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BACTERIA IN COMBINATION WITH FERTILIZERS IMPROVE GROWTH, PRODUCTIVITY AND NET RETURNS OF WHEAT (*Triticum aestivum* L.)

Mubshar Hussain[1], Zeshan Asgher[1], Muhammad Tahir[2], Muhammad Ijaz[3], Muhammad Shahid[4], Hakoomat Ali[1] and Abdul Sattar[3,*]

[1]Department of Agronomy, Bahauddin Zakariya University, Multan, Pakistan; [2]Department of Environmental Sciences, COMSATS Institute of Information Technology Vehari-Pakistan; [3]College of Agriculture, BZU, Bahadur Campus Layyah- Pakistan; [4]Department of Bioinformatics and Biotechnology, Government College University, Faisalabad-38000, Pakistan.
[*]Corresponding author's e-mail: abdulsattar04@gmail.com

Plant growth promoting rhizobacteria (PGPR) associate with roots of plants and improve plant growth by utilizing diverse mechanisms like nitrogen fixation, phosphorus solubilization and phytohormone production. Current study was conducted to isolate, characterize, and identify the wheat associated PGPR and investigate the inoculation effect of selected strains for growth promotion of wheat in combination with different levels of chemical fertilizer at two different ecological locations (Multan and Layyah, Pakistan). Among the total 22 bacterial isolates, 11 were positive for acetylene reduction assay (ARA), 9 isolates exhibited P solubilization activity and 19 bacterial isolates produced growth hormone indole-3-acetic acid (IAA) in culture medium. From these, two bacterial isolates with maximum potential to fix nitrogen, solubilize phosphorous and to produce IAA were identified through 16S rRNA gene sequence analysis as *Pseudomonas* sp. LYT-1 (accession no. KT933231) and *Bacillus* sp. MWT-14 (accession no. KT933232). Their performance as PGPR along with different levels of nitrogen (N) and phosphorous (P) chemical fertilizers (0-0, 105-75 and 150-100 NP kg ha^{-1}) was evaluated under field conditions at two different locations (Multan and Layyah) in 2014-15. The experiments were laid out in Randomize Complete Block Design (RCBD). Results indicated that bacterial strain *Pseudomonas* sp. LYT-1 (with maximum *in vitro* nitrogenase activity), increased the growth parameters like plant height, chlorophyll contents, productive tillers, spike length and straw yield of wheat at both the locations. The inoculation of *Bacillus* strain MWT-14 (with highest in vitro phosphate solubilization and IAA production) in combination with 0-0, 105-75 and 150-100 kg ha^{-1} N-P fertilizer improved the 1000-grain weight by 9.8%, 5.4%, 4.6% and 2.1%, 6.6%, 4%, respectively at Multan and Layyah, and grain yield by 16.3%, 12.3%, 4.7% and 17.1%, 14.2%, 8.5%, respectively at Multan and Layyah over their respective non-inoculated control treatments. These results concluded that plant growth promoting strains *Pseudomonas* sp. LYT-1 and *Bacillus* sp. MWT-14 enhance the growth, productivity and net returns of wheat when used as bio-inoculant along with 30% reduced of the recommended NP fertilizer dose.

Keywords: Phosphate solubilization, nitrogen fixation, hormone production, nutrient use efficiency, rhizosphere, root exudates.

INTRODUCTION

Wheat is cereal grain food for various countries of the world. The contribution of wheat to the world diet in the form of higher number of calories and protein makes it a more secure diet to human population (Abd-El-Haleem *et al*., 2009). Increase in the number of people using wheat as staple food has increased its area under cultivation, but average yield is still lower than the potential of wheat cultivars due to many factors including late sowing (Sattar *et al*., 2010; Hussain *et al*., 2012 a,b), poor irrigation facilities (Kibe *et al*., 2006; Rehim *et al*., 2012) and un-availability of nutrients like nitrogen (Rehman *et al*., 2014) and phosphorous (P) (Richardson *et al*., 2009; Rehim *et al*., 2012; Hussain *et al*., 2016). Phosphorus, a major among plant essential nutrients, is applied to soil as synthetic fertilizer produced from the mining of rock phosphate and chemical processing (Richardson *et al*., 2009). After application to alkaline soil, P binds with calcium ions to form complex tricalcium phosphate and becomes unavailable for plants. Because of less availability to the plant, it is one the most important limiting factor for growth and development of plant than other macro nutrients (Richardson *et al*., 2009). Thus, farmers have to apply more and more P to meet the requirement of the crop productivity. This practice increases the production cost of the farmer and environmental pollution in general. Another option available to farmers is the use of phosphate solubilizing bacteria (PSB) as bio-fertilizer; as these bacteria have the ability to produce organic acids like gluconic, malic, lactic, acetic acids and lower the pH of surroundings, thereby

detaching the cations attached to phosphates (Richardson 2001; Khan *et al*. 2009; Tahir *et al*., 2013). Likewise N, being macro-nutrient, plays an imperative function in formation of plant cell proteins and other essential bio-molecules and improves the plant vegetative as well as reproductive growth (Shridhar, 2012). Availability of atmospheric N to plants depends on its conversion into combined form e.g., NH_4^+ through a process of industrial N fixation at very high temperature and pressure for synthesis of BNF. Of the total atmospheric N, 60% is converted to available form through BNF which is not only economically but also environmental friendly than chemical fertilizers (Ladha *et al*., 1997; Shridhar 2012).

Phytohormone production is another mechanism which PGPR utilize to improve plant growth (Tahir *et al*. 2013). Auxins, especially IAA play a prime role in growth and development by escalating root area, increasing the moisture and nutrients uptake. Rhizosphere bacteria including *Azospirillum, Azotobacter, Bacillus, Enterobacter* and *Pseudomonas* produced significant amount of IAA as major property to improve plant productivity(Tahir *et al*., 2013). Isolation of *Bacillus* strains from the rhizosphere of various crops, phosphate solubilization, IAA production *in vitro* and plant growth promoting potential of *Bacillus* strains under field conditions have been reported (Tahir *et al*., 2015; Tahir *et al*., 2013). In addition, *Pseudomonas* spp. equipped with plant-beneficial traits like phosphate solubilization, N fixation, IAA production and bio-control of plant pathogens have been isolated from the rhizosphere of various crops (Babana *et al*., 2012; Kumar *et al*., 2012; Pastor *et al*., 2014; Tahir *et al*., 2015). Keeping in view all these problems, the objective of the present study was to isolate, characterize the rhizosphere bacteria on the basis of plant growth promoting qualities (phosphate solubilization, N fixation and IAA producing ability) and to identify the selected isolates through *16S rRNA* sequencing. Furthermore, to evaluate the PGPR potential of the selected isolates as bio-inoculant for improvement of growth, productivity and net returns from wheat, field experiments were conducted at two different location (Multan and Layyah) with varying levels of chemical fertilizer (N and P).

MATERIALS AND METHODS

***Extraction of wheat associated diazotrophs*:** Samples of rhizospheric soil collected from wheat field grown at Multan and Layyah were kept under storage at 4°C to use for further processes. Diazotrophs were isolated by adding 0.1 g of root and attached soil into eppendorf tubes (1.5 mL) poured with 1.0 mL of semi-solid nitrogen free medium i.e. NFM (Rennie *et al*., 1982.). Enrichment was carried out by repeated (5 to 6 times) transfer of grown bacterial culture (25.0 µL) after 48 h to fresh NFM containing eppendorf tubes. Afterwards, to get single colonies, cultures were splashed on plates poured with NFM agar plates.

