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Isolation and Characterization of Plant Growth Promoting Rhizobacteria (PGPR) and Their Effects on Improving Growth of Wheat

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Abstract

Plant Growth Promoting Rhizobacteria (PGPR) is commonly used as an inoculants for improving the growth and yield of agricultural crops. So, screening for the selection of effective PGPR strains is very critical. In this study, 180 bacteria were isolated from the rhizosphere of different wild plants collected from the vicinity of Erzurum and Kırsehir, Turkey. These isolates were tested for their nitrogen fixing (NF) and Phosphate Solubilizing (PS) capacity. Sixteen isolates were found to have both NF and PS potential at different levels and designated as AS1, AS2, AS3…AS16). These 16 isolates were also analysed in their auxin (IAA) and ACC deaminase production potentials. To investigate the effects of PGPR isolates on the growth of spring wheat (Triticum aestivum L.) a pot and a field experiment was conducted. According to the results of pot experiments only the most effective six isolates were tested at field trials and after harvest, some growth and yield parameters were analysed. Results showed that 6 isolates (AS1, AS3, AS4, AS6, AS8 and AS15) have significant ($P<0.05$) positive effect on plant height, dry matter and protein content. On the basis of some cultural, cytological characteristics and fatty acid profiles, these effective isolates were identified as Cellulomonas turbata (AS1), Pseudomonas putida (AS3), Bacillus cereus (AS4), Enterobacter cloacae (AS6), Bacillus megaterium (AS8) and Bacillus megaterium (AS15). As a result, it can be said that inoculation of wheat with this strains may lead both the increases in yields and substitute costly nitrogen-phosphate fertilizer in wheat production.

Keywords: Plant Growth Promoting Rhizobacteria (PGPR), Phosphate solubilization, nitrogen fixation, IAA production, ACC deaminase production, wheat

INTRODUCTION

It is well known that rhizosphere contains many useful microorganisms for plant growth. In both managed and natural ecosystems beneficial plant associated bacteria play a key role in supporting and/or increasing plant health and growth. Rhizospheric bacteria are involved in various biotic activities of soil ecosystem to make it dynamic for nutrient turnover and sustainable for crop production. Rhizobacteria (root colonizing bacteria) that exert the beneficial effects on the growth of the host plant via direct or indirect mechanisms are termed as plant growth promoting rhizobacteria (PGPR).

Strains of PGPR use one or more direct or indirect mechanisms to enhance the growth and health of plants. It was reported that PGPR directly enhance plant growth by a variety of mechanisms such as fixation of atmospheric nitrogen, solubilization of phosphorus and other minerals, production of siderophores and planth growth hormones [1-5].

It was reported that plant beneficial rhizobacteria may decrease the global dependence on hazardous agricultural chemicals such as fertilizers which destabilize the agro-ecosystems [6]. Various species of bacteria belonging to the genera of Azospirillum, Pseudomonas, Klebsiella, Azotobacter, Enterobacter, Alcaligenes, Bacillus, Burkholderia and Serratia have been reported to enhance the plant growth [7, 8].

Because of the adverse effects of chemical fertilizers on environment, bio-fertilizers being essential components of organic farming play vital role in maintaining long term soil fertility and sustainability. One of the most important components of biofertilizers is the members of PGPR. Besides above facts, the long term use of biofertilizers containing PGPR is economical, eco-friendly, more efficient, productive and accessible to marginal and small farmers over chemical fertilizers [9, 10]. So, use of microbial inoculants of PGPR for the enhancement of sustainable agricultural production is becoming a more widely accepted practice in intensive agriculture in many parts of the world [11].

