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Isolation and identification of phosphate solubilizer *Azospirillum*, *Bacillus* and *Enterobacter* strains by 16SrRNA sequence analysis and their effect on growth of wheat (*Triticum aestivum* L.)

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Abstract

The aim of the present study was to isolate phosphate solubilizing bacteria from wheat rhizosphere and investigate their potential for plant growth promotion. Three phosphate solubilizing bacterial strains were isolated by serial dilution method from the rhizosphere of wheat grown under wheat-cotton and wheat-rice crop rotation. 16S rRNA gene sequence of the isolates WS-1, T-34 and T-41 showed 98% similarity to those of *Azospirillum*, *Bacillus* and *Enterobacter* sequences in NCBI data base, respectively. The bacterial strain WS-1 clustered with *Azospirillum brasilense* strains in the phylogenetic tree constructed using NCBI data base sequences of the genus *Azospirillum*. The bacterial isolate T-34 formed cluster with *Bacillus licheniformis* strains in the phylogenetic tree constructed using 16S rRNA sequences of the genera *Bacillus* and *Paenibacillus*. In the phylogenetic tree constructed using 16S rRNA gene sequences of genus *Enterobacter*, the isolate T-41 clustered with *Enterobacter amnigenus* strains. Phosphate solubilizing activity of the bacterial strains in Pikovskaya medium was investigated by molybdate blue color method using spectrophotometer while organic acids produced in the medium were detected on HPLC. In a growth medium containing insoluble tri-calcium phosphate and supplemented with sucrose, maltose, glucose or galactose as single C-source, all the three isolates produced organic acids like acetic acid, citric acid and gluconic acid for phosphate solubilization. Among the organic acids detected, acetic acid was produced in highest amounts by all strains tested in the media containing different sugars. Maximum acetic acid (56.7 µg/ml) was produced by *Bacillus* strain T-34 in medium supplemented with sucrose and citric acid (36.2 µg/ml) by the same strain on glucose supplemented medium. Oxalic acid was produced by *Bacillus* strain T-34 (2.89 µg/ml) and *Enterobacter* strain T-41 (5.93 µg/ml) only in the medium containing galactose as C-source. Maximum amount of gluconic acid (25.4 µg/ml) was produced by *Azospirillum* strain WS-1 in the medium containing glucose as C-source. Highest P solubilization activity (298.3 µg/ml) was observed in *Bacillus* strain T-34 grown on sucrose supplemented medium. Maximum P solubilization was detected in *Enterobacter* strain T-41 (292.2 µg/ml) in medium containing maltose. *Azospirillum* strain WS-1 showed maximum P solubilization (218.1 µg/ml) in medium containing glucose as single C-source. All the three bacterial strains produced growth hormone IAA in the growth medium supplemented with tryptophan. Quantification on HPLC indicated maximum IAA production (31.15 µg/ml) by *Bacillus* strain T-34. A pot experiment conducted to study the effect of bacterial isolates on growth of wheat showed that inoculation with *Azospirillum*, *Bacillus* and *Enterobacter* strains increased the grain yield of wheat by 9.3%, 14.8%, 13.1%, respectively over non-inoculated control. Under field conditions, increase in grain yield of plants inoculated with *Azospirillum*, *Bacillus* and *Enterobacter* was 11.2%, 15.7% and 5.6%, respectively compared with non-inoculated plants.These results indicated that bacterial isolates having plant-beneficial traits like phosphate solubilization and IAA production and capable of improving growth of wheat when used as inoculants qualify for production of biofertilizer for wheat crop.

Keywords: insoluble phosphorous; organic acids; PGPR; plant growth hormones; rhizosphere; sugars.
Abbreviations: A.A- acetic acid; ANOVA- analysis of variance; C.A- citric acid; cfu/g soil-colony forming units per gram of soil; DAP- diammonium phosphate; G.A-gluconic acid;HPLC- High performance liquid chromatography; IAA- Indol-3-acetic acid; L.A-lactic acid; LB- Luria Bertani; LSD- Least significant difference; M.A- malic acid; N.D- not detected; OA- oxalic acid;PCR-polymerase chain reaction; ppm- parts per million; PGPR-plant growth promoting rhizobacteria; Rev/min- revolutions per minute; RCBD- randomized complete block design.

Introduction

Rhizospheric bacteria can improve plant growth by utilizing diverse mechanisms including phosphate solubilization, phytohormone production, nitrogen fixation and bio-control of plant pathogens (Bhattacharyya and Jha, 2011; Vessey, 2003). A single plant growth promoting rhizobacterium (PGPR) may possess one or more than one of these plant beneficial traits (Vessey, 2003). PGPR are important for agriculture as best alternative to chemical fertilizers due to the fact that application of PGPR as biofertilizer reduces the cost of crop production. A large number of PGPR like *Azospirillum*, *Azotobacter*, *Bacillus*, *Enterobacter*, *Pseudomonas*, *Klebsiella* and *Paenibacillus* has been isolated from rhizosphere of various crops (Saharan and Nehra, 2011; Vessey, 2003). Application of PGPR as biofertilizer has resulted in improved growth and grain yield of various crops such as wheat, rice, maize and sugarcane (Bhattacharyya and Jha 2011; Moutia et al., 2010; Saharan and Nehra 2011; Mirza et al., 2001).

Table 1. Colony morphology and 16S rRNA identification of bacteria isolated from wheat rhizosphere under different crop rotations

Isolate	16S rRNA Identification	Gram staining	Size	Motility	Colony color	Colony figure
WS-1	*Azospirillim* sp.	Negative	Short plump rods	Spiral motility	Whitish to light pink	
T-34	*Bacillus* sp.	Positive	Thin long rods	Motile	White or light brown colony	
T-41	*Enterobacter* sp.	Negative	Medium size rods	Motile	yellowish white colony	

To study the colony and cell-morphology, pure bacterial colonies were grown in LB broth medium overnight at 30°C. 20 µl of bacterial culture was examined under light microscope. For identification of bacteria, 16S rRNA gene was amplified through PCR and PCR product was sequenced.