***Isolation of wheat associated phosphate solubilizing bacterial isolates*:** For the isolation of wheat associated phosphate solubilizing bacterial isolate, 1.0 g of rhizosphere soil was inoculated in test tube filled with 10.0 mL of sterilized sodium chloride solution (0.85%, w/v) and thoroughly mixed. This mixture was further diluted (10X) up to 10^{-3}–10^{-5} by transferring 1.0 mL from each tube to the next tube containing NaCl solution (0.85%, w/v), and 100 µL of the dilution were equally distributed on plates poured with Pikovskaya agar medium (supplemented with insoluble form of phosphorous i.e. tri-calcium phosphate TCP) plates (Pikovskaya, 1948). The plates were kept under incubation at 30°C for 7 days. After several hours of incubation, halo-zones were formed around the bacterial colonies which indicate P-solubilization (solubilization of TCP) activity of the bacterial colonies. For further processing, the halo-zones forming bacterial colonies were selected, picked and purified on plates containing fresh Pikovskaya agar medium.

***Estimation of nitrogen fixed by wheat associated rhizosphere bacteria*:** Determination of diazotrophic potential of the rhizosphere bacteria was made through acetylene reduction assay in partially-solid combined carbon medium (CCM; Rennie *et al*., 1982) and NFM (Okon *et al*., 1977). Glass tubes (sterilized) of capacity 16.0 mL were poured separately with 5.0 mL of semisolid NFM and CCM, inoculated with single bacterial colonies and were closed with rubber stoppers. These tubes were kept under incubation at 30°C for 16 hours. When bacterial growth become visible in vials, injection of acetylene C_2H_2 (10%) was made into vials with grown bacterial culture and were kept under incubation at 30°C for 16 hours. Samples (triplicate) of each bacterial colony were processed for ARA. The tubes with bacterial culture but without C_2H_2 were considered as inoculated control. An additional non-inoculated (no bacterial culture) control which consists of tubes injected with acetylene (10%) was also processed. On a Gas Chromatograph (Gasukurokogyo model 370, Tokyo, Japan) using Porapak N column (Supelco Inc., Bellefonte, Pennsylvania), for the estimation of ethylene gas produced by bacterial cultures, peak height of the samples was compared with that of standard (1% C_2H_4).

***Estimation of phosphate solubilization by rhizosphere bacteria*:** Phosphate solubilized by bacterial isolates was quantified using phosphomolybdate blue color method (Murphy and Riley, 1962) with following modifications. Single colonies of the bacterial isolates were inoculated in Luria-Bertani (LB) broth medium and grown overnight on arbitrary shaker (150 rpm) at 30±2°C. From these overnight grown bacterial cultures, 1.0 mL was inoculated to Pikovskaya broth medium (Pikovskaya, 1948) for 15 days on arbitrary shaker (150 rpm) at 30±2°C. Supernatant (Cell-free growth medium) was extracted by centrifugation at 6000 *xg*

for 15 min. and pH of the supernatant was measured after the removal of residues through filtration. Optical density of the solutions containing bacterial culture after the development of blue color was recorded on Spectrophotometer (CamspecM350-Double Beam UV-Visible Spectrophotometer, UK) at 882 nm and compared with standard curve made using KH_2PO_4 2, 4, 6, 8, 10 and 12 ppm solutions to measure the amount of solubilized phosphate (primary and secondary orthophosphate) by the bacterial isolates.

***Estimation of indol-3-acetic acid produced by wheat associated bacteria*:** Single bacterial colonies were developed in LB broth medium overnight at 30±2°C to estimate the concentration of bacterially produced IAA in culture medium. One mL of the overnight grown bacterial cultures was inoculated to 150 mL of growth medium (LB broth) with tryptophan (100 mg L^{-1}) and without tryptophan (IAA biosynthesis precursor) and were grown for 15 days. By centrifugation at 6000 rpm for 15 min, cell-free supernatant was extracted. Acidification of the supernatant (pH 2.8) was made by adding some drops of HCl (1.0 N) and extraction of this acidified supernatant was made by adding equivalent volumes of ethyl acetate (Tien *et al*., 1979). The extract was dried through evaporation and suspended in ethanol (1.0 mL). For the quantification of bacterial culture produced IAA in *vitro*, pure indole-3-acetic acid (Sigma, USA) was used as standard and analysis of the samples was performed through High-performance liquid chromatography on HPLC (Perkin-Elmer, USA; Series 200). Ultra Violet (UV) detector and Techsphere 5-ODS C-18 column were used. The retention time and peak area of the samples was compared with that of the standard for quantification of bacterial culture produced IAA by software (Turbochrom) using an interface (Perkin-elmeer, USA).

***Molecular identification of selected isolates*:** For isolation of genomic DNA, bacterial cultures of selected isolates (LYT-1 and MWT-14) were allowed to grow for 12 h at 30±2ºC in LB broth medium. Centrifugation was performed at 10,000 x g to get bacterial cell pellets and total genomic DNA extraction was then performed according to manufacturer's protocol using FastDNA SPIN Kit (MP Biomedicals, USA) and was stored at -20ºC. The extracted DNA was used as tempelate to amplify the gene *16S rRNA* using primers 27F (forward primer) and 1492R (reverse primer) as described earlier (Weisburg *et al*., 1991). PCR amplification was performed in GS0001 thermocycler (Gene Technologies, Braintree, United Kingdom) using 50 µL deionized nuclease free water, 5.0 µL of 2.0 mM dNTPs, Taq buffer 5.0 µL (Fermentas), 0.75 µL of 5.0 U μL^{-1} Taq DNA polymerase (Fermentas) and 1.5 µL of each primer (forward and reverse). The extracted DNA (40 ng) was used as tempelate. Temperature conditions were also modified as initial denaturation was performed at 95°C for 5 minutes, 35 cycles of 95°C for 60 s, 55°C for 30 s, and for 60 s at 72°C. The final extension was performed at 72°C for 10 minutes. Purification of amplified PCR product was done by using high pure PCR Clean-up Micro Kit (Roche Applied Science, USA). Sequencing was performed commercially (Eurofins, Germany). The 16S rRNA gene sequences were analysed and compared with available sequences in the GenBank database using the NCBI BLASTn tool. Cleaned nucleotide sequences were deposited in Genbank to get accession numbers i.e. *Pseudomonas* sp. LYT-1 (accession no. KT933231) and *Bacillus* sp. MWT-14 (accession no. KT933232). Phylogenetic trees were prepared by downloading the closely related sequences from GenBank database. These sequences were aligned by CLUSTAL X and analyzed using neighbor joining method.

***Inoculum preparation and field experiments*:** Selected bacterial isolates *Pseudomonas* sp. LYT-1(with nitrogen fixation and IAA synthesis ability) and *Bacillus* sp. MWT-14 (with phosphate solubilization activity) were grown in LB broth (150 mL) on arbitrary shaker (150 rpm) at 30±2°C overnight. Serial dilution method was used to measure the strength of the inoculums. On LB agar medium plates, 100 µL aliquots from the serial dilutions were spread and the plates were kept under incubation for 12 h at 30°C. Colony forming units (CFU) was counted and inoculum of each strain was adjusted to 10^9 CFU mL^{-1} of saline solution. A field study to examine the effectiveness of PGPR strains *Pseudomonas* sp. LYT-1 and *Bacillus* sp. MWT-14 as sole inoculation and in combination with different levels of N-P fertilizer (0-0, 105-70 and 150-100 kg ha^{-1}) was performed during winter season 2014-15 at two different locations (Multan and Layyah). Inoculum was mixed with sterilized filter-mud and seeds of wheat variety Galaxy-2013 were pelleted with this mixture containing filter mud and inocula (10^9cfu/g). Inoculated seeds (120 kg ha^{-1}) were sown using hand drill. The N and P were applied on the bases of treatment. All other agronomic practices were performed equally throughout experiment. At harvest maturity (140-150 days after sowing), harvesting of the crop was done. Physico-chemical characteristics of experimental soil were determined before conducting and after the harvesting of experiment (Table 1).