The survival of inoculated PGPR in the plant rhizosphere is in most cases a precondition for potential plant stimulation effect during the vegetation time or at least during early plant development [12, 13]. Knowledge of plant growth promoting effects and the survival of PGPR at different regions have adverse climatic conditions such as high altitude, low oxygen pressure and low temperature may be important for successful root inoculation. Indigenous isolates may be preferred in the selection of PGPR for inoculation of crop plants as they are adapted in the environment and can be more competitive than the non-indigenous bacterial flora [14, 15]. Erzurum is an important city and plateau situated 1957 meters above sea level in Eastern Anatolia, Turkey. Approximately 18.5 % of total surface area of Erzurum is arable land. A large portion of the agricultural produce consists of cereals. Furthermore, the lands in Erzurum and North Eastern Anatolia have managed to stay intact and chemical free. The fact that the ecological system of this region is intact creates an environment that has a high potential for organic agriculture. Both Erzurum province and Eastern Anatolia and other similar regions with high altitude are characterized by short growing seasons and cold climatic conditions. So, if a bacterium will be used as inoculant fort the growth of crops in this region it must be competitive and resistant to these adverse conditions. Therefore 180 bacterial isolates from the rhizosphere of different wild plants collected from the regions with high altitude were isolated. They were screened for their plant growth promoting characteristics. Six of them were found to be effective isolates and the effects of inoculation with these six indigenous bacteria on growth and yield of wheat were investigated under greenhouse and field conditions.

MATERIALS and METHODS

Plant material

Triticum aestivum cv. Kirik, predominant wheat cultivar in the region, obtained from the Eastern Anatolia Agricultural Research Institute, Erzurum, was used as the plant material in the experiments.

PGPR strains and culture conditions

One hundred and twelve soil samples were collected from the mountains of Erzurum and Kırsehir province in Turkey. Soil samples (approximately 300 g) were taken from the rhizosphere of different wild plants at the depth of 15 cm and brought immediately to laboratory, Department of Biology, Ataturk University, Erzurum. Soil samples were maintained in refrigerator at 5 °C. Soil suspensions were prepared in sterilized water under aseptic conditions and used to inoculate Nutrient Agar (NA, Oxoid) medium. One hundred and eighty bacterial isolates having different morphological appearance on agar medium were selected and stored. All of the 180 isolates were tested for their nitrogen fixing and phosphate solubilizing activities.

Phosphate Solubilization

Fifty microliter inoculum (approximately 1-2 × 109 cfu/mL) was transferred in the glass tubes containing on mL of sterilized and brom phenol supplemented National Botanical Research Institues's Phosphate Growth Medium (NBRIP-BPB). This medium contained (g/L): 10 glucose, 5 Ca3(PO4)2 , 5 MgCl2 , 0.25 MgSO4 , 0.2 KCl , 0.1 (NH4)2SO4 and 0.025 BPB (brom phenol blue). The pH of the medium was adjusted to 7.0 before autoclaving. Uninoculated medium served as control. The tubes were incubated at 30 °C in shaking incubator at 180 rpm for 3 d. At the end of incubation period tubes were centrifuged at 5000 rpm for 10 min. Absorbances of obtained supernatants were assayed at 600 nm using a spectrophotometer [16,17]. Soluble phosphate was estimated by Vanadomolybdate Method and expressed as equivalent phosphorus (mg/L) [18].

Acetylene Reduction assay (ARA)

Nitrogen fixation was determined in nitrogen free medium by ARA [19]. For this purpose cultures were prepared according to Holguin and Bashan [20] and incubated at 30 °C for 48 h without agitation. Ethylene production was measured using Hewlett Packard Gas Chromatograph (model 6890, USA).

Sixteen of 180 isolates were found to be have both nitrogen fixation and phosphate solubilization activity. Further studies were done with this sixteen isolates.

Identification of bacterial isolates

After isolation procedures, selected isolates were identified according to fatty acid methyl ester (FAME) analysis. FAMEs were separated by GC with a fused-silica capillary column (25 m x 0.2 mm) with cross-linked 5% phenyl methyl silicone. The FAME profile of the bacterial strain was identified by comparing the commercial databases with the MIS software package [21].

Auxin Production

Indole 3-acetic acid (IAA) production was determined according to the method of Brick et al. [22]. For this purpose cultures of the isolates were grown in Luria Bartani (LB) broth amended with 500 µg/mL of tryptophan at 27 °C for 120 h at 200 rpm. After incubation period cells were removed by centrifucation at 5000 rpm and supernatant was assayed for IAA production.