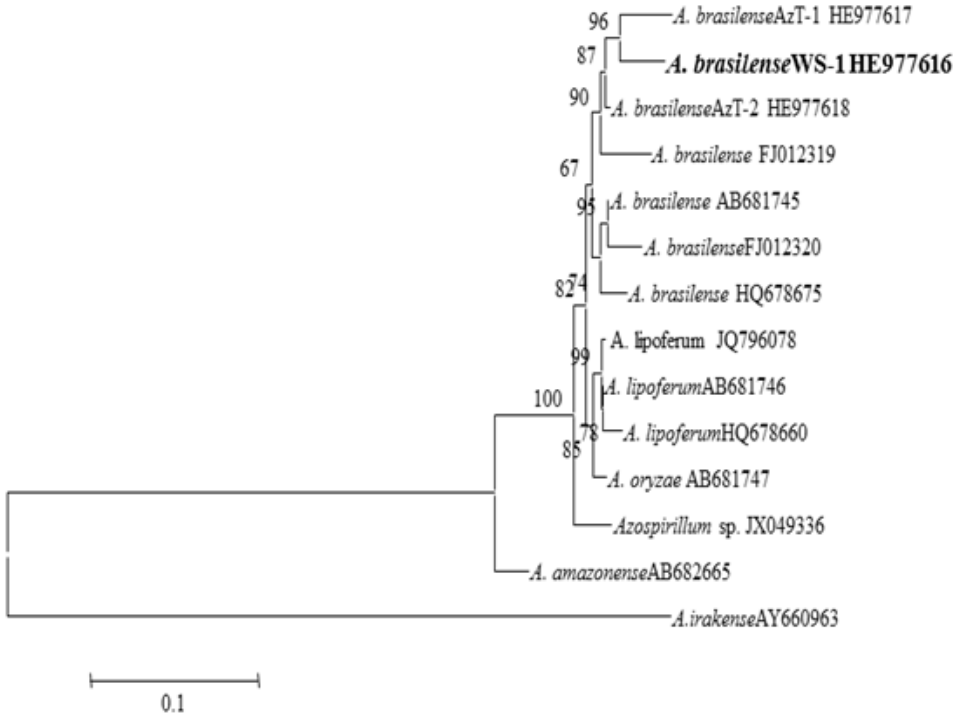

Fig 1. Amplified 16S rRNA gene fragment from the isolated strain *Azospirillum brasilense* WS-1 was sequenced and blast searched though NCBI database. Closely related sequences were downloaded and aligned using CLUSTAL X. These sequences were analyzed using maximum likelihood (ML) method. The bootstrap replicates (BS) values of 70% or greater represent well supported nodes and thus only those were retained. *Azospirillum irakense* with accession number AY660963 was taken as outgroup. *Azospirillum* strain WS-1 is present in the group containing *A.brasilense* strains.

Phosphorous is a major plant nutrient often present in soil in suboptimal concentrations. In most soils, concentration of available phosphorous is very low due to its existence as insoluble forms like phosphates of calcium and iron (Bhattacharyya and Jha 2011; Gyaneshwar et al., 2002; Park et al., 2009; Richardson et al., 2009). P-solubilization activity has been reported in many genera of PGPR like *Azospirillum, Bacillus, Enterobacter, Paenibacillus and Pseudomonas* (Bhattacharyya and Jha 2011; Goes et al., 2012; Hu et al., 2006; Perrig et al., 2007; Saharan and Nehra 2011; Vazquez et al., 2000;; Vessey 2003). PGPR carry out phosphate solubilization by secretion of organic acids for converting insoluble phosphorous into available forms (primary and secondary orthophosphates). Organic acids produced by PGPR include malic acid, acetic acid, citric acid, oxalic acid, lactic acid, formic acid, gluconic acid and 2-Keto-gluconic acid (Chen et al., 2006; Gulati et al., 2010; Park et al., 2009; Vazquez et al.,2000). In pure bacterial cultures, organic acid production has been demonstrated by utilizing different sugars like sucrose, glucose, fructose, D-xylose and manitol (Hu et al., 2006; Perrig et al., 2007; Rodriguez et al., 2004). Organic acids especially 2-ketogluconic acid, citric acid, malic acid and oxalic acid have been detected in rhizosphere of various crops and vegetables (Jaeger et al., 1999). In addition, different sugars like sucrose, glucose and maltose have also been detected in rhizosphere of wheat and other crops (Derrien et al., 2004; McRae and Monreal 2011; Moghimi et al., 1978). Phosphate-solubilizing microbes are

considered important members of PGPR and their application as biofertilizer has been shown to improve growth of cereals and other crops (Bhattacharyya and Jha 2011; Gyaneshwar et al., 2002; Hu et al., 2006; Shahab et al., 2009; Vessey 2003). Another mechanism utilized by PGPR for plant growth improvement is production of phytohormones (Saharan and Nehra 2011; Shahab et al., 2009; Vessey 2003). Plant growth hormones like auxins play a vital role in plant growth promotion by increasing root area and enhancing uptake of water and nutrients. Production of plant growth hormones like IAA is a major property of soil microorganisms including *Azospirillum*, *Azotobacter*, *Bacillus*, *Enterobacter* and *Pseudomonas* (Bhattacharyya and Jha 2011; Vessey 2003). Among these, *Azospirillum* strains are well known PGPR for producing hormone IAA in significant concentration and as a result improving plant growth (Saikia et al., 2012). The objective of the current study was the isolation of phosphate solubilizing bacteria from the rhizosphere of wheat grown under different crop rotations, as well as the identification of the isolates by sequencing their 16S rRNA gene. Furthermore, quantification of organic acids produced by these isolates utilizing different sugars was carried out along with estimation of P-solubilizing activity in these growth media. Effect of inoculation of wheat plants with these bacterial isolates was also investigated to find potential PGPR for wheat crop.