***Crop allometric and yield parameters*:** Data on plant allometric parameters such as leaf area index (LAI), leaf area duration, crop growth rate (CGR), net assimilation rate (NAR) and chlorophyll contents was recorded fortnightly started from 55 days after sowing (DAS) at tillering of the crop. A homogenous area (0.5 m^2) from each treatment was cut randomly and leaves were separated. Total area of the separated leaves was measured by using leaf area meter (DT Area Meter, Model MK2, Delta T Devices, Cambridge, UK). The LAI was then calculated by dividing total area of the leaves to the total ground area. Thereafter, LAD, CGR and NAR were calculated by following the protocol described by Hunt (1978). Chlorophyll contents were measured using chlorophyll meter (SPAD 502, Spectrum Technologies, Inc, Aurora, IL). At maturity, yield related parameters were

Table 1. Physico-chemical analysis of experimental soil before sowing and after harvesting at two different locations.

Determinant	Before sowing		After harvesting	
	Layyah	Multan	Layyah	Multan
Texture	Sandy loam	Silt loam	Sandy loam	Silt loam
EC (dsm^{-1})	0.52	2.6	0.57	2.0
pH	8.4	7.7	8.2	7.5
Organic Matter (%)	0.30	0.84	0.39	0.90
P (ppm)	4.00	8.98	6.00	9.00
N (%)	0.050	0.080	0.080	0.095
K (ppm)	117	19	131	23

measured by standard procedure following by Hussain *et al*. (2016).

***Statistical analysis*:** The collected data was analyzed using software Statistics 8.1 version. Fischer's analysis of variance technique (ANOVA) was followed during analysis, and comparison among treatments' means was performed by LSD test at 5% probability (Steel *et al*., 1997). Further, graphical presentation of data was accomplished by using Microsoft Excel 2010 computer program along with ± S.E.

***Economic analysis*:** To evaluate the feasibility of using bacterial strains with NP levels in accelerating wheat yield economic analysis was conducted. Total expenses incurred on wheat production from sowing to harvesting were computed. The expenses included the rent of land, preparation of seedbed, sowing of crop, cost of fertilizers, cost of irrigation water, cost on bacterial strains and plant protection measures. Further, gross income was estimated by considering the current prices of the wheat grains and straw. Net profit was figured by subtracting expenses from total income (CIMMYT, 1988).

RESULTS

***Isolation and screening of rhizosphere bacteria for plant beneficial traits*:** Among the growth media tryptophan supplemented and without tryptophan, maximum IAA production was observed in LB broth medium supplemented with tryptophan. Among the bacterial isolates, significantly ($P<0.05$) higher concentration (618±11mg/L and 2.8±0.3 mg/L) of IAA was produced by the isolate *Bacillus* sp. MWT-14 (from Multan) in culture media supplemented with tryptophan and without tryptophan respectively. Eleven bacterial isolates were ARA positive in both NFM and CCM media. Maximum ARA (1820±43 and 811±21n mol C_2H_2/h/mg protein in CCM and NFM, respectively) was observed in vial inoculated with the bacterial isolate *Pseudomonas* sp. LYT-1 (from Layyah). Of the total, nine bacterial isolates obtained from Pikovskaya agar medium plates were tested for P solubilization in Pikovskaya broth medium supplemented with tri-calcium phosphate (TCP). All these bacterial isolates reduced the pH of the medium (pH 7.0 to pH<6) and solubilized phosphate (58±4-355±14µg/mL) in culture medium (Table 4). Maximum amount of solubilized P (355±14µg/mL) was obtained in Pikovskaya broth medium inoculated with bacterial isolate *Bacillus* sp. MWT-14.

***Identification through 16S rRNA gene sequencing*:** 16S rRNA gene sequence analysis of the bacterial isolate LYT-1 and MWT-14 showed 98% sequence similarity with *Pseudomonas florescence* strain G33 (accession no. KT767930) and *Bacillus* sp. LS-063 (accession no. KF870442), respectively. Phylogenetic analysis showed that among the clusters, the bacterial isolate LYT-1 formed a cluster with *Pseudomonas* florescence strains and the isolate MWT-14 is present in cluster containing the *Bacillus thuringiensis* strains (Fig. 1a,b).

Table 4. Effect of bacterial inoculation and different levels of NP fertilizer on plant height, number of productive tillers and spike length of wheat at two different locations.

Bacterial strain	Plant height (cm)						Number of productive tillers (m^{-2})						Spike length (cm)					
	Multan			Layyah			Multan			Layyah			Multan			Layyah		
NP levels (kg ha^{-1})	0-0	105-70	150-100	0-0	105-70	150-100	0-0	105-70	150-100	0-0	105-70	150-100	0-0	105-70	150-100	0-0	105-70	150-100
Non-inoculated	63.6e	90.7b	95.1ab	60.6e	85.4b	92.1a	143.8g	296.0 d	277.3 e	145.8 f	294.0 d	281.0d	7.07 e	9.59bc	9.47 c	7.01 e	9.47bc	9.34 c
B. subtilis	69.9d	97.6a	98.4a	65.4d	93.9a	93.4a	158.0g	304.0 cd	316.0 c	160.9 f	325.7 bc	324.0c	7.77 d	9.92 a-c	10.12 ab	7.17 d	9.81a-c	10.0 ab
P. aurentiaca	77.7c	95.9a	96.9a	75.3c	91.2a	90.7a	176.0f	345.3 b	456.0 a	190.9 e	342.0 ab	356.0 a	8.01 d	9.98 a-c	10.26 a	7.92 d	9.84a-c	10.12 a
LSD at 5%		4.80			3.93			17.04			17.54			0.48			0.47	

Plant height (cm) of twenty randomly selected plants from each treatment was measured with the help of measuring tape starting from the base to tip of the plant and averaged. Total spike bearing tillers were counted from randomly selected area of 1 m2 from five different locations within a treatment. The counted tillers were averaged for recording the number of productive tillers. Twenty random selected spikes were used to record average spike length (cm), number of spikelets per spike and number of grains per spike. Each value in the Table 6 is the mean of 3 values