ACC(1-aminocyclopropane-1carboxylic acid) deaminase activity

ACC deaminase activity of cell free extracts was determined by estimating the amount of α ketobutyrate (α-KB) generated by the enzymatic hydrolysis of ACC according to the method of Honma and Shimomura [23].

Plant growth promotion

Plant growth promotion capacities of isolates were tested in both greenhouse and field experiments.

Bacteria were grown in nutrient broth (NB) medium for 24 h and diluted with sterile distilled water to a final concentration of 108 cfu/mL [24]. Wheat seeds were placed in culture suspensions for 30 min before sowing.

Pot experiments were carried out in the greenhouse with wheat cultivar Kirik. Sixteen indigenous isolates were used for pot experiments. The pots containing uninoculated seeds were used as control. All of the pots were distributed in a completely randomized design. The soil was taken from the uncultivated region of the campus of Ataturk University. This soil was filled in eight liter pots and eight seeds were sown in each pot and thinned to five plants per pot after the full emergence of the first leaf. The pots were regularly irrigated to maintain a proper moisture level [24].

A private field, uncultivated for five years, in Pusudere Village, Pasinler was used for field experiments. Some characteristic of the soil of this field were as follows: organic matter content 1.32 %; available P and K contents 14.9 and 455 kg/ha, respectively, pH 7.51.

Two years replicated field trials were conducted. Field experiment consisted of 8 treatments and two replications. Six of treatments were selected isolates (AS1, AS3, AS4, AS6, AS8 and AS15) according to results of pot experiments; 1 was N fertilization (4kg/da ammonium sulphate) and 1 was control (without inoculation and N fertilization). Plots were distributed in randomized complete block design. Plants were irrigated at the beginning of stem elongation, heading and milky ripening stages. Harvesting was done on the 5th of september in both years.

RESULTS and DISCUSSION

Isolated PGPR and their characteristics

As mentioned before cultivable lands of both Erzurum and other similar regions are characterized by cold climatic conditions and short growing seasons. If an inoculant will be used as a biofertilizer agent in such area it must be resistant to cold and more competitive. Based on the idea that bacteria isolated from the environments with adverse conditions (with high altitude, low oxygen pressure and cold temperature) may be more competitive and resistant as inoculants, we isolated bacteria from the different habitats of mountains except for AS1, AS2, AS10 and AS14 which were isolated in our previous study and tested as inoculants for chickpea [25].

Many researchers have reported that PGPR are able to exert a beneficial effect upon plant growth and N2 fixing and P-solubilizing bacteria may be important for plant nutrition by increasing N and P uptake by the plants [2, 26, 27]. So, we tested 180 bacteria for their N2 fixing and

P-solubilizing activity. As a result 16 of 180 isolates were found to have both NF and PS activity and these isolates were identified using FAME profile analysis and shown in Table 1. Of the N2 fixing and P-solubilizing 16 bacteria 12 were from different mountains (5 Palandoken Mountain, 4 Cicekdagi Mountain, 2 Kervansaray Mountain and 1 Baranli Mountain) and other 4 species from the localities with lower altitude. According to the results of FAME identification, among the isolated bacteria 9 (AS2, AS9, AS13, AS15, AS4, AS8, AS12, AS16 and AS15) were belong to group of Firmicutes, 4 (AS3, AS6, AS11 and AS10) to group of Gammaproteobacteria, 1 (AS1) to group of Actinobacteria and 1 (AS5) to group of Proteobacteria. AS7 could not be identified by FAME analysis. Our results also indicated that among the isolated bacteria the most effective isolates in their plant growth promoting traits were belong to the genera of Bacillus, Pseudomonas and Cellulomonas. Bacillus was the most predominant genus (56.25 %). This is an expected situation because aerobic endospor formers have long been considered to be important components of soil bacterial community. Many researchers have also reported that the members of Bacillus are often identified as dominant taxa of cultivable microbial populations from rhizosphere of various crop plants [4, 28, 29]. Furthermore endospor forming bacteria are preferred as inoculants as they are adapted in the environment and can be more competitive than the other rhizobacteria.