Results

Isolation and identification of bacteria on the basis of 16S rRNA gene sequence analysis

Three phosphate solubilizing bacterial isolates were obtained from rhizosphere of wheat grown in different crop rotations. Typical spirilla like cell morphology and motility (short plump rods, with spiral motility) was exhibited by the isolate WS-1. Morphology of colonies and cells of isolate T-34 and T-41 showed resemblance to *Bacillus* and *Enterobacter* spp, respectively (Table 1). Isolate T-34 was obtained from rhizosphere of wheat grown under wheat-cotton rotation (Mailsi), T-41 was isolated from the rhizosphere of wheat grown under wheat-rice rotation (Hajeera, AJK) and WS-1 was isolated from the rhizosphere of wheat grown under wheat-rice rotation (Sahiwaal). 16S rRNA gene sequence analysis of the bacterial isolate WS-1 showed 98% similarity with *Azospirillum brasilense*. The isolate T-34 showed 98% similarity with *Bacillus* sp. Sequence of the isolate T-41 showed 98% similarity with *Enterobacter amnigenus.* Phylogenetic analysis showed that bacterial isolate WS-1 clustered with *Azospirillum brasilense* strains (Fig. 1). Within genus *Bacillus*, *Bacillus* strain T-34 closely clustered with the *Bacillus licheniformis* strains (Fig. 2). The cluster was supported through high bootstrap value which suggests that this strain belongs to *Bacillus licheniformis*. Bacterial isolate T-41 was closely related to the group containing *Enterobacter amnigenus* strains (Fig. 3).

Organic acid production

We investigated utilization of sugars for organic acid production and phosphate solubilization by bacterial cultures simultaneously. Four sugars were used as single carbon source in the growth media to quantify organic acid produced by the bacterial isolates. The results suggested that *Bacillus* strain T-34 used all sugars (glucose, galactose, maltose and sucrose) and produced citric acid, malic acid, acetic acid and lactic acid in considerably higher concentrations compared

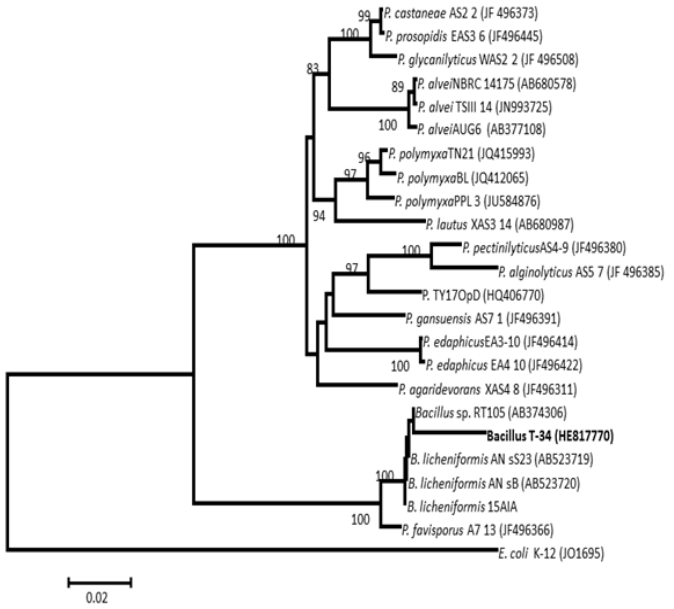

Fig 2. Amplified 16S rRNA gene fragment from the isolated strain *Bacillus* T-34 was sequenced and blast searched though NCBI database. Closely related sequences were downloaded and aligned using CLUSTAL X. These sequences were analyzed using maximum likelihood (ML) method. The bootstrap replicates (BS) values of 70% or greater represent well supported nodes and thus only those were retained. *E. coli* K-12 was taken as outgroup. *Bacillus* strain T-34 is present in the group containing *B. licheniformis* strains.

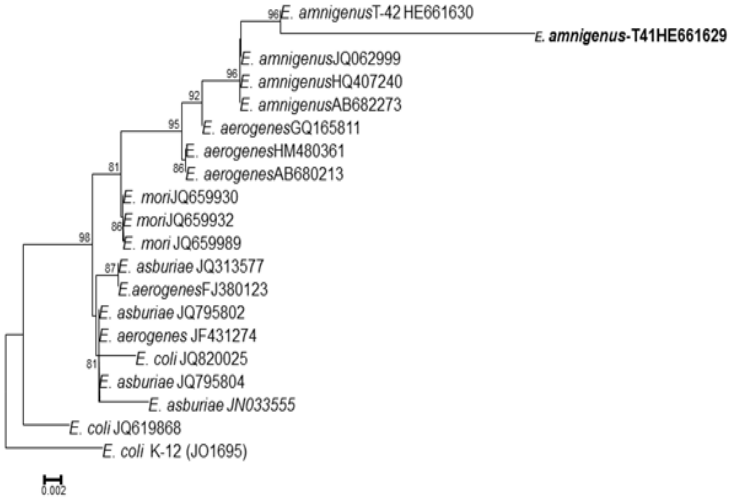

Fig 3. Amplified 16S rRNA gene fragments from the isolated strain *Enterobacter* strain T-41 was sequenced and blast searched though NCBI database. Closely related sequences were downloaded and aligned using CLUSTAL X. These sequences were analyzed using maximum likelihood (ML) method. The bootstrap replicates (BS) values of 70% or greater represent well supported nodes and thus only those were retained. *E. coli* K-12 was taken as outgroup. *Enterobacter* strain T-41 is present in the group containing *Enterobacter amnigenus* strains.

with the *Azospirillum* WS-1 and *Enterobacter* T-41 strains. All the bacterial isolates produced acetic acid, citric acid and gluconic acid on all sugars tested in the present study. In the present study acetic acid was produced in significantly higher concentration by the tested bacterial strains with all the carbon sources used (Fig.4; A, B, C and D). Among the isolates *Bacillus* strain T-34 produced maximum amount of acetic acid (56.6 µg/ml) when grown in the medium containing sucrose as a single carbon source (Fig. 4A). *Enterobacter* strain T-41 produced higher amount of acetic acid (42.5 µg/ml) on media containing maltose (Fig. 4B) while *Azospirillum* strains WS-1 produced higher amount of this acid (14.3 µg/ml) in medium supplemented with glucose as a single C-source (Fig. 4C). Citric acid was the second dominant organic acid produced by the isolates after acetic acid. Oxalic acid was produced by *Bacillus* strain T-34 (2.89 µg/ml) and *Enterobacter* strain T-41 (5.93 µg/ml) with only galactose as single C-source in the medium (Fig. 4D). In glucose supplemented growth medium, *Azospirillum* WS-1 produced highest amounts of gluconic acid (25.4 µg/ml) compared to other strains (Fig. 4C).

