Environmental Sustainability
https://doi.org/10.1007/s42398-018-0014-2

ORIGINAL ARTICLE

Endophytic *Azospirillum* for enhancement of growth and yield of wheat

Nasrin Karimi[1] · Mohammad Javad Zarea[1] · Samina Mehnaz[2]

Received: 4 April 2018 / Revised: 22 June 2018 / Accepted: 23 June 2018
© Society for Environmental Sustainability 2018

Abstract

The present study was aimed at the isolation of nitrogen fixing bacteria belonging to genus *Azospirillum* from wild wheat and their growth promoting effect on the bread wheat, under pot culture and filed conditions in semi-arid environment. Two bacterial strains were obtained on the NFb (N-free malate medium) medium with ability to produce phytohormone (indoleacetic acid; IAA) and fix atmospheric nitrogen. Based on 16S rRNA sequence analysis, one isolate (Sp1) showed 99% homology with *Azospirillum brasilense* and the second isolate (Sp2) showed 99% homology with *Azospirillum zeae*. Effect of these two isolated strains on bread wheat was examined under pot experiment. Both bacterial strains were found to be effective in enhancing wheat growth. However, best results were obtained with isolate Sp2. Therefore, only this strain was introduced as inoculum in field experiment, to investigate its effect on wheat yield under semi arid conditions during winter growing season 2016–2017. Based on the obtained field results, wheat inoculated with isolate Sp2 displayed an increased total grain yield by up to 18% as compared to un-inoculated plants. These results show the potential of these bacteria to be used as biofertilizer.

Keywords *Azospirillum* · Wild wheat · IAA · Dryland farming · *Triticum aestivum*

Introduction

Microorganisms play an important role in biogeochemical cycles and recently have been assumed to have a considerable potential for agriculture use (Robe et al. 2003). Most of soil microorganisms have the ability to colonize plants, creating an association with them and increasing growth and yields. These microorganisms are commonly called plant growth promoting rhizobacteria (PGPR) (Maddonni et al. 2004). Among PGPR members of the genus *Azospirillum* are well known and commonly used bacteria in agriculture (Naiman et al. 2009). Bacteria with ability to fix nitrogen are frequent colonizer of grasses and cereal crops belonging to the genera *Azospirillum, Acetobacter, Azoarcus, Enterobacter,* and *Herbaspirillum* (Mehnaz et al. 2001). Recently much more attention has been paid to analyze the role of PGPR in improving plant growth under stress conditions (Díaz-Zorita et al. 2012; Zarea et al. 2012, Zarea 2017).

Azospirillum a Gram-negative, microaerophilic, non-fermentative soil bacterium lives in soil or found in association with plant. They are free-living nitrogen-fixing rhizosphere bacteria from the family Rhodospirillaceae. *Azospirillum* belongs to the group of bacteria known as associated bacteria with plant growth-promoting properties. At present, sixteen species of bacterial genus *Azospirillum* have been described (Zarea 2017); including 15 N-fixing and one non-N-fixing species (Reis et al. 2015). Mutualistic association between *Azospirillum* and various plant species, especially grain crops, has generated lots of interest. Growth promoting effect of *Azospirillum* on yield of staple crop under field conditions in different soils and climates has been elucidated (Zarea 2017). Positive response of various crops such as wheat, corn, soybean and rice to *Azospirillum* inoculation has been observed (Naiman et al. 2009). Positive impacts on plant growth following *Azospirillum* inoculation has been attained through several mechanisms including enhancement of macro- and micro nutrient content of plant, promotion of root development, production of growth regulators, nitrogen fixation and modulation of leaf anatomy (Okon 1994; García de Salamone et al. 1996; Naiman et al. 2009; Jafarian and

✉ Mohammad Javad Zarea
zareamj@yahoo.com; mj.zarea@ilam.ac.ir

[1] Faculty of Agriculture, Ilam University, P.O. Box 69315516, Ilam, Iran

[2] Department of Biological Sciences, Forman Christian College (A Chartered University), Lahore, Pakistan

Published online: 03 July 2018

Zarea 2016; Zarea 2017). A variety of *Azospirillum* species and other beneficial bacteria have been reported to have ability to colonize the root systems and above ground parts of plants such as rice (Khammas et al. 1989), wheat (Kopylov et al. 2009), maize (Mehnaz et al. 2007a, b), sugarcane, and other plants (Steenhoudt and Vanderleyden 2000). These bacteria benefit plant through several direct and indirect ways. Beneficial effects of PGPR have been attributed to the biological nitrogen fixation and productions of phytohormones such as IAA which results in promoting root development and consequently enhancing water uptake and nutrient absorption (Tien et al. 1979; Hartmann et al. 1983; Sharma et al. 2015; Puente et al. 2017).

Wheat (*Triticum aestivum* L.) is the world's most cultivated and important cereal crop (Beche et al. 2014; Li et al. 2016; Feng et al. 2018). Wheat is the second most extensively grown crop throughout the world (Li et al. 2016). It is also one of the most important crops in arid and semi-arid areas of Iran. Genus *Aegilops* belong to the family *Poaceace* and have been assumed to have an important role in the evolution of bread wheat (Niranjana 2017).

The aim of our investigation was to isolate the native strains of *Azospirillum* from roots of wild wheat growing on mountainous regions of west Iran. This study was aimed to investigate and to determine the effect of the inoculation of these bacteria on grain yield and the nutrient content (N and P) of wheat plants grown under pot culture and dryland farming. Bacterial isolates were characterized and screened for plant growth promoting potential. The hypothesis of this study is that inoculation with indigenous PGPR can be an advantage and support establishment since native bacteria can easily adapt to the natural conditions and enhance the plant growth in this region (Zahid et al. 2015).