In recent years, it has been found that some rhizobacteria contain an enzyme ACC-deaminase that hydrolysis ACC into ammonia and α-ketobutyrate and decreases the amount of ACC, as well as ethylene, outside the germinating seeds, thereby acting as a sink for ACC [30]. On the other hand Glick et al. [31] and Yuhashi et al. [32] have reported that decreased levels of ACC results in lower levels of endogenous ethylene, which eliminate the potential inhibitory effects of higher ethylene concentrations. Furthermore, ethylene is also known to effect several aspects of root development and nodule formation including its action as an inhibitor of nodulation [33, 34]. Again, one of the mechanisms of plant growth stimulation in environments could be bacterial production of phytohormone IAA [36]. Because of above mentioned reasons, in this study ACC deaminase and IAA production capacities of bacteria were also determined and results were given in Table 2.

As seen in Table 2, all of the isolates were able to reduce acetylene (have nitrogenase activity). Phosphate solubilization activity was also exhibited by all isolates. The best isolates for NF and PS activities were AS2 and AS1, respectively. In both NF and PS activities of isolates there were significant ($P<0.05$) differences in comparison with their corresponding controls. Except for AS12, AS15 and AS16, all of the other 13 isolates had the capacity to produce IAA. Again, AS1, AS2, AS3, AS5, AS6 and AS9 also showed ACC deaminase activity. Maximum IAA and ACC deaminase producers were AS13 (118.55 μg/mL) and AS3 (32.92 μM/mg/h), respectively.

Plant growth promoting potentials of isolated bacteria

Plant growth promoting potentials of isolated bacteria were tested in both pot experiments and field trials. Results were given Table 3 and Table 4.

As seen in Table 3, the growth of wheat was influenced by bacterial inoculation in pot experiments. AS1 (Cellulomonas turbata), AS3 (Pseudomonas putida), AS4 (Bacillus cereus) and AS8 (Bacillus megaterium) were the most effective strains and they stimulated shoot growth of wheat as 24.32 %, 20.94 %, 13.51 % and 33.78 %, respectively. These four isolates enhanced the growth in all measured parameters. Maximum root elongation (136.44 mm) was also obtained from the AS8 inoculation (25.92 % over control). The effects of inoculation on the growth parameters were found to be statistically important ($P<0.05$). According to the results of pot experiments the most effective six strains (AS1, AS3, AS4, AS6, AS8 and AS15), which were found to be effective on growth at least measured two parameters, were chosen and field trials were conducted with these strains.

According to the results of pot experiments inoculation of wheat seeds with the isolates of AS2, AS5, AS7, AS9, AS10, AS11, AS12, AS13, AS14 and AS15 reduced the shoot length. These ineffective isolates also caused important decreasing in other growth parameters, except for AS9 (in root length) and for AS6 (in dry weight). These negative effects on different growth and yield parameters might be due to production of some kind of phytotoxins that inhibited the growth of inoculated plants [1, 36].

Results of field experiment showed that although growth yields obtained from the PGPR inoculants were lower than that of nitrogen application, inoculation significantly ($P<0.05$) enhanced both biomass and grain yields and protein content. Strain AS8 provided 18.27 % and 17.58 % biomass and grain yields, respectively, more than uninoculated control. All of the other tested bacteria provided significant increments in both biomass and grain yields which ranged from 10.07 to 16.11 % and 9.34 to 15.38 %, respectively. AS8 also enhanced the protein content.

In our pot and field experiments, it was observed that inoculation with the isolates of AS1, AS3, AS4, AS6, AS8 and AS15 significantly promoted growth and yield of wheat under non axenic conditions. On the other hand, inoculation resulted in early seedling growth and development in pot experiments. Similar findings were also reported by Dobbelaere et al. [37]. Generally, there was a positive correlation between the increments in all measured parameters in pot experiments. We also observed a positive correlation between the increase in biomass and grain yields in field trials.