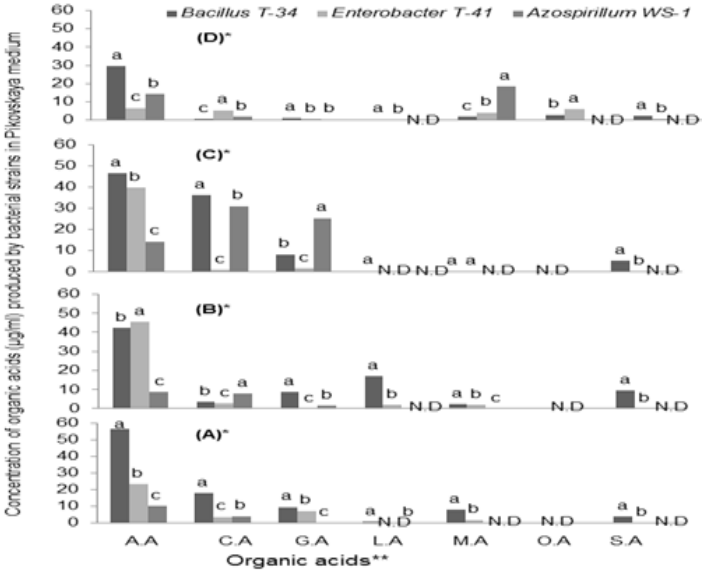

Fig 4. Organic acid production (µg/ml) by bacterial isolates grown in Pikovskaya medium supplemented with different C-sources. Bacterial cultures were grown for two weeks in growth medium containing insoluble tri-calcium phosphate and different sugars as a single carbon and energy source. The values given are an average of 3 replicates. N.D (Not detected). *Fig. 4A (organic acid production in sucrose supplemented medium), Fig. 4B (organic acid production in maltose supplemented medium), Fig. 4C (organic acid production in glucose supplemented medium), Fig. 4D (organic acid production in galactose supplemented medium). ** Organic acids produced by bacterial isolates grown in Pikovskaya medium supplemented with different sugars. Abbreviations used along X-axis are; A.A- Acetic acid; C.A-Citric acid; G.A-Gluconic acid; L.A- Lactic acid; M.A- Malic acid; O.A- Oxalic acid; S.A- Succinic acid
Small letters like a, b, c on the graph bars represent statistically different values at 5% level.

Phosphate solubilization

Maximum reduction in pH and P-solubilization was observed in *Bacillus* strain T-34 (298.3 µg/ml) and *Enterobacter* strain T-41 (258.6 µg/ml) when grown in media containing insoluble tri-calcium phosphate (Fig.5). Maximum reduction in pH and increased P-solubilization by *Azospirillum brasilense* strains WS-1 (218.1 µg/ml) was observed in the medium containing glucose as C-source.

Indol-3-acetic acid production

The isolates were also investigated for the ability to produce plant growth hormone indol-3-acetic acid (IAA) in pure culture. Bacterial strains *Azospirillum*, *Bacillus* and *Enterobacter* produced IAA in culture media containing tryptophan. Among these strains, *Bacillus* strain T-34 produced significantly higher amount of IAA (31.15 µg/ml) compared with *Enterobacter* strain T-41 (26.37 µg/ml) and *Azospirillum brasilense* strain WS-1 (7.29 µg/ml).

Grain and straw yield

To study the effect of bacterial inoculation, three bacterial strains were used as inoculants for wheat. All bacterial inoculants positively affected the plant growth and yield as compared to non-inoculated control plants (Fig.6 and 8). In pot experiment maximum increase in grain and straw yield was observed in plants inoculated with the most efficient P-solubilizing *Bacillus* strain T-34 compared to non-inoculated plants. Compared with non-inoculated control plants, increase in grain yield of plants inoculated with *Azospirillum*, *Bacillus* and *Enterobacter* was 9.3%, 14.8% and 13.1%, respectively. Under field conditions maximum increase in grain and straw yield was observed in plants inoculated with *Bacillus* strain T-34 compared to non-inoculated plants. Increase in grain yield was 11.2%, 15.7% and 5.6% in plants inoculated with *Azospirillum*, *Bacillus* and *Enterobacter*, respectively when compared with non-inoculated plants (Fig. 8). Significant variation in bacterial population (cfu/g soil) was observed in pot and field experiment at different growth stages. Bacterial population was maximum at booting stage but it decreased at harvesting stage (Fig. 9) in field experiment. In the pot experiment bacterial population (cfu/g) soil was maximum at tillering stage and it declined at maturity (Fig. 7).

Discussion

Three phosphate solubilizing bacterial isolates were obtained from rhizosphere of wheat grown in different crop rotations. Morphology of colonies and cells of isolate WS-1, T-34 and T-41 showed resemblance to *Azospirillum*, *Bacillus* and *Enterobacter* spp, respectively. On the basis of the 16S rRNA gene sequence analysis, the bacterial isolate WS-1 was identified as *Azospirillum brasilense*. The isolate T-34 and T-41 were identified as *Bacillus* and *Enterobacter* spp., respectively. Isolation of *Azospirillum*, *Bacillus* and *Enterobacter* strains from the rhizosphere of wheat, rice and sugarcane has been reported previously from the same cropping area (Mehnaz et al., 2001; Mirza et al., 2001). Production of organic acids has been implicated in P-solubilizing activity of microbes (Vazquez et al., 2000). It has been reported that different sugars like glucose, galactose, maltose and sucrose are present in exudates of wheat rhizosphere (Derrien et al., 2004; McRae and Monreal 2011;