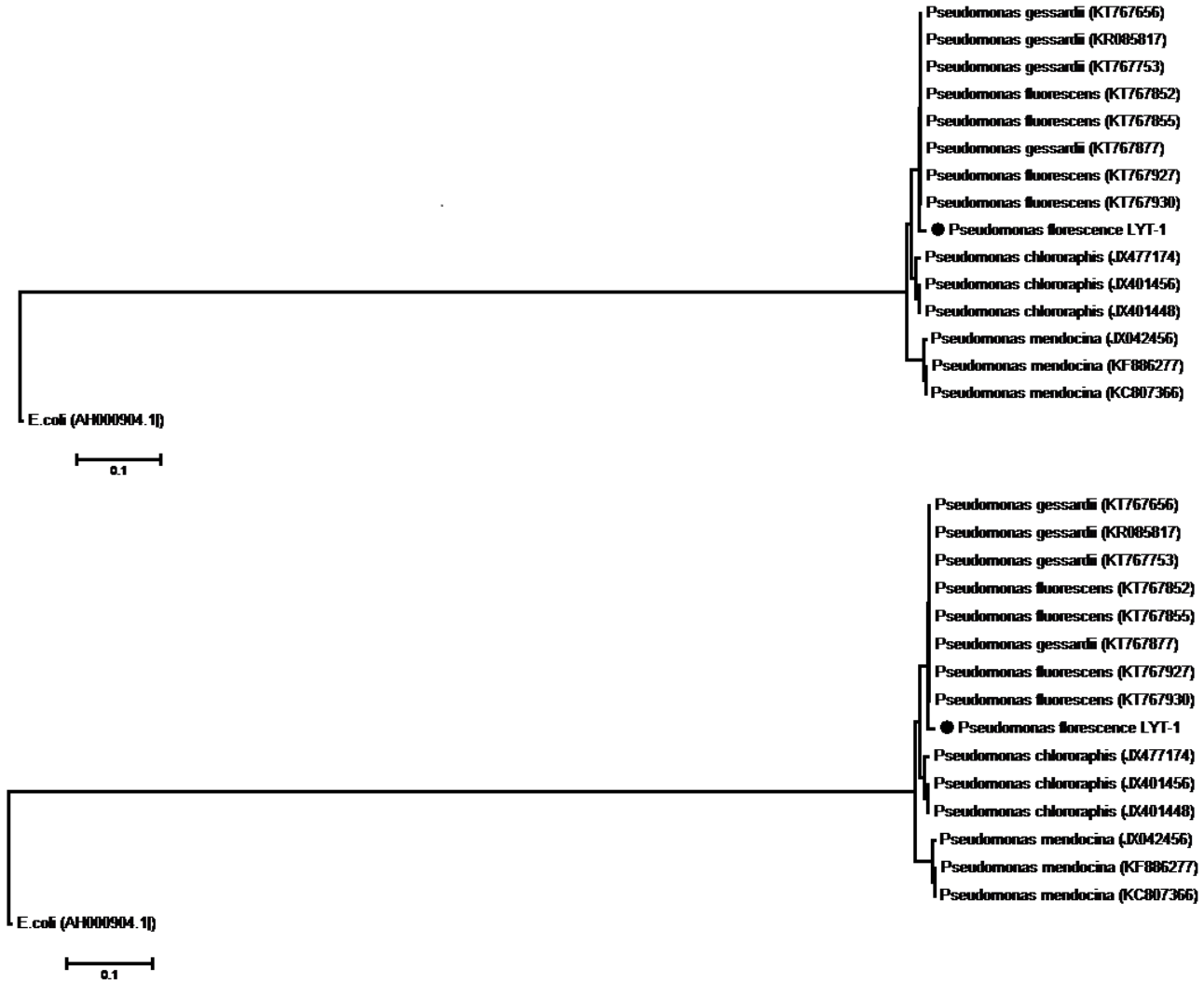

Figure 1. Amplified 16S rRNA gene fragments from the isolated strain *Bacillus* strain MWT-14 and *Pseudomonas* strain LYT-1 was sequenced and blast searched though NCBI database. Closely related sequences were downloaded and aligned using CLUSTAL X. These sequences were analyzed using neighbor-joining method. The bootstrap replicates (BS) values of 50% or greater represent well supported nodes and thus only those were retained. *E. coli* (AH000904) was taken as out-group.

***Effect of PGPR inoculation on growth and productivity of wheat*:** In field experiments, interactive effect between bacterial strains and NP levels was significant ($P<0.05$) on chlorophyll contents, leaf area, leaf area index (LAI), leaf area duration (LAD), crop growth rate (CGR) and net assimilation rate (NAR) of wheat. Both the bacterial strains, specifically *Pseudomonas* sp. LYT-1, increased chlorophyll contents of wheat at all fertilizer levels over non-inoculated control treatments at both the experimental sites (Fig. 2a,b). However, results were non-significant at 90 DAS with NP rate of 150-100 kg $ha^{-1}$with respect to bacterial strains. Similarly, LAI (Fig. 3a,b) and LAD (Fig. 4a,b) was significantly higher (P<0.05) in treatments inoculated with *Pseudomonas* sp. LYT-1 at all fertilizer levels as compared to their respective non-inoculated control during the entire growth period at both experimental locations. However, LAI and LAD was significantly ($P<0.0.5$) higher only at 60 DAS in treatment inoculated with *Bacillus* sp. MWT-14 as compared to non-inoculated control and the treatment inoculated with *Pseudomonas* sp. LYT-1 with all fertilizer levels. Inoculation of wheat with *Bacillus* sp. MWT-14 improved CGR at 55-70 DAS and 75-80 DAS in combination with all fertilizer doses but at 40-55 DAS, CGR was higher in the treatments inoculated with *Pseudomonas* sp. LYT-1 at both the experimental sites (Fig. 5a,b). Application of NP fertilizer at the rate of 150-100 kg ha^{-1} along with bacterial

inoculation observed higher NAR at both locations (Fig. 6a,b) over non-inoculated control.

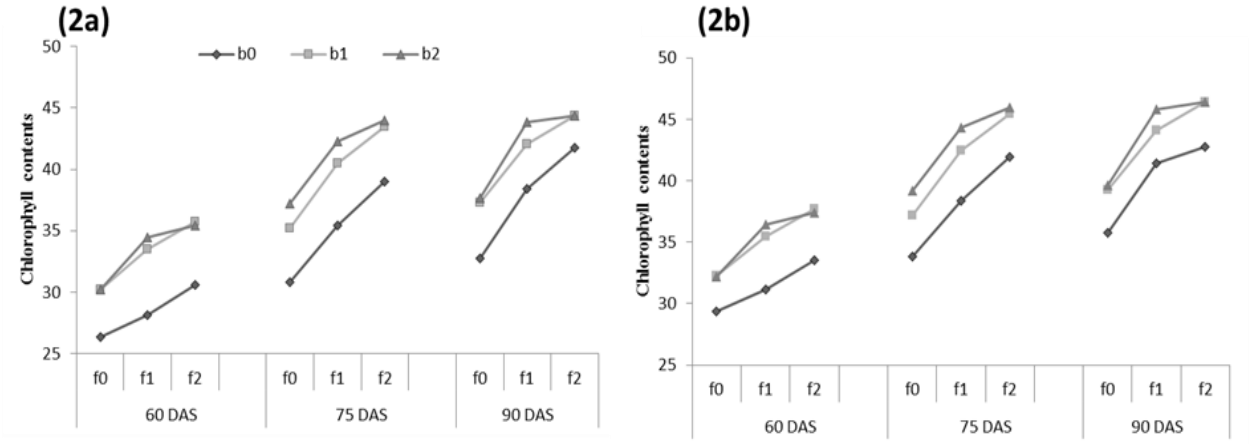

Figure 2. Chlorophyll contents of wheat as influenced by PGPR inoculation and different levels of NP fertilizer at two different locations; 2a) Multan, 2b) Layyah.

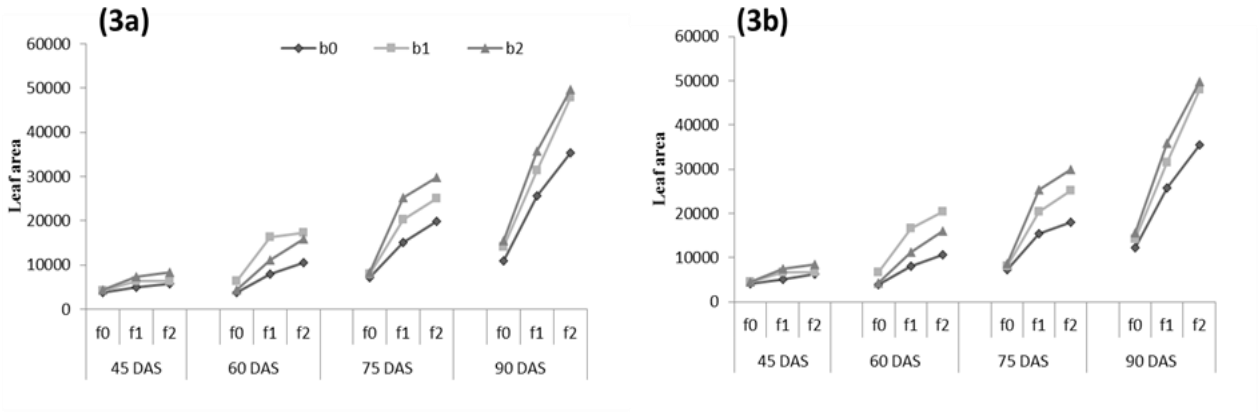

Figure 3. Leaf area of wheat as influenced by PGPR inoculation and different levels of NP fertilizer at two different locations; 3a) Multan, 3b) Layyah.

