Journal of Biodiversity and Environmental Sciences (JBES)
ISSN: 2220-6663 (Print) 2222-3045 (Online)
Vol. 9, No. 6, p. 31-38, 2016
http://www.innspub.net

RESEARCH PAPER OPEN ACCESS

Characterization and identification of wheat (*Triticum aestivum* L.) rhizospheric bacteria by using 16s rRNA gene sequencing

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Article published on December 30, 2016

Key words: Chemotaxonomic characterization, PGPR, Wheat, Yield, Yield attributes

Abstract

Bacterial strains were isolated from wheat rhizospheric soil with the aim to explore potential strains for the biofertilizer technology suitable to the local climate and conditions. All the isolates were characterized for their plant growth promoting (PGP) activities such as IAA production, P-solublization, *nif*H gene presence, catalase and oxidase production. The promising and potential strains based on their PGP activities were tested in two experiments of wheat. In the first growth chamber experiment, ten strains were inoculated with the seeds and sown in small pots filled with the soil. All the strains showed positive results in yield attributes of the plant like shoot length, root length, fresh and dry weight. Further, six strains which performed best in the growth chamber experiment were tested in a pot experiment under controlled conditions and the results showed that each strain significantly increased like shoot length (7.6-25%), root length (19.7-34.1%), number of tillers (33.3-100%), fresh weight (17.8-31.3%), dry weight (19.3-48.6%), spike length (4.3-28.2%) and grain yield (7.6-41.5%) of wheat plants over control. The strains used in the pot experiment were identified by standard procedure of 16s rRNA gene sequencing which showed that two of strains were from genus *Bacillus*, two from *Pseudomonas*, one from *Acinetobacter* and one from *Jeotgalicoccus*.

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Introduction

Wheat is the main staple food for most of the population of Pakistan and largest grain source of the country. It adds 13.1 percent to the value added in agriculture and 2.7 percent to GDP. The estimated yield obtained in 2010 was 24.2 million tons (Economic Survey of Pakistan 2010-11). Average wheat yield in rainfed area is 1.5 tons per hectare which is dependent on the amount of rainfall. This is below the potential yield and the major reasons for low productivity in rainfed areas are low soil fertility, shortage of irrigation water and inefficient fertilizer use. Soils of Pot war are low in organic matter contents which affect soil fertility and soil structure badly (PARC). Therefore for sustainable agriculture, the use of plant growth promoting rhizobacteria (PGPR) has increased throughout the world. Inoculation of PGPR has reported the increase in the yield of many agronomically important field crops (Biswas *et al.*, 2000a,b; Asghar *et al.*, 2002 and Khalid *et al.*, 2003).

Plant growth promoting rhizobacteria are a group of bacteria living free in the soil or in association with plants and enhance plant growth and yield by different mechanisms of action. They may produce different hormones which stimulate plant growth, solubilize nutrients including phosphorous solubilization and iron chelation, fix atmospheric nitrogen, act as bio-control agents and improve soil structure (Hayat *et al.*, 2010). PGPRs are often termed as "biofertilizers" as they increase the availability and uptake of nutrients for the plants (Vessey, 2003). Biofertilizers are an important component of integrated plant nutrient management as they elevate the circulation of plant nutrients and lessen the need of chemical fertilizers (Rodriguez and Fraga, 1999).

These biofertilizers include bacterial strains from genera such as *Pseudomonas, Azospirillium, Azotobacter, Bacillus, Burkholderia, Enterobacter, Rhizobium, Erwiniaand Flavobacterium, Acinetobacter and Jeotgalicoccus* (Rodriguez and Fraga, 1999).

Several studies clearly demonstrated the positive and beneficial effects of PGPR on growth and yield of different crops. Increases in agronomic yield due to PGPR inoculation have been correlated to production of growth-stimulating phytohormones as well as phosphate solubilisation (Kohler *et al.*, 2006). It has been reported that wheat yield was enhanced up to 30% with *Azotobacter* inoculation and up to 43% with Bacillus inoculation (Kloepper *et al.*, 1991). Similar results were obtained when barley seed was inoculated with different PGPRs (Canbolat *et al.*, 2006). Root weight was increased by 8.9–16.7% and shoot weight by 28.6–34.7% over the control. According to Wu *et al.* (2005) microbial inoculum *Bacillus megaterium* and *Bacillus mucilaginous* increased the plant growth as well as improved nutritional assimilation of plant (total N, P, and K). Egamberdiyeva (2007) inoculated maize with bacterial strains *Pseudomonas alcaligenes*, *Bacillus polymyxa*, and *Mycobacterium phlei* and reported a significant increase in root dry weight (19–52%) and total dry matter of maize was increased up to 38%. There was 15 % increase in wheat yield due to inoculation of PGPR as reported by Chen *et al.* (1996). Keeping in view the beneficial effects of PGPR, a lab study was conducted in to isolate the bacterial strains from wheat rhizosphere, identified them and evaluated their efficiency as PGPR on growth of wheat in growth pouches under controlled conditions in growth chamber.