Moghimi et al., 1978). Therefore we investigated utilization of sugars for organic acid production and phosphate solubilization by bacterial cultures simultaneously. Four sugars were used as single carbon source in the growth media to quantify organic acid produced by the bacterial isolates. The results suggested that *Bacillus* strain T-34 used all sugars (glucose, galactose, maltose and sucrose) and produced citric acid, malic acid, acetic acid and lactic acid in considerably higher concentrations compared with the *Azospirillum* WS-1 and *Enterobacter* T-41 strains. All the bacterial isolates produced acetic acid, citric acid and gluconic acid on all sugars tested in the present study. Similar results have been reported in studies (Archana et al., 2012; Chen et al., 2006; Vazquez et al., 2000) in which *Azospirillum*, *Bacillus* and *Enterobacter* produced citric acid, oxalic acid, gluconic acid and 2-Keto-gluconic acid. In the present study acetic acid was produced in significantly higher concentration by the tested bacterial strains with all the carbon sources used. *Bacillus* strain T-34 efficiently utilized sucrose as C-source and produced maximum amount of acetic acid while *Azospirillum brasilense* WS-1 strain consumed glucose for its growth as single C-source and produce acetic acid. Similar production of higher amount of acetic acid by *Bacillus* and *Enterobacter* utilizing the carbon sources like glucose and sucrose has been reported (Chen et al., 2006; Vazquez et al., 2000). Oxalic acid was produced by *Bacillus* strain T-34 and *Enterobacter* strain T-41 with only galactose as single C-source in the medium. These strains utilize galactose as single C-source and produced oxalic acid. Oxalic acid production by *Bacillus* and *Enterobacter* strains has also been reported (Vazquez et al., 2000). In glucose supplemented growth medium, *Azospirillum brasilense* WS-1 used glucose for its growth as single C-source and produced highest amounts of gluconic acid compared to other strains. Our results are in well corroboration with the findings (Bashan and de-Bashan 2010; Perrig et al., 2007; Rodriguez et al., 2004) that in medium supplemented with glucose as single C-source, azospirilla utilized glucose and secreted gluconic acid. Maximum reduction in pH and P solubilization was observed in *Bacillus* strain T-34 and *Enterobacter* strain T-41 when grown in media containing insoluble tri-calcium phosphate. It has been reported that rhizospheric bacteria secrete organic acids like acetic acid, citric acid and gluconic acid to lower the pH of medium to solubilize the phosphorous (Archana et al., 2012; Chen et al., 2006; Kumar et al., 2011; Vazquez et al., 2000; Nahas 1996). Maximum reduction in pH and P-solubilization by *Azospirillum brasilense* strain WS-1 was observed in the medium containing glucose as C-source. It has been reported that azospirilla secreted gluconic acid for phosphate solubilization (Bashan and de-Bashan 2010; Perrig et al., 2007; Rodriguez et al., 2004). Results of present study indicate that production of gluconic acid by *Azospirillum* alone is not responsible for P-solubilization rather different organic acids are accountable for lowering of pH and P-solubilization. Other studies indicated involvement of different organic acids produced by different PGPR in P-solubilization (Kpomblekou and Tabatabai 1994; Park et al., 2009; Vessey 2003). The isolates were also investigated for the ability to produce plant growth hormone indol-3-acetic acid (IAA) in pure culture as useful trait utilized by microbes in plant growth promotion (Bhattacharyya and Jha 2011; Shahab et al., 2009; Vessey 2003). In the present study, bacterial strains *Azospirillum*, *Bacillus* and *Enterobacter* produced IAA in culture media containing tryptophan as a precursor of IAA. Production of IAA in pure culture of other strains of *Azospirillum*, *Bacillus*

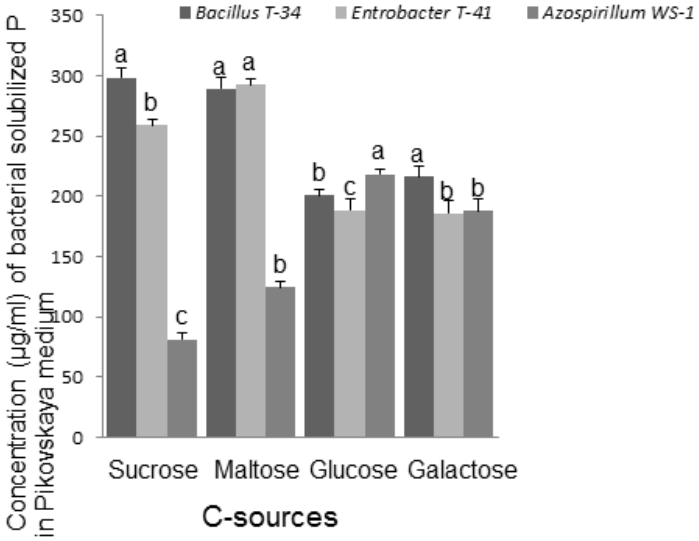

Fig 5. Phosphate solubilization (µg/ml) by bacterial isolates on Pikovskaya media supplemented with different C-sources. For P-solubilization, bacterial cultures were grown for two weeks in growth medium containing insoluble tri-calcium phosphate and different sugars as a single carbon and energy source. The values are an average of three determinations. Small letters like a, b, c on graph bars represent statistically different values at 5% level

and *Enterobacter* has been reported previously (Kumar et al., 2011; Mehnaz et al., 2001; Mirza et al., 2001; Vessey 2003). To study the effect of bacterial inoculation, three bacterial strains were used as inoculants for wheat. All bacterial inoculants positively affected the plant growth and yield as compared to non-inoculated control plants. Maximum increase in grain and straw yield was observed in plants inoculated with the most efficient P-solubilizing *Bacillus* strain T-34 compared to non-inoculated plants. Compared with non-inoculated control plants increase in grain yield in plants inoculated with *Azospirillum*, *Bacillus* and *Enterobacter* was 9.3%, 14.8% and 13.1%, respectively. Under natural field conditions but with reduced fertilizer dose, *Azospirillum* WS-1, *Bacillus* T-34 and *Enterobacter* T-41 increased the grain yield by 11.2%, 15.7% and 5.6% respectively. The results of present study indicate that inoculation of wheat with phosphate solubilizing and phytohormone producing bacterial strains improved the growth and grain yield of wheat. Several studies have reported that application of *Azospirillum*, *Bacillus* and *Enterobacter* strains as bio-inoculants improve plant growth (Kumar et al., 2011; Mirza et al., 2001; Moutia et al., 2010; Saikia et al., 2012; Vessey 2003). Significant variation in bacterial population (cfu/g soil) was observed in pot and field experiment at different growth stages. Bacterial population was maximum at booting stage but it decreased at harvesting stage in the field experiment. In the pot experiment, maximum cfu/g dry soil was detected at tillering stage and minimum at plant maturity. It has been reported that rhizosphere bacterial populations are maximum during fast growth stages of plants compared with plant maturity stage (Lucy et al., 2004; Roesti et al., 2006).