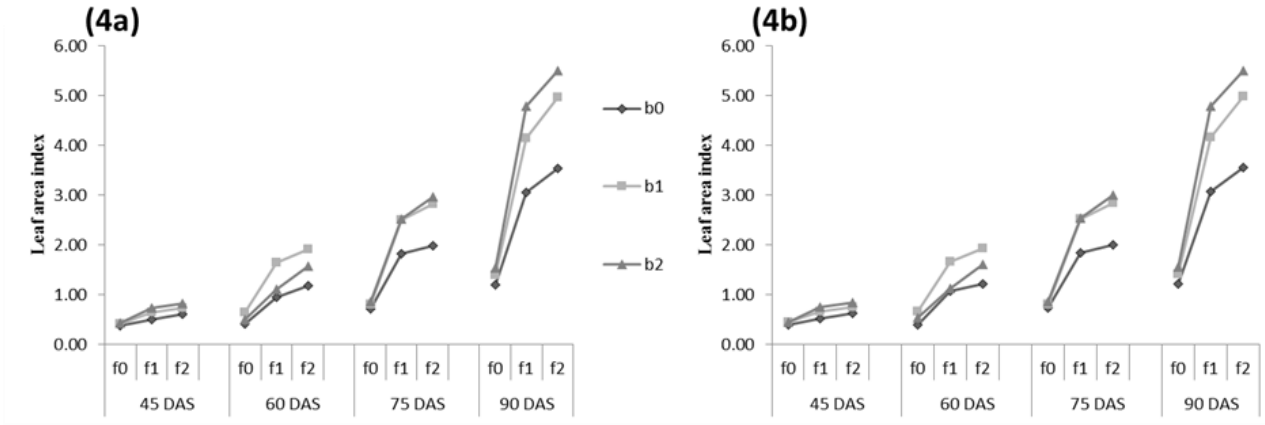

Figure 4. Leaf area index of wheat as influenced by PGPR inoculation and different levels of NP fertilizer at two different locations; 4a) Multan, 4b) Layyah.

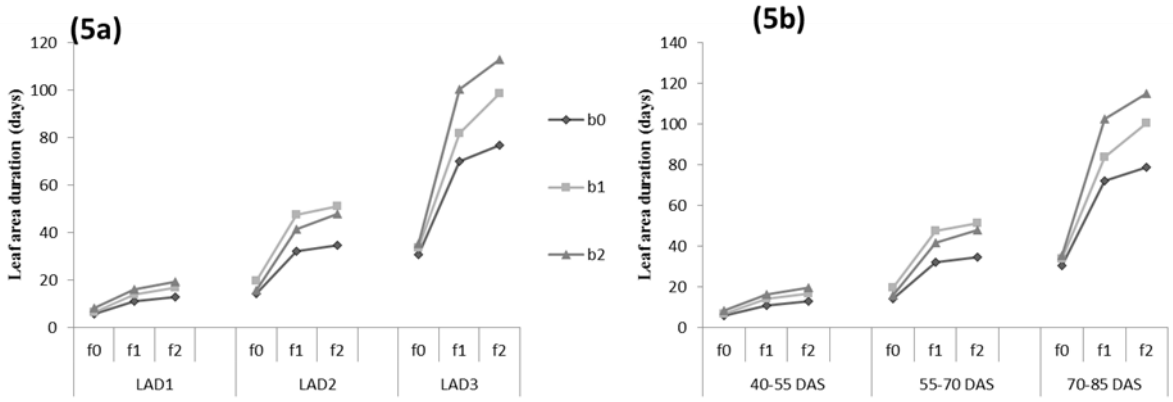

Figure 5. Leaf area duration of wheat as influenced by PGPR inoculation and different levels of NP fertilizer at two different locations; 5a) Multan, 5b) Layyah.

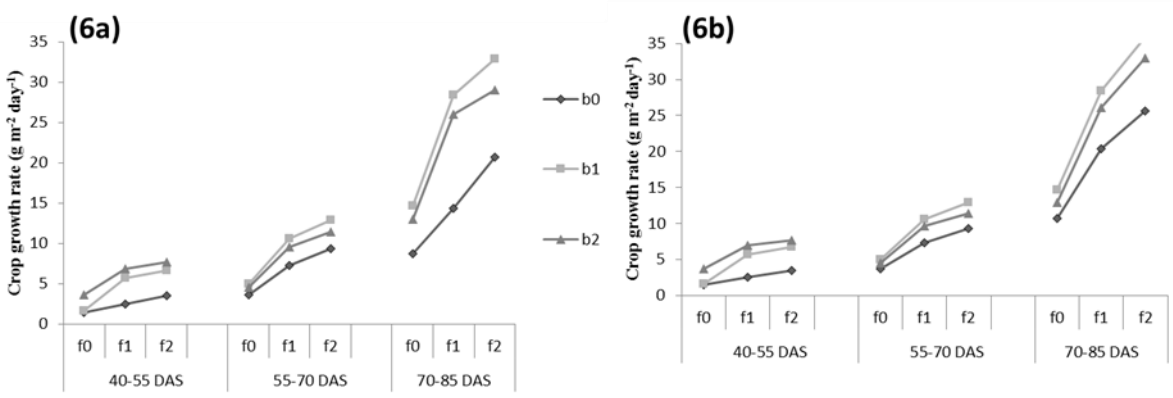

Figure 6. Crop growth rate of wheat as influenced by PGPR inoculation and different levels of NP fertilizer at two different locations; 6a) Multan, 6b) Layyah.

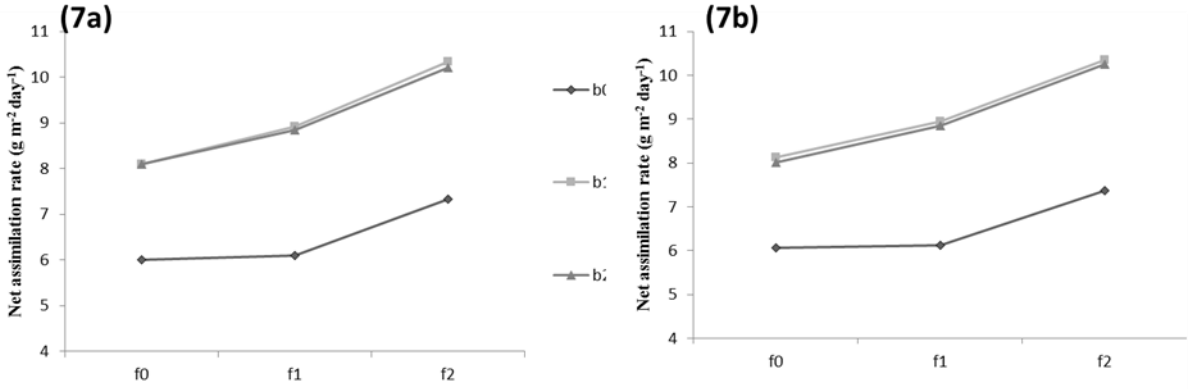

Figure 7. Net assimilation rate of wheat as influenced by PGPR inoculation and different levels of NP fertilizer at two different locations; 6a) Multan, 6b) Layyah.