In both plant growth traits and plant growth promoting potentials we can suggest the using of AS8 (Bacillus megaterium), AS15 (Bacillus megaterium), AS1 (Cellulomonas turbata) and AS3 (Pseudomonas putida). Although AS4 and AS6 also caused important increments in growth parameters, we are not suggesting them as inoculants because AS4 (Bacillus cereus) and AS6 (Enterobacter cloacae) may be an insect and human pathogens, respectively, and bacteria have this kind of characteristics are known to be inconvenient. These inconvenient isolates must be tested about their pathogenity before use an inoculant.

In the light of present results, it may be concluded that strains of AS1, AS3, AS8 and AS15 can be suitable inoculants for spring wheat cultivations in areas with similar conditions as in Erzurum and Eastern Anatolia. These inoculants should be tested with their combinations.

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Table 1. Isolated bacteria which have both NF and PS activity and their FAME identification

Code	From rhizosphere of	Locality	FAME Identification	Taxonomic group
AS1	*Scorzonera hispanica*	University Campus	*Cellulomonas turbata*	Actinobacteria
AS2	*Xeranthemum inapertum*	University Campus	*Bacillus megaterium*	Firmicutes
AS3	*Astragalus microcephalus*	Palandoken Moun*	*Pseudomonas putida*	Gammaproteobacteria
AS6	*Echium italicum*	Palandoken Moun	*Enterobacter cloacae*	Gammaproteobacteria
AS9	*Centaurea sp.*	Palandoken Moun	*Bacillus mycoides*	Firmicutes
A13	*Dispacus sylvestris*	Palandoken Moun	*Bacillus cereus*	Firmicutes
A15	*Euphorbia sp.*	Palandoken Moun	*Bacillus megaterium*	Firmicutes
A4	*Xeranthemum annum*	Kervansaray Moun	*Bacillus cereus*	Firmicutes
A8	*Artemisia absinthium*	Kervansaray Moun	*Bacillus megaterium*	Firmicutes
A5	*Verbascum sp.*	Cicekdagi Moun	*Neisseria mucosa*	Proteobacteria
AS11	*Alcanna sp.*	Cicekdagi Moun	*Vibrio furnissii*	Gammaproteobacteria
AS12	*Onosma sp.*	Cicekdagi Moun	*Bacillus cereus*	Firmicutes
AS16	*Verbascum cicekdagensis*	Cicekdagi Moun	*Bacillus megaterium*	Firmicutes
AS7	*Verbascum vulcanicum*	Cicekdagi Moun	*Couldn't be identified*	-
AS10	*Mentha longifolia*	Graveyard	*Enterobacter cloacae*	Gammaproteobacteria
AS14	*Senecio pseudoorientalis*	Hospital Campus	*Bacillus cereus*	Firmicutes

*: mountain

Table 2. Some Plant Growth Promoting Traits of Isolated Bacteria

Code	Acetylene reduction (nmol/ethylene/ml/h)	Phosphate solubilization (mg phosphate/L)	IAA Production (mg/ml)	ACC deaminase production (µM α ketoglutarate/mg/h)
AS1	128.65 ± 9.66 *	222.5 ± 3.41 *	30.08 ± 1.52	6.38 ± 0.05
AS2	165.11 ± 7.61*	51.4 ± 2.52 *	46.48 ± 0.39*	4.64 ± 0.01
AS3	77.29 ± 4.41 *	31.6 ± 1.95	94.67 ± 3.82*	32.92 ± 2.35*
AS4	68.27 ± 3.94 *	45.8 ± 2.20*	13.21 ± 1.93*	-
AS5	47.40 ± 2.28 *	40.7 ± 1.33*	105.46 ± 2.77*	17.66 ± 2.71*
AS6	35.82 ± 2.26	37.8 ± 2.84*	112.94± 4.38*	24.52 ± 3.30*
AS7	50.24 ± 3.25*	70.8± 2.54*	47.34 ± 3.66*	-
AS8	22.17 ± 2.10	29.9 ± 3.36	79.57± 5.35*	-
AS9	37.31 ± 2.60*	25.7 ± 2.76	90.07 ± 2.39*	8.24 ± 1.74
AS10	42.53 ± 3.32*	22.4 ± 2.34	102.44 ± 3.57 *	-
AS11	18.28 ±1.05	20.8 ± 3.72	105.61 ± 3.95	-
AS12	13.20 ± 1.22	19.2 ± 1.36	-	-
AS13	16.61 ± 2.48	22.4 ± 1.88	118.55 ± 4.11*	-
AS14	8.11 ± 0.50	16.7 ± 0.85	116.97 ± 4.33	-
AS15	7.24 ± 1.22	11.3 ± 0.33	34.50 ± 3.96	-
AS16	14.92 ± 2.11	10.2 ± 0.39	-	-