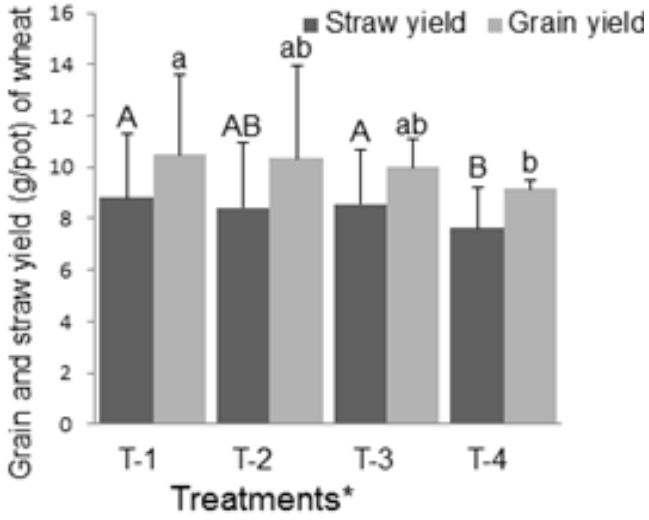

Fig 6. Effect of bacterial inoculation on straw and grain yield (g/pot) of wheat grown in earthen pots. *Treatments: T-1- *Bacillus* T-34; T-2- *Enterobacter* T-41; T-3- *Azospirillum* WS-1; T-4- Non-inoculated control. Four seedlings were maintained in each pot and there were 5 replications for each treatment. Plants were harvested at maturity Small and capital letters like A, B, C and a, b, c on graph bars represent statistically different values at 5% level.

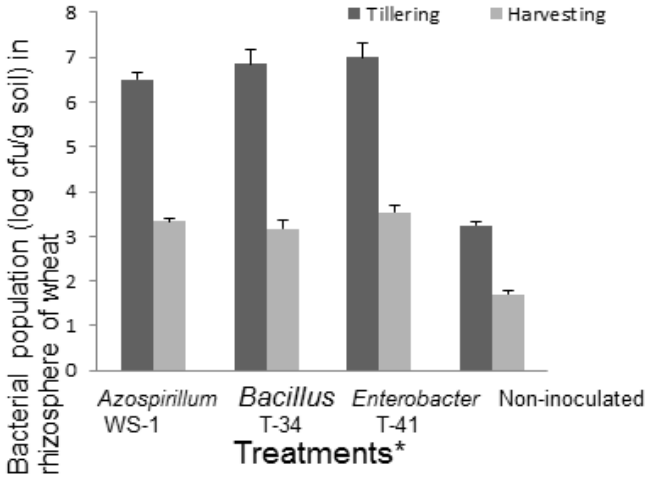

Fig 7. Bacterial population (log cfu/g soil) in the rhizosphere of wheat grown in earthen pots at different stages. *Treatments: T-1- *Azospirillum* WS-1; T-2- *Bacillus* T-34; T-3- *Enterobacter* T-41; T-4- Non-inoculated control Rhizosphere soil samples were obtained and bacterial population (log cfu/g soil) was measured by serial dilution method on LB agar medium plates. Values are the mean of three replicates.

Materials and Methods

Isolation of bacteria from wheat rhizosphere

Rhizosphere soil samples were collected by taking the soil attached with roots of wheat plants grown under wheat-rice rotation (Hajira, AJK latitude 33°46'16.58"N, longitude 73° 53'46.67"E and an elevation of 965.6 m; Sahiwal, located at 30°39′52″N73°6′30″E) and wheat-cotton crop rotations (Mailsi District Vehari, located at 29°48'1N 72°10'33E at an altitude of 126 m) by uprooting the plants carefully. The root samples along with adhering soil were stored at 4°C and used for further studies. Rhizosphere soil (1.0 g) was added to 9.0 ml saline solution (0.8%). Serial dilutions (10X) were made by adding 1.0 ml soil suspension to next tube in the series and up to 10^{-5} dilutions were prepared. 100 µl aliquots from 10^{-3}–10^{-5} dilutions were spread on LB agar medium plates (Maniatis et al., 1982). The plates were incubated overnight at 30ºC and morphologically different colonies appearing on the growth medium were selected for further purifications. Isolated colonies were streaked on fresh LB agar plates to get single-cell colonies. Bacterial growth (40 µl) from LB broth and single colonies from agar plates were examined under the microscope (Microscope, LaboPhot 2 Nikon, Japan) to record cell morphology and motility. Strain T-34 was isolated from wheat-cotton rotation; strain T-41 was isolated from wheat-rice rotation. For isolation of WS-1, 0.1 g soil with roots (1.0 mm) was added to 1.5 ml Eppendorf tubes containing 1.0 ml of semi-soild Nitrogen-free malate medium i.e. NFM (Rennie 1981). For purification and enrichment of cultures, 50 µl from each tube were transferred to fresh Eppendorf tubes containing NFM after 48 hours and the procedure was repeated up to 5-6 times. Bacterial growth was examined at each step under microscope (Microscope, LaboPhot 2 Nikon, Japan). After this, cultures were streaked on NFM solid plates to get single colonies. Single colonies were again inoculated to semi-solid NFM as well as to LB broth and LB agar plates at 30°C for 24h and used for studying colony and cell-morphology. Bacterial growth (40 µl) from LB broth and single colonies from agar plates were examined under the microscope (Microscope, LaboPhot 2 Nikon, Japan) to record cell morphology andmotility.

Identification of bacteria using 16S rRNA sequence analysis

For isolation of DNA, single bacterial colonies from LB agar plates were inoculated to LB broth (5.0 ml) and incubated overnight at 30ºC on an arbitrary shaker (150 rev/min). Bacterial cell pellets were obtained by centrifugation at 10,000 rev/min. Genomic DNA from bacterial cell pellets was then extracted using FastDNA SPIN Kit for bacteria (MP Biomedicals, USA) according to manufacture's protocol. DNA quality and quantity was measured using a Nanodrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc-USA). 16S rRNA gene of the isolates was amplified by PCR. PCR amplification was performed in a GS0001 thermocycler (Gene Technologies, Braintree, United Kingdom) in a total volume of 50 µl, containing 1.0 µl (40 ng) of DNA, 10 µl of 5X Phire reaction buffer, 200 µM of each dNTP (1.0 µl), 10 µM of each primer (2.0 µl) , 0.5 µl of Phire Hot Start II polymerase (Thermo Scientific, USA) and nuclease-free water (Promega) up to 50 µl. Primers used for 16S rRNA gene amplification were 27F (5,-GGGGTTTGATCCTG GCTCAG) and 1492R 5,-CGGCTACCTTGTTACGAC (Weisburg et al., 1991). The following PCR cycle conditions were used. First denaturation and enzyme activation at 98ºC for 2 min, followed by 35 cycles of 98ºC for 10 s, 52ºC for 10 s and 72ºC for 30 s, and a final extension step at 72ºC for 10 min. PCR reactions were analysed on 1 % (w/v) agarose