Interactive effect between bacterial strains and NP levels was significant on plant height, population of productive tillers and spike length of wheat at both experimental locations (Table 4). Both bacterial strains, *Pseudomonas* sp. LYT-1 in particular, improved plant height, population of productive tillers and spike length of wheat at all fertilizer levels but the effect was more outstanding at higher level of NP (Table 4). Non-inoculated control treatment with all N-P_2O_5 fertilizer levels resulted in significantly lower plant height, number of productive tillers and spike length at both the locations (Table 4). Number of spikelets per spike, number of grains per spike and 1000-grain weight was significantly (P<0.05) affected

due to interactive outcome of different NP fertilizer levels and bacterial strain inoculation (Table 5). Both the bacterial strains, *Pseudomonas* sp. LYT-1 specifically, improved number of spikelets per spike and number of grains per spike of wheat at all NP fertilizer levels but the impact was more outstanding at higher levels of NP fertilizer (Table 5). Interestingly, the 1000-grain weight was obtained maximum in the treatment where the bacterial strain *Bacillus* sp. MWT-14 was inoculated along with all levels of NP fertilizer. However, the 1000-grain weight was recorded maximum with MWT-14 inoculation along with higher levels of NP fertilizer at both the experimental sites (Table 5). Minimum numbers of spikelets per spike, number of grains per spike and 1000-grain weight were obtained in non-inoculated control treatment with application of N-P_2O_5 at 0-0 kg ha^{-1} at both experimental locations (Table 5).

Interaction between the different NP fertilizer levels and bacterial strains inoculation showed significant effect on straw and grain yield of wheat at both locations (Table 6). Straw yield was improved with increased NP fertilizer levels, particularly with higher fertilizer dose. Both the strains, *Pseudomonas* sp. LYT-1 in particular, improved the straw yield of wheat at all fertilizer levels at both the experimental sites (Table 6). However, the impact was more distinct with higher NP fertilizer levels at both the experimental locations. Grain yield was increased due to *Bacillus* sp. MWT-14 inoculation with all NP fertilizer levels at both the experimental sites (Table 6). Minimum straw yield and grain yield was obtained in non-inoculated control treatments with zero application of NP fertilizer.

Interactive effect between bacterial strains and NP fertilizer levels was significant on biological yield and harvest index of wheat at both experimental locations (Table 7). Both the bacterial strains, *Pseudomonas* sp. LYT-1 in particular, improved biological yield of wheat at all fertilizer levels but the effect was more prominent at higher level of NP (Table 7). Inoculation of bacterial strain *Bacillus* sp. MWT-14 increased the harvest index (%) over all other treatments at

Table 5. Effect of bacterial inoculation and different levels of NP fertilizer on number of spikelets per spike, number of grains per spike and 1000 grain weight of wheat at two different locations.

Bacterial strain	Number of spikelets per spike						Number of grains per spike						1000 grain weight (g)					
	Multan			Layyah			Multan			Layyah			Multan			Layyah		
NP levels (kg ha^{-1})	0-0	105-70	150-100	0-0	105-70	150-100	0-0	105-70	150-100	0-0	105-70	150-100	0-0	105-70	150-100	0-0	105-70	150-100
Non-inoculated	12.9 d	15.9 b	15.9 b	11.11 c	13.0ab	13.02a b	21.1 g	27.9 de	29.1cd	19.1 b	19.60 b	19.9 b	39.9 c	42.5 a-c	43.3 ab	37.6 d	39.03 cd	39.5 b-d
B. subtilis	14.2 c	16.5 ab	17.2 a	13.1a	13.4 a	14.1 a	23.1fg	30.1 b-d	32.3 b	19.8 b	23.70 a	24.3 a	43.8 ab	44.8 a	45.3 a	38.4 d	41.6a	41.1 ab
P. aurentiaca	14.6 c	16.7 ab	17.2 a	11.2 bc	13.3 a	13.7 a	25.1ef	31.3bc	37.1a	20.2 b	23.5 a	25.3 a	41.3bc	43.4 ab	43.8 ab	38.2 d	41.7 a	40.5 a-c
LSD at 5%		0.93			1.88			2.81			1.87			2.99			2.02	

For recording 1000-grain weight, three random samples each of 1000 grains were taken from seed lot of every plot and observed weights (g) were averaged. Each value in the Table 6 is the mean of 3 values.

Table 6. Effect of bacterial inoculation and different levels of NP fertilizer on straw yield and grain yield of wheat at two different locations.

Bacterial strain	Straw yield (t ha^{-1})						Grain yield (t ha^{-1})					
	Multan			Layyah			Multan			Layyah		
NP levels (kg ha^{-1})	0-0	105-70	150-100	0-0	105-70	150-100	0-0	105-70	150-100	0-0	105-70	150-100
Non-inoculated	2.05 e	4.47 d	5.42 c	1.85 d	3.10 c	3.56 b	2.40 f	3.50 d	4.00 bc	2.21 e	3.50 c	4.00 b
B. subtilis	2.15 e	6.10 b	5.52 c	1.90 d	3.57 b	4.30 a	2.79 e	3.93 bc	4.40 a	2.59 d	4.03 b	4.34 a
P. aurentiaca	2.37 e	6.25 b	7.43 a	1.89 d	3.64 b	4.35 a	2.53ef	3.87c	4.20 b	2.40 d	4.00 b	4.20 a
LSD at 5%		0.57			0.25			0.26			0.14	

To record biological yield, each plot was harvested manually, sun dried for a week, tied into bundles and weighed for getting biological yield. After recording biological yield, the bundles were threshed manually and the grains were weighed to record grain yield. Straw yield was recorded by weighing the left over straw. The yields (biological, grain and straw) from the harvested areas were converted into kg ha-1 by unitary method. Each value in the Table 6 is the mean of 3 values.

Table 7. Effect of bacterial inoculation and different levels of NP fertilizer on Biological yield and Harvest index of wheat at two different locations.

Bacterial strain	Biological yield (t ha^{-1})						Harvest index (%)					
	Multan			Layyah			Multan			Layyah		
NP levels (kg ha^{-1})	0-0	105-70	150-100	0-0	105-70	150-100	0-0	105-70	150-100	0-0	105-70	150-100
Non-inoculated	4.45 e	7.97 d	9.42 c	4.06 e	7.10 c	7.56 b	53.9ab	43.9 c	42.5 c	54.4 ab	56.3 a	52.9 b
B. subtilis	4.94 e	10.03b	9.92 bc	4.49 d	7.59 b	8.64 a	56.5 a	39.2 d	44.4 c	57.7 a	53.1 a	56.8 a
P. aurentiaca	4.90 e	10.12b	11.53 a	4.29 d	7.57 b	8.55 a	51.6 b	38.2 d	36.1 e	55.9 a	52.0 b	49.1 c
LSD at 5%		0.61			0.24			3.98			3.40	

Harvest index (HI) was taken as ratio of grain yield to biological yield expressed in percentage. Each value in the Table 7 is mean of three values.

both the experimental sites. Non-inoculated control treatment with all N-P_2O_5 fertilizer levels resulted in significantly lesser biological yield and harvest index at both the locations (Table 7).

