseudomonas* significantly increased the plant growth of chickpea plant. Similarly, *Pseudomonas* and other PGPR exhibited enhanced nodulation, nodule dry weight and plant growth in chickpea **(Verma *et al.*, 2014). Hameeda *et al.* (2010)** have reported that the dual inoculation of *Rhizobium* with *Pseudomonas* significantly increased the nodule number, nodule weight and nitrogenase activity for chickpea under glasshouse conditions.

Stefan *et al.* (2013) reported that co-inoculation of two rhizobacterial strains significantly increased nodule number, nodule weight, total biomass and yield of runner bean compared to single strain application. Similar results were also reported by **Malik and Sindhu (2011)** using *Pseudomonas* with *Mesorhizobium* sp. that significantly improved chickpea growth and its yield components as compared with the sole application.

The results observed on the N_2 fixer, *Mesorhizobium* and PGPR in culture medium promoted to study the interaction of these organisms in soils for chickpea. Co-inoculation, increased growth and yield, compared to single inoculation, provided the plants with more balanced nutrition and improved absorption of nitrogen, phosphorus and mineral nutrients **(Sánchez *et al.*, 2014).** *Azospirillum* is also considered to be a *Rhizobium* "helper" stimulating nodulation, nodule activity and plant metabolism, all of which stimulate many plant growth variables and plant resistance to unfavorable conditions **(Bashan *et al.*, 1993; Bashan *et al.*, 2004).** In the present study when symbiotic nitrogen fixing bacteria (*Mesorhizobium* sp. for chickpea), and PGPR (*Bacillus*, *Azotobacter*, flourescent *Pseudomonas*, *Achromobacter* sp., and *Paenibacillus* sp. [PNF_{16}]) were used in different combinations, a high level of plant growth promotion was recorded in certain treatments with the added benefit of greater yield under unsterilized soil conditions.

Further advantages of mixed cultures over single strains have been well documented in literature for eaxmple: i) *in vitro* studies have shown that *Azospirillum* can produce more phytohormones when grown in mixed culture **(Spaepen *et al.*, 2007; Dardanelli *et al.*, 2008),** ii) mixed cultures provide conditions more suitable for nitrogen fixation than pure cultures **(Holguin and Bashan 1996; Remans *et al.*, 2008b)** and iii) mixed inoculation of biocontrol microorganisms is more efficient in controlling pathogens than the use of single strain inoculants, e.g., combinations of *Pseudomonas* with *Serratia* **(Frommel *et al.*, 1991; Hameeda *et al.*, 2010; Verma *et al.*, 2014).**

CONCLUSION

The co-inoculation of *Paenibacillus durus* (PNF_{16}) and *Mesorhizobium* sp. followed by other PGPR were found highly effective for significant enhancement in growth, nodulation and yield of chickpea. The findings of the present investigation are encouraging and needs further investigation on mechanisms of interaction in the rhizosphere as well as field performance of this new isolate *Paenibacillus durus* (PNF_{16}) which has demonstrated promising PGP activities and compatible with other tested PGPRs.

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