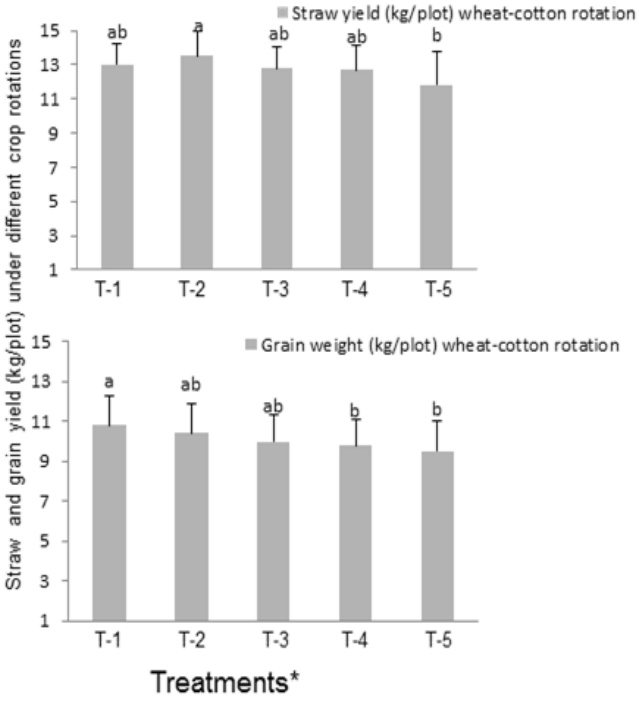

Fig 8. Effect of bacterial inoculation on straw and grain yield of wheat under field conditions.
*Treatments: T-1- *Azospirillum* WS-1; T-2- *Bacillus* T-34; T-3- *Enterobacter* T-41; T-4- Non-inoculated control (Full recommended fertilizer); T-5- Non-inoculated control (reduced fertilizer). Wheat seeds were sown in field with plot size 6 m x 3 m. Inoculated plants were fertilized with reduced nitrogen and phosphorous fertilizer. Grain and straw yield was recorded at maturity. Values are the mean of three replicates. Small letters like a, b, c on graph bars represent statistically different values at 5% level.

gel electrophoresis and visualized under U.V. light after SYBRSafe (Invitrogen) staining. PCR products were purified with High Pure PCR Cleanup Micro Kit (Roche Applied Science, USA) and sent for sequencing. Sequencing was performed by Baseclear, Leiden, The Netherlands on capillary sequencers.

Nucleotide sequence accession numbers

The 16S rRNA sequences obtained were trimmed (Bio-Edit 7.1), identified using BLAST at the National Centre for Biotechnology Information web site (www.ncbi.nlm.nhi.gov), and submitted to GenBank EMBL with accession number; *Azospirillum* WS-1(HE977616) *Bacillus* T-34 (HE817770) and *Enterobacter* T-41 (HE661629).

Phylogenetic analysis

Closely related sequences were downloaded and aligned using CLUSTAL X (Thompson et al., 1997). These sequences were analyzed using maximum likelihood (ML) method as outlined by Mirza et al., (2009). The bootstrap replicate (BS) values of 70% or greater represent well supported nodes and thus only those were retained (Hillis and Bull 1993).

Extraction and analysis of organic acids produced by bacterial isolates

Single bacterial colonies from LB agar plates were inoculated to LB broth (5.0 ml) and incubated overnight at 30ºC on an arbitrary shaker (150 rev/min). From these overnight-grown cultures, 1.0 ml bacterial cultures were inoculated to150 ml of Pikovskaya broth medium (Pikovskaya 1948) supplemented with (5.0 g/l) a single filter-sterilized sugar (sucrose, glucose, maltose or galactose) as carbon and energy source. Growth was maintained for two weeks on arbitrary shaker (150 rev/min) at 30ºC. Bacterial cultures were centrifuged at 6,000 rev/min for 6 min to get cell-free growth medium. This cell-free supernatant was collected in a new tube and 10 ml of this cell-free supernatant was concentrated to 1.5 ml in a concentrator (eppendorf Concentrator 5301, Germany) and filtered using 0.2 µm filter (Orange Scientific GyroDisc CA-PC, Belgium) for detection of organic acids. The remaining cell-free supernatant was stored at -40 ºC for the estimation of solubilized phosphorous by the bacterial cultures. Samples were analyzed on HPLC (PERKIN ELMER series 200 with 20 µl auto-sampler PE NELSON 900 series interface), using Diode-array detector at 210 nm and their UV spectra (190-400 nm), Microgaurd Cation-H Precolumn and an Aminex HPx-87H analytical column was used for separation. Sulfuric acid (0.001N) was used as mobile phase with flow rate of 0.6 ml/min. Solutions (100 ppm) of citric acid, malic acid, acetic acid, succinic acid, gluconic acid, lactic acid and oxalic acid were used as standard. Peak areas and retention time of samples were compared with those of standards for quantification of organic acids produced by bacterial isolates.

Phosphate solubilization

Quantitative analysis of phosphate solubilization by bacteria was done by molybdate blue color method (Watanabe and Olsen 1965). The cell-free supernatant stored in previous step was used for detection of phosphate solubilization. To check the drop in pH of media by the bacterial isolates due to production of organic acids, pH of this cell-free supernatant was measured with a pH meter. This cell-free supernatant was filtered with 0.2 µm filter (Orange Scientific GyroDisc CA-PC, Belgium) to remove residues. For determination of solubilized phosphate (primary orthophosphate and secondary orthophosphate) optical density was recorded on spectrophotometer (Camspec M350-Double Beam UV-Visible Spectrophotometer, UK) at 882 nm and preparation of standard curve of KH_2PO_4 using 2, 4, 6, 8, 10, 12 ppm solutions.