DISCUSSION

Domination of crop rhizosphere by variety of bacteria enforced the researchers to isolate the bacteria exhibiting plant growth promoting traits. Bacterial growth become visible after 48 h as a veil like pellicle in NFM growth medium inoculated with roots and attached soil. This pellicle formation in NFM semisolid was initially considered a characteristic of *Azospirillum*, which finds suitable oxygen concentration just below the surface due to its microaerophilic nature (Tarrand *et al.*, 1978). However, other studies (Gupta *et al*., 2014; Haahtela *et al*., 1981) proved the isolation of other microaerophilic bacteria including species with nitrogen fixing activities from nitrogen-free semisolid media. Isolation of diazotrophs from the rhizosphere of various crops using nitrogen-free semisolid media have also been reported (Malik *et al*., 1997; Mehnaz, 2015; Mehnaz *et al*., 2001; Mirza *et al*., 2014; Rennie *et al*., 1982.; Tahir *et al*., 2013). Among the 13 isolates obtained from Pikovskaya agar medium plates, phosphate solubilization was observed in 8 bacterial isolates. Pikovskaya medium contain insoluble P in the form of tricalcium phosphate (TCP) and is commonly used medium for the isolation of P-solubilizing bacteria (Perez *et al*., 2007; Pikovskaya, 1948; Shahid *et al*., 2014; Tahir *et al*., 2013).

Among all bacterial isolates, the isolate *Pseudomonas* sp. LYT-1 from Layyah region showed significantly higher (*P<0.05*) ARA activity (1820±43 and 811±21 n molC_2H_4/h/mg protein in CCM and NFM, respectively), this strain also produced higher concentration (521±10µg/mL) of IAA in tryptophan supplemented growth medium (Table 2). Association of strains belongs to genus *Pseudomonas* with grasses and N fixing ability of the strains (Bahulikar *et al*., 2014; Baldani *et al*., 2014) has been reported. Previous studies have proved the *Pseudomonas* as IAA producing bacteria. The strain *Bacillus* sp. MWT-14 from Multan region produced significantly higher amount of IAA in tryptophan supplemented medium (Table 2). The same strain also

Table 2. Acetylene reduction activity and indole-3- acetic acid production of the associated bacteria with the rhizosphere of wheat grown on two different agro-climatic conditions of southern Punjab.

Bacterial isolates	IAA production (mg/L)		ARA (n mol C2H4/h/mg protein)	
	Without tryptophan	With tryptophan	NFM	CCM
LYT-1	2.5±0.2	521±10	811±21	1820±43
LYT-2	0.4±0.1	150±6	ND	ND
LYT-3	1.2±0.2	110±11	11±1.0	105±10
LYT-4	ND	ND	ND	ND
LYT-5	1.5±0.2	418±17	12±1	125±12
LYT-6	2.0±0.1	492±5	5±0.2	72±6
LYT-7	1.8±0.1	50±7	ND	ND
LYT-8	2.3±0.1	21±1.5	ND	ND
MWT-9	0.8±0.1	18±1.5	10±1.5	62±4
MWT-10	0.5±0.1	12±1.0	ND	ND
MWT-11	0.8±0.2	20±21	ND	ND
MWT-12	2.0±0.2	55±8	21±1.0	132±8
MWT-13	2.0±0.1	115±8	24±1.0	93±7
MWT-14	2.8±0.3	618±11	ND	ND
MWT-15	1.9±0.2	21±2	3.0±0.5	51±2
MWT-16	1.5±0.2	16±4	3.0±0.2	35±3
MWT-17	1.2±0.1	32±4	8±0.8	85±3
MWT-18	1.3±0.1	12±1.2	21±2	63±4
MWT-19	1.8±0.3	125±12	ND	ND
MWT-20	2.8±0.4	281±12	ND	ND
MWT-21	ND	ND	ND	ND
MWT-22	ND	ND	ND	ND

Bacterial cultures were grown in semi-solid NFM and CCM media for estimation of nitrogen fixation. After 48 h, acetylene (10%) was injected and samples were again incubated at 30°C for 16 hours. ARA activity was observed through gas chromatography; For the estimation of IAA produced, bacterial cultures were grown in medium supplemented with and without tryptophan for two weeks. Supernatant was extracted, acidified with HCl and analyzed on HPLC ; ND = not determined; Each value is the mean of three replicates ± Standard deviation.

reduced pH from 7.0 to 4.8±0.2 of the Pikovskaya broth medium supplemented with TCP (Table 3) and solubilised maximum amount of phosphorous (355±14 µg/mL).

Table 3. Phosphate solubilization and pH of the broth medium supplemented with tricalcium phosphate by the associated bacterial isolates with the rhizosphere of wheat grown on two different agro-climatic conditions of southern Punjab.

Bacterial isolates	pH of the medium	P-solubilization (µg/mL)
LYT-1	6.8±0.2	ND
LYT-2	5.2±0.1	58±4
LYT-3	6.9±0.2	ND
LYT-4	5.8±0.2	62±3
LYT-5	7.0±0.1	ND
LYT-6	7.0±0.2	ND
LYT-7	5.0±0.2	183±5
LYT-8	5.1±0.2	151±3
MWT-9	6.8±0.2	ND
MWT-10	5.5±0.1	136±3
MWT-11	5.4±0.1	140±6
MWT-12	6.9±0.2	ND
MWT-13	7.0±0.1	ND
MWT-14	4.8±0.2	355±14
MWT-15	7.0±0.1	ND
MWT-16	6.9±0.1	ND
MWT-17	6.9±0.1	ND
MWT-18	7.0±0.1	ND
MWT-19	5.7±0.2	110±7
MWT-20	5.6±0.2	122±2

Bacterial cultures were grown in Pikovskaya broth medium for 15 days. Supernatant was extracted by centrifugation and phosphate solubilization was determined on spectrophotometer using molybdate blue color method; ND = not determined; Each value is the mean of three values; ± Standard deviation.

Association and isolation of phosphate solubilising *Bacillus* strains from the rhizosphere of various crops like wheat, rice, sugarcane and maize has been frequently reported (Tahir *et al*., 2015; Tahir *et al*., 2013). Two bacterial strains *Pseudomonas* sp. LYT-1 (with highest ARA activity) and *Bacillus* sp. MWT-14 (possessed highest P-solubilization and IAA production) were selected to investigate their PGPR potential for wheat under field conditions at two different locations in combination with different levels of NP fertilizer. Inoculation of wheat with PGPR either as sole application or in combination with all levels of NP fertilizer increased the chlorophyll contents, LAI, LAD, CGR and NAR during the entire crop growth period at both locations (Fig. 2a-6b). Among the bacterial strains, the strain *Bacillus* sp. MWT-14 performed extraordinary best in improving growth parameters at 60 DAS while the performance of the strain *Pseudomonas* sp. LYT-1 was better at 45, 75 and 90 DAS. The reason for this may be the soil factors like temperature, moisture, aeration, root exudates nature and indigenous bacterial population at that specific plant growth stage was more suitable for the growth, survival and activity of the *Pseudomonas* strain. Previous studies have reported that the population, diversity, survival and activity of bacteria is affected due to nature of root exudates and indigenous rhizosphere bacteria (Shridhar, 2012; Tahir *et al*., 2015; Walker *et al*., 2003).

Both the bacterial strains, *Pseudomonas* sp. LYT-1 in particular, improved plant height, number of productive tillers, spike length, grains count, straw yield and biological yield of wheat at all fertilizer levels but the effect was more prominent at higher level of NP. This might be due to the fact that the strain *Pseudomonas* sp. LYT-1 had higher ARA activity; so its inoculation as bio-inoculant along with chemical fertilizer might improve N and P availability to the plants. This has been proved that N, being macro-nutrient, plays an important role in formation of plant cell proteins and other essential bio-molecules and improves the plant vegetative as well as reproductive growth (Shridhar, 2012). In previous studies it has been reported that inoculation of N fixing bacteria increased the uptake of N and improved growth of wheat (Abbasdokht and Gholami 2010; Baldani *et al*., 2014).