Material and methods

Isolation, screening and phenotypic characterization of soil bacteria

Bacterial strains were isolated from wheat rhizospheric soil obtained from Abdullah Shah Ghazi Bukhari, Attock (33°14'26.38" N and 72°23'10.29" E)which has sandy loam soil. Isolation of the stains was carried out by dilution plate technique using phosphate buffer saline as a saline solution. Tryptic Soya Agar (TSA; Difco) medium was used and the plates used for isolation were incubated at 28°C for 48-72 hours depending upon the growth of the bacterial colonies.

Then single bacterial colonies were picked and streaked on TSA medium plates with the aim to achieve single colonies. When single colonies were achieved, these were preserved in 35% glycerol (w/v) at -80°C and further characterization was carried out. The morphological characteristics of the strains were observed and noted using light microscope (Olympus BX 60).

Plant growth promoting assay

Plant growth promotion activities like IAA production, phosphorus solubalization and presence of nifH gene were evaluated following different standard procedures. For IAA production, bacterial cultures were grown in Tryptic Soya Broth (TSB) at 28±2 °C for 24-36 hours. Then 100 uL of this bacterial suspension was inoculated in 5 ml of autoclaved Luria Broth medium (LB medium) with and without L-tryptophan. L-tryptophan was added 500 µg ml^{-1} in each test tube. These test tubes with the LB medium and bacterial suspension were allowed to grow at 28±2 °C in an incubator shaker for 48 hours. After 48 hours, these cultures were centrifuged at 10000 rpm for 10 minutes. 2 ml supernatant was then mixed with two drops of Orthophosphoric acid and 4ml of the Salkowski reagents (50ml, 35% of Perchloric acid, 1ml 0.5M $FeCl_3$ solution) and the optical density was determined at 530 nanometer using spectrophotometer. Development of pink color was an indicator of IAA production. IAA production by strains was measured by standard curve graph where standards range was upto 10µg ml-1 (Brick *et al.*, 1991).

P-solublization was determined qualitatively by measuring the halo zone formation by bacteria on Pikovskaya (PKV) agar medium (Pikovskaya, 1948; Gaur, 1990) and the P-solublizer strains were further evaluated quantitatively. Quantitative P-solubalization was measured by inoculation of bacterial broth culture in a 250 ml flask containing 100 ml of PKV medium and 5 g L-1 of insoluble tri-calcium phosphate. The pH of the media was adjusted at 7.0 and was autoclaved at 121°C for 15 minutes.

After the inoculation of bacterial culture, the flasks were placed in the incubator shaker for 8 days at 30°C. The pH of the media was measured followed by centrifugation at 8500 rpm for 25 min. The supernatant was measured for available phosphorus by the protocol given by Watanabe & Olsen (1965). The optical density of the supernatant was determined at 700 nm using spectrophotometer and the values were determined by standard curve graph and standards range was up to 1 µg ml^{-1}.Nitrogen fixing characteristic of the bacterial strains was checked by amplification of nifH gene by PCR amplifications. Universal forward and reverse primers PolF[b] (TGC GAY CCS AAR GCBGACTC) and PolR[b] (ATSGCCATCATYTCRCCG GA) were used, respectively.

Identification of bacterial strains using 16SrRNA gene sequencing

Standard method of 16S rRNA gene sequencing was used to identify the strains. Singe colony of each strain was picked and DNA template was prepared followed by 16S rRNA amplification with help of PCR. Universal primers 9F (5́-GAGTTTGATCCTGGCTCAG-3́) and 1510R (5́-GGCTACCTTGTTACGA-3́) were used for PCR amplification. For the 16S rRNA gene amplification, the reaction mixture (25 µL) was prepared by initial denaturation for 2 minutes at 94°C. Further,30 cycles of denaturation at 94 °C for one minute was carried out followed by primer annealing at 52°Cfor half a minute. After annealing, the primer extension was carried out at 72°C for 2 min and at last final extension at 72°C for 10 min in a thermo cycler.

Amplified PCR products of 16S ribosomal gene were separated on 1% agarose gel in 0.5X TE (Tris-EDTA) buffer containing 2 µL ethidiumbromide (20 mg/mL). λ Hind-III ladder was used as a size marker. The gel was viewed under UV light and photographed using gel documentation system. Amplified PCR products of full-length 16S rRNA genes were purified using PCR purification kit (QIAGEN) according to the standard protocol recommended by the manufacturer.