Indole-3-acetic acid production

For the estimation of Indol-3-acetic acid (IAA), single bacterial colonies from LB agar plates were inoculated in LB broth (5.0 ml) and incubated overnight at 30ºC on an arbitrary shaker (150 rev/min). From this overnight grown culture, 1.0 ml was inoculated in L.B medium (50 ml) supplemented with L-tryptophan (0.1 g/l) for two weeks. The cell-free supernatant of the culture medium was obtained by centrifuging the cultures at 5,000 rev/min for 7 min and the pH of the supernatant was adjusted to 2.8 with HCl (1N). Indol-3-acetic acid from the acidified culture medium was extracted with equal volumes of ethyl acetate (Tien et al., 1979), evaporated to dryness and re-suspended in 1.0 ml of ethanol. The samples were analyzed by HPLC (Varian Pro star) using UV detector and C-18 column. Methanol: acetic

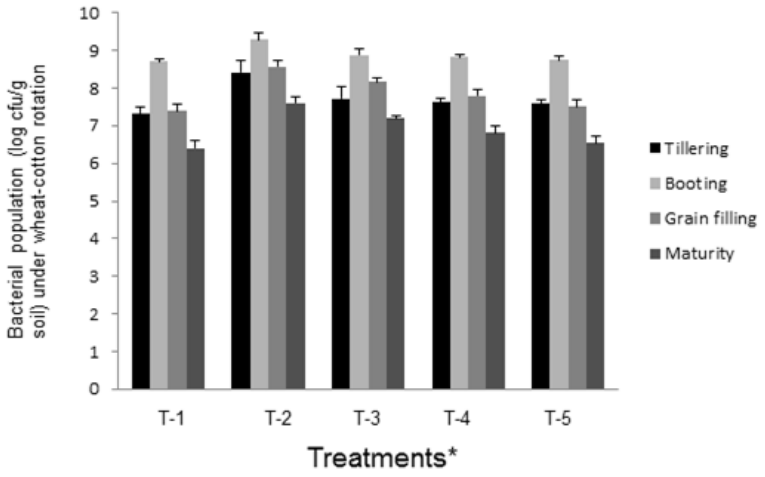

Fig 9. Bacterial population (log cfu/g soil) in wheat rhizosphere at different stages. *Treatments: T-1- *Azospirillum* WS-1; T-2- *Bacillus* T-34; T-3- *Enterobacter* T-41; T-4- Non-inoculated control (Full recommended fertilizer); T-5- Non-inoculated control (reduced fertilizer). Rhizosphere soil samples were obtained and bacterial population (log cfu/g soil) was measured by serial dilution method on LB agar medium plates. Values are the mean of three replicates

acid: water (30:1:70 v/v/v) was used as a mobile phase at the rate of 1.0 ml/min. Pure indole-3-acetic acid (1000 ppm) was used as standard. The IAA of the samples was identified and quantified by comparing the retention time and peak area with that of the standard by using computer software (Varian).

Inoculation of plants with bacterial cultures

Wheat plants of variety Sehar-2006 were grown in earthen pots containing sandy loam non-sterilized soil (EC 2.5 ds/m, pH 8.2, organic matter 0.6%, available phosphorous 7.5 mg/kg and total nitrogen 0.06%). Four plants were maintained in each pot with five replicates. Bacterial cultures were grown overnight at 30ºC in LB medium (30 ml) and centrifuged at 10,000 rev/min for 10 min. The cell pellet was washed with 0.8% saline solution and re-suspended in saline. One ml of bacterial culture (10^7c.f.u) was applied directly to partly exposed root system of each seedling after 3 days of germination. To each pot 1.2 g each of urea and DAP (equivalent to 80% of the recommended dose i.e. 250 kg/ha of DAP and 250 kg/ha urea) was applied. This fertilizer level was equivalent to 0.61 g/pot (nitrogen) and 0.44 g/pot (phosphorous).Plants were harvested at maturity and data regarding grain yield and straw yield was recorded.

The bacterial isolates were also inoculated to wheat crop under field conditions. Single bacterial colonies from LB agar plates were inoculated in LB broth (100 ml) and incubated overnight at 30ºC on an arbitrary shaker (150 rev/min). Inoculum (50 ml) with cell density of $\simeq 10^7$cfu/ml was mixed with 50 g filter-sterilized mud and seeds (0.66 kg) were pelleted with this mixture. Inoculated seeds (0.22 kg/plot) equivalent to recommended seed rate i.e. 123.5 kg/ha were sown in field plots of size 6 m x3 m. Experiment was laid out in Randomized Complete Block Design (RCBD) with three replicates.To each plot 0.47 kg each of urea and DAP (equivalent to 80% of the recommended dose i.e. 250 kg/ha of DAP and 250 kg/ha urea) was applied. This fertilizer level was equivalent to 0.39 kg/plot (nitrogen) and 0.22 kg/plot (phosphorous).Plots were irrigated four times during the plant growth. Crop was harvested at maturity and grain and straw yield was recorded. Bacterial population was estimated by taking rhizosphere soil samples from wheat grown in soil- filled earthen pots as well as field-grown plants. Rhizosphere soil (1.0 g) was added to 9.0 ml saline solution (0.8%). Serial dilutions (10X) were made by adding 1.0 ml soil suspension to next tube in the series and up to 10^{-5} dilutions were prepared. 100 µl aliquots from 10^{-3}–10^{-5} dilutions were spread on LB agar medium plates. The plates were incubated overnight at 30ºC. Colony forming units (cfu/g of dry soil) were calculated by counting the colonies and their log values were calculated.

Statistical analysis

Experiment was laid out in Randomized Complete Block Design (RCBD) with three replicates. Results were subjected to analysis of variance (ANOVA) and significance at the 5% level was tested by Least Significance Difference Test (LSD) by using a computer software program MSTATC. Mean values and the standard error were calculated.

Conclusions

All the three PGPR investigated in the present study utilized sugars reported to be present in wheat rhizosphere, produced organic acids and exhibited maximum plant-beneficial traits like P-solubilization, IAA production and improvement of growth of wheat plants with reduced fertilizer dose. Therefore these strains of *Azospirillum,Bacillus* and *Enterobacter* qualify for further field testing and may be considered promising candidates for biofertilizer production for wheat.

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