Values and standard deviations are the averages of three seperate experiments. An asterisk denotes a value significantly greater than the corresponding control value ($P<0.05$).

Table 3. The Effects of Inoculation With Isolated Bacteria on Shoot and Root Lenght and Dry Weight of Wheat in Pot Experiments

Code	Shoot lenght (mm)	Root lenght (mm)	Dry weight (g/plant)
Control	148.14 ± 3.10	108.68 ± 1.29	0.48 ± 0.072
AS1	184.22 ± 2.66 *	123.57 ± 2.18*	0.53 ± 0.064*
AS2	141.54 ± 1.94	115.25 ±2.41*	0.46 ± 0.017
AS3	179.63 ± 3.30 *	124.19 ± 1.91 *	0.54 ±0.083*
AS4	168.82 ± 2.71*	115.65 ± 1.22*	0.51 ± 0.039*
AS5	140.19 ± 2.00	111.77 ± 1.77	0.71 ± 0.071*
AS6	159.77 ±1.96	128.73 ± 1.28 *	0.58 ± 0.024*
AS7	124.19 ± 2.63	85.92 ± 2.27	0.37 ± 0.012
AS8	198.19 ± 3.*	136.44 ± 2.19 *	0.60 ±0.033*
AS9	95.66 ± 2.25	112.61 ± 2.63	0.29 ± 0.011
AS10	115.28 ± 2.39	81.20 ±2.55	0.54 ± 0.028*
AS11	117.13 ± 3.31	80.48 ± 1.73	0.36 ± 0.041
AS12	116.66 ± 1.83	78.69 ± 1.37	0.35 ± 0.037
AS13	114.81 ± 2.99	118.24 ± 2.49*	0.34 ± 0.058
AS14	122.36 ± 3.14	84.00 ± 2.13	0.55 ± 0.084*
AS15	111.98 ± 2.66	118.53 ± 2.82 *	0.53 ± 0.092*
AS16	17.12 ± 2.11	80.84 ±0.92	0.52 ± 0.048*
Values and standard deviations are the averages of three seperate experiments. An asterisk denotes a value significantly greater than the corresponding control value (P<0.05).			

Table 4. Effects of Inoculation of PGPR on the Biomass, Grain Yield and Protein Content of Wheat at Field Conditions

Applications	Biomass (kg/da)	Grain yield (kg/da)	Grain protein (%)
Control	695.55±0.40h	182.13±1.35f	11.90±0.35bc
4 kg N/da	862.96±2.22a	224.38±1.11a	14.20±0.52a
AS1	797.19±0.70d	208.19±0.56c	12.40±0.23bc
AS3	786.73±0.89e	205.11±0.62d	12.20±0.40bc
AS4	765.92±1.18g	199.39±0.50e	11.80±0.29c
AS6	772.13±0.41f	201.24±0.80e	11.90±0.06bc
AS8	822.82±1.36b	214.52±0.69b	13.90±0.11a
AS15	807.34±0.57c	210.48±0.85c	12.80±0.00b
Values and standard deviations are the averages of three seperate experiments. Values with same letter are not significant (P<0.05).			