The purified PCR product samples were sequenced using DNA sequencing service of MACROGEN, Korea (www.dna.macrogen.com/eng) using universal 16Sr RNA gene sequencing primers. The sequence results were blast through NCBI/Eztaxon (Chun *et al.*, 2007) and sequence of all the related species were retrieved to get the exact nomenclature of the isolates. Phylogenetic analyses were performed using bioinformatics software MEGA-5 (Tamura *et al.*, 2007). Other software used for sequence alignment and comparisons were CLUSTAL X and Bio-Edit. DNA accession numbers of each strain were obtained from DNA Data Bank of Japan (DDBJ).

Wheat inoculation

Among all the isolates, potential strains on the basis of PGP tests were selected for evaluation of their effect on wheat crop. Initially ten strains were selected and were inoculated in the growth chamber experiment (controlled conditions) which was conducted in small pots having sterilized soil. Un-inoculated pots were treated as control. There were 11 treatments including the control with four replications with CRD as the experimental design as the experimental units were homogeneous. Plant parameters like shoot length, root length, fresh and dry weight (oven dried at 65 ^{o} C for 24 hours) were measured after a month of germination and most promising six strains were further evaluated in pot experiment using CRD design. There were seven treatments in the pot experiments including the un-inoculated treated as control with four replications. Plant yield and yield attributes like shoot length, root lengths, number of tillers, fresh weight (gm/plant), dry weight (gm/plant), spike length were measured at the harvest maturity of the crop. The obtained data was analyzed statistically by ANOVA and the means were compared using LSD test with significance level of ≤0.05 (Steel *et al.*, 1997).

Results and discussion

Among all the strains isolated from wheat rhizosphere, the potential strains which produced maximum indole acetic acid and solubilized phosphorus were checked for growth enhancing parameters on wheat crop in two experiments.

Table 1. Impact of Inoculum on plant growth (Growth chamber experiment).

S. No.	Strain ID	Treatment	Shoot length (cm)	Root length (cm)	Fresh weight (gm)	Dry weight (gm)
1	Control	T_1	15.9±2.23	5.13±0.93	1.47±0.46	0.76±0.15
2	RW-45	T_2	24.8±3.15	7.54±1.03	2.17±1.21	1.24±0.45
3	RW-14	T_3	22.1±2.96	7.69±1.13	3.99±1.94	2.09±1.09
4	RW-46	T_4	24.4±3.49	10.25±2.21	3.75±1.90	2.14±1.11
5	RW-43	T_5	24.7±3.41	10.81±2.06	2.99±1.14	1.68±1.34
6	RW-17	T_6	22.3±3.06	8.96±1.04	2.64±1.19	1.37±1.22
7	RW-34	T_7	23.2±3.45	13.37±3.17	4.01±3.54	1.98±2.06
8	RW-13	T_8	21.9±3.55	10.34±2.03	2.40±1.09	1.16±1.06
9	RW-35	T_9	25.7±3.97	11.25±1.45	2.53±1.57	1.35±1.17
10	RW-33	T_{10}	22.9±3.19	9.68±1.24	3.23±2.06	1.81±1.31
11	RW-42	T_{11}	27.7±4.09	12.91±2.19	4.26±2.19	2.29±1.09

The first experiment was carried out in growth chamber in controlled conditions for one month and ten strains were inoculated with seed and sown. All the parameters taken showed the positive results with increase in shoot length, root length, fresh and dry weight of plants (Table 1). Significant increase in shoot length was observed in all the treatments over control.

Maximum increase was observed in T11, which gave 74% increase in shoot length followed by 61%, 56%, 55%, 53% by T9, T2, T5 and T4, respectively. Khalid *et al.*, 2003 observed 70.5% increase in shoot length of wheat by application of a strain isolated from wheat rhizosphere. This increase in the shoot length can be due to release of metabolites by PGPRs (Van Loon, 2007) and mineralization of nutrients which are easily available for plants.

An increase of 159% in root length was observed by T7 followed by 151%, 119%, 110% and 100% by T11, T9, T5 and T8, respectively when compared to control (Fig. 1). Shaharooma *et al.*, 2008 reported that there is significant increase in root length due to application of PGPRs; they also state that phyto-hormones production by PGPRs can be major cause of increase in root length of plants. Increase in fresh and dry weight of the plants was observed in all the treatments over control.

Table 2. Impact of Inoculum on plant growth (Pot experiment).