Grain yield was surprisingly higher due to interaction between different NP levels and inoculation of *Bacillus* sp. MWT-14. This strain solubilized more concentration of insoluble phosphate and produced IAA in *vitro*. Inoculation of *Bacillus* as bio-inoculant might have resulted in increased root area, P availability and uptake of other nutrients which resulted in accumulation of more food in grain and ultimately the 1000-grain weight and grain yield was increased in *Bacillus* inoculated plants. It has been reported previously that inoculation of phosphate solubilizing bacteria improved the P uptake by solubilizing insoluble P and increased the growth and yield of wheat and other crops (Shahid *et al*., 2012; Tahir *et al*., 2013; Gupta *et al*., 2014; Panhwar *et al*., 2014).

The data about economic analysis (Tables 8, 9) revealed that there was an overall increase in the net benefits from inoculated treatments (with 150-100 kg ha^{-1} N-P as well as 105-70 kg ha^{-1} N-P) as compared to both the non-inoculated control treatments at both the experimental sites. Among different treatments the highest net benefits (269.91 and 251.45 US$ ha^{-1} at Layyah and Multan, respectively) were obtained from the treatment where PGPR strain *Bacillus* sp. MWT-14 was used as inoculum along with 105-70 kg NP ha^{-1}. The same strain generated highest net income of 262.97 US$ ha^{-1} at Multan and 240.01 US$ ha^{-1} at Layyah with NP fertilizer dose of 150-100 kg ha^{-1}. The new innovated technology adapted on the bases their economic feasibility for the farmer (Shah *et al*., 2013) Minimum net income was produced from non-inoculated treatment with zero NP

Table 8. Variable and fixed cost for economic analysis, Multan.

Treatment	T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9	Remarks
Grain yield	2.4	2.79	2.53	3.5	3.93	3.87	4	4.4	4.2	t.ha^{-1}
Adjusted grain yield	2.16	2.51	2.28	3.15	3.54	3.48	3.6	3.96	3.78	10% less than actual to bring at farmer's level
Gross benefit (a)	636.12	739.48	670.58	927.68	1041.65	1025.74	1060.2	1166.22	1113.21	294.5 US$/ton
Straw yield	2.05	2.15	2.37	3.10	6.1	6.25	5.42	6.52	7.43	t.ha^{-1}
Adjusted straw yield	1.85	1.94	2.13	2.79	5.49	5.63	4.88	5.87	6.69	10% less than actual to bring at farmer's level
Gross benefit (b)	6.51	6.83	7.53	9.85	19.38	19.86	17.21	20.71	23.60	3.53 US$/ton
Gross benefit ©	642.63	746.32	678.11	937.52	1061.02	1045.6	1077.42	1186.93	1136.82	Gross benefit (a+b)
Cost of DAP	0	0	0	220.83	220.83	220.83	315.62	315.62	315.62	36.32 US$/bag
Cost of Urea	0	0	0	33.84	33.84	33.84	53.43	53.43	53.43	17.18 US$/bag
Cost of PGPR	0	4.91	4.91	0	4.91	4.91	0	4.91	4.91	4.91 US$/ha
Fixed cost	550	550	550	550	550	550	550	550	550	US$./ha
Cost that vary	0	4.91	4.91	254.67	259.58	259.58	369.05	373.96	373.96	US$./ha
Total cost	550	554.91	554.91	804.67	809.58	809.58	919.05	923.96	923.96	US$./ha
*Net benefit	92.63	191.41	123.19	132.85	251.45	236.02	158.37	262.97	212.85	US$./ha

T1=Non-inoculated control along with 0-0 kg ha^{-1} N-P; T2=Inoculated with *Bacillus* sp. MWT-14 and 0-0 kg ha^{-1} N-P
T3=Inoculated with *Pseudomonas* sp. LYT-1and 0-0 kg ha^{-1} N-P; T4=Non-inoculated control with 105-70 kg ha^{-1} N-P
T5=Inoculated with *Bacillus* sp. MWT-14 and 105-70 kg ha^{-1} N-P; T6=Inoculated with *Pseudomonas* sp. LYT-1 and 105-70 kg ha^{-1} N-P
T7=Non-inoculated control along with 150-100 kg ha^{-1} N-P; 8= Inoculated with *Bacillus* sp. MWT-14 and 150-100 kg ha^{-1} N-P
T9= Inoculated with *Pseudomonas* sp. LYT-1 and 150-100 kg ha^{-1} N-P

Table 9. Variable and fixed cost for economic analysis, Layyah.

Treatment	T_1	T_2	T_3	T_4	T_5	T_6	T_7	T_8	T_9	Remarks
Grain yield	2.21	2.59	2.4	3.5	4.03	3.94	4	4.34	4.2	t.ha^{-1}
Adjusted grain yield	1.99	2.33	2.16	3.15	3.63	3.55	3.60	3.91	3.78	10% less than actual to bring at farmer's level
Gross benefit (a)	585.76	686.48	636.12	927.68	1068.15	1044.30	1060.20	1150.32	1113.21	294.5 US$/ton
Straw yield	1.85	1.90	1.89	3.10	3.57	3.64	3.56	4.30	4.35	t.ha^{-1}
Adjusted straw yield	1.67	1.71	1.70	2.79	3.21	3.28	3.20	3.87	3.92	10% less than actual to bring at farmer's level
Gross benefit (b)	5.88	6.04	6.00	9.85	11.34	11.56	11.31	13.66	13.82	3.53 US$/ton
Gross benefit ©	591.64	692.52	642.12	937.52	1079.49	1055.86	1071.51	1163.98	1127.03	Gross benefit (a+b)
Cost of DAP	0	0	0	220.83	220.83	220.83	315.62	315.62	315.62	36.32 US$/bag
Cost of Urea	0	0	0	33.84	33.84	33.84	53.43	53.43	53.43	17.18 US$/bag
Cost of PGPR	0	4.9	4.9	0	4.91	4.91	0	4.91	4.91	4.91 US$/ha
Fixed cost	550	550	550	550	550	550	550	550	550	US$./ha
Cost that vary	0	4.91	4.91	254.67	259.58	259.58	369.05	373.96	373.96	US$./ha
Total cost	550	554.91	554.91	804.67	809.58	809.58	919.05	923.96	923.96	US$./ha
*Net benefit	41.64	137.61	87.21	132.85	269.91	246.28	152.46	240.02	203.07	US$./ha

T1=Non-inoculated control along with 0-0 kg ha^{-1} N-P; T2=Inoculated with *Bacillus* sp. MWT-14 and 0-0 kg ha^{-1} N-P
T3=Inoculated with *Pseudomonas* sp. LYT-1and 0-0 kg ha^{-1} N-P; T4=Non-inoculated control with 105-70 kg ha^{-1} N-P
T5=Inoculated with *Bacillus* sp. MWT-14 and 105-70 kg ha^{-1} N-P; T6=Inoculated with *Pseudomonas* sp. LYT-1 and 105-70 kg ha^{-1} N-P
T7=Non-inoculated control along with 150-100 kg ha^{-1} N-P; T8= Inoculated with *Bacillus* sp. MWT-14 and 150-100 kg ha^{-1} N-P
T9= Inoculated with *Pseudomonas* sp. LYT-1 and 150-100 kg ha^{-1} N-P

fertilizer application which was far less when compared with that was obtained from inoculated treatment with zero NP fertilizer application at both the experimental sites. Inoculation of *Pseudomonas* LYT-1 in combination with all fertilizer levels gave higher net benefits as compared to the respective non-inoculated control treatments at both the experimental locations.

***Conclusions*:** The bacterial strain *Pseudomonas* spp. LYT-1 along with different levels of NP improved the wheat productivity due to significant expansion in chlorophyll contents, crop allometric traits and along with elevated yield components. The *Bacillus* strain improved the 1000-grain weight and grain yield of wheat. Therefore these bacterial strains with NP fertilizer even at reduced level qualify for

further field testing and may be considered as promising candidates for commercial biofertilizer.

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