S. No.	Treatment	Strain ID	Shoot length (cm)	Root length (cm)	No. of Tillers	Fresh weight (gm/plant)	Dry weight (gm/plant)	Spike length (cm)	Grain yield (kg/ha)
1	T_1	Control	71.26±7.16	16.56±2.35	3	17.72±2.54	6.64±1.23	6.70±0.99	2900.43±203.15
2	T_2	RW-46	76.67±8.16	19.82±3.15	4	21.98±4.19	8.63±2.65	7.55±1.27	3119.82±249.25
3	T_3	RW-43	82.49±9.68	20.97±4.15	4	20.87±2.15	7.92±1.19	6.99±1.09	3158.98±293.65
4	T_4	RW-45	76.09±8.49	21.87±4.58	5	22.52±2.19	8.32±2.19	7.19±2.14	3612.36±353.98
5	T_5	RW-35	84.82±8.98	21.56±4.19	4	22.55±2.48	8.36±2.22	7.51±2.06	3906.76±393.65
6	T_6	RW-42	89.05±9.58	22.21±5.16	6	23.27±3.78	8.66±2.14	8.59±2.58	4103.22±416.35
7	T_7	RW-34	85.46±8.47	21.57±4.78	5	22.48±2.98	9.87±3.09	7.85±2.77	3705.54±347.85

The maximum increase in fresh weight was 189% by T11 followed by 172%, 171%, 155% by T7, T3 and T4. Pras hant D *et al.*, 2009 reported an increase in dry weight of wheat plants by application of PGPRs.

The second experiment was carried out in plastic pots in glass house (Table 2). Six strains which showed maximum growth in the first experiment were selected and inoculated with seed. The data taken at harvest showed positive results in every parameter by all the PGPRs application over control. The results correlate significantly with findings of Saber *et al.,* 2012.Shoot length was measured at the time of harvest.

The results showed that T6 had an increase of 25% in shoot length over control followed by T7, T5 and T3 which showed an increase of 20%, 19% and 16% respectively. There was significant increase in shoot length of wheat plants sue to application of PGPRs when applied with combination of chemical fertilizers and composts (Akhtar *et al.*, 2009). Along with the increase in shoot length, there was increase in root length too. An increase of 34% was observed in root length by inoculation of T6 in the pot experiment followed by 30% by T5 and T7. The root length was increased significantly when different rhizobacteria were inoculated on wheat plants in the pot experiment (Khalid *et al.*, 2004).

The increase in the root length can be due to the interaction of PGPRs with the roots and increased cell division in the root tips (Levanony and Bashan, 1989). Similar results have been observed in maize (Kapulnik and Okon, 1983) and tomato (Hadas and Okon, 1987).

The PGPRs also had positive effect on the number of tillers as reported by Afzal *et al.*, 2005. A 100% increase in number of tillers plant $^{-1}$ was showed by T6 followed by 66% increase by T4 and T7 and 33% by T2, T3 and T5. Sisie *et al.*, 2011 reported an increase of 25% in number of tillers in wheat by the application of PGPRs. The production of IAA by the rhizobacteria has been discussed as the cause of increase in tillers of the plant but still this factor cannot be the only one reason. The other reason can be the general growth enhancement of the plants by PGPRs Assuero and Jorge, 2010.

Fresh and dry weight plant $^{-1}$ is a major parameter to determine the growth of the plants. Inoculation of PGPRs significantly enhanced the fresh and dry weight of the plants. Maximum increase in fresh weight plant $^{-1}$ was observed by T6 which was 31% more than control. An increase of 27% in fresh weight was observed by T4 and T5 followed by 26% by T7 and 24% by T1.

Dry weight of the plants also showed similar results to the fresh weight with only one variation as maximum increase was showed by T7 followed by T6 and T2. Increase in dry weight of the wheat plants was stated by Egamberdieva, 2010, Narula *et al.*, 2006. Spike length was also measured and was found correlated to other parameter findings. T6 exhibited longest spikes as compared to all other treatments.

Fig. 1. Impact of inoculum application on Plant height (cm).

There was 28% increase in the length of the spikes in the T6 when compared with the control. T7 showed 17% increase, followed by 12% by T2 and T5. Afzal and Asghar, 2008 and Agamy *et al.*, 2012 reported increase in length of the spike by application of PGPRs. Grain yield of the wheat plants was positively influenced by application of PGPR strains.

All the strains showed significant increase over control. Maximum increase in grain yield was observed by T6 which was 41% more than the control. Other treatments had 34%, 27% and 24% increase n grain yield by T5, T7 and T4 respectively. Increase in grain yield of wheat plants due to application of PGPRs has been stated by Faramarzi *et al.*, 2012, Khalid *et al.*, 2004 and Ozturk *et al.*, 2003. As the number of tillers plant $^{-1}$ were increased by application of PGPRs so it can have effect on grain yield.

Acknowledgement

The research article is a part of Ph.D. research work, and the first author profoundly acknowledges the full cooperation of supervisory committee and their valuable suggestions during the whole experimental durations. Financial support from the Higher Education Commission Pakistan (PhD. Indigenous Scholarship) is also greatly acknowledged.

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