Materials and methods

Isolation of nitrogen fixing bacteria from wild wheat

Bacterial isolates were procured from the roots of wild wheat, *Aegilops triuncialis* and *Aegilops speltoides,* naturally growing in mountainous regions of west Iran. Roots, collected from different regions, were thoroughly washed with distilled sterile water to detach adhering soil. Roots were then cut into 1 cm long pieces and immersed in 5% NaCl solution for 10 min. We used 5% NaCl solution in order to cause plasmolysis of root cortex cells. This procedure led to better release of endophytic bacteria from inside of the root tissues. Root segments were then submerged into 10 cm vial of semi-solid NFb (N-free malate medium) (Döbereiner and Day 1976) and incubated at 25 °C for 7 days. After incubation, a loopful of the pellicle which formed beneath the surface of the medium was streaked on plates of modified RC medium (Table 1). DL-malic acid served as carbon sources for *Azospirillum* spp. as previously advised by Caceres (1982) for *Azospirillum* isolation. Yeast extract used at the rate of 0.5 g mL^{-1} in the medium supplies bacteria with vitamins and other growth factors. Some bacteria require vitamins and other growth factors for optimum growth and yeast extract is rich in vitamins, amino acids such as glycine, serine, and tyrosine, and minerals (Fujita and Hashimoto 1985; Chen et al. 2007). Two distinct colonies (named Sp1 and Sp2) were streaked on modified Rojo Congo (Congo red; RC), medium (Table 1) supplemented with 0.01 g L^{-1} yeast extract. RC medium was first introduced by Caceres (1982) as proper medium for isolation of *Azospirillum* spp. Both bacteria strains absorbed Congo red dye and became scarlet and dried with time.

Indoleacetic acid (IAA) production assay

The ability of bacterial isolates to produce IAA was determined qualitatively on NFb medium. A colorimetric technique was performed using the Salkowski's method (Ehmann 1977). Both bacterial strains were grown in NFb medium amended with 100 mg L^{-1} tryptophan. After incubation for 24 h at 28 °C with continuous shaking at 125 rpm, the broth was centrifuged at 10,000 rpm, for 15 min. One mL aliquot of the supernatant was mixed with Salkowski's reagent at the ratio of 1:2 and incubated for 20 min under darkness at room temperature (Gordon and Weber 1951). Development of pink-red color was the sign of IAA production. Absorbance of the pink-red color

Table 1 Modified RC medium used for the distinction between isolated colonies

Content	g L^{-1}
DL-malic acid	5
K_2HPO_4	0.5
$MgSO_4*7H_20$	0.2
NaCl	0.1
yeast extract	0.5
$CaCl_2*2H_2O$	0.02
$FeCl_3*6H_20$	0.015
Congo red solution (1:400 aqueous solution)[a]	15 ml
Micro nutrient	2 ml
Agar	18
Adjust pH to 6.8 with KOH	
Micronutrient solution:	1 L
$CuSO_4 \bullet 5H_2O$	0.4 g
$ZnSO_4 \bullet 7H_2O$	0.12 g
$MnSO_4 \bullet H_2O$	1.5 g

[a]Added to the medium before autoclaving

was measured at 530 nm. The concentration of IAA was quantified using a standard curve of pure IAA solution.

PCR-amplification and 16S rRNA sequence analysis

Selected isolates were subjected to 16S rRNA sequencing analyses. The crude DNA was extracted according to the method described by Cheng and Jiang (2006) with some modifications. Strains were cultured in NFb medium supplemented with 1.5 mg L^{-1} yeast extract. Bacterial cultures were then incubated at 27 °C for 4 days with continuous shaking at 120 rpm. To extract the bacterial DNA, 1 ml cell suspension was centrifuged at 10,000 rpm for 3 min. After removing the supernatant, the cells were washed with 300 µl STE buffer twice (Cheng and Jiang 2006). The cells were then centrifuged at 10,000 rpm for 3 min. The pellets were re-suspended in 200 µl TE buffer. 400 µl phenol saturated with Tris (pH 8.0) was added to the tubes to lyse the cells, followed by a vortex-mixing step of 60 s. To separate the aqueous phase from the organic phase, the samples were subsequently centrifuged at 13,000 rpm for 10 min at 4 °C. 200 µl upper aqueous phase was then mixed with 200 µl chloroform and centrifuged for 5 min at 13,000 rpm at 4 °C. Amplification was carried out using the Az16S–D sequencing primers (Forward, 5′CCGCGGTAATACGAAGGGGGC; Reverse, 5′GCCTTCCTCCGGCTTGTCACCGGC) (Shime-Hattori et al. 2011). Amplified PCR products were separated on 1% agarose gel stained with ethidium bromide. A reaction mixture of 25 µL, containing 3 µL (15 ng) of template DNA, 15 µL of Master Mix, and 1.5 µL of each 10 µM forward and reverse primers, was prepared. The PCR was performed in a thermal cycler with an initial denaturation at 94 °C for 5 min followed by 33 cycles with denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s, and extension at 72 °C for 1 min and final extension at 72 °C for 7 min. Sequence similarity was determined by comparing the sequences from GenBank (National Center for Biotechnology Information; NCBI) with the BLAST program. Phylogenetic analysis was performed using the software package MEGA program, version 6 (Tamura et al. 2013). Bootstrap analysis was performed to test the statistical reliability of the topology of the neighbour-joining tree with 1000 bootstrap resamples of the data. Phylogenetic trees were constructed by the maximum-likelihood method using MEGA program, version 6 (Tamura et al. 2013). The sequences were deposited in the NCBI databank under the accession numbers MG282188 (Sp1) and MG384967 (Sp2).

Pot experiment

A pot experiment was designed to evaluate the plant growth promoting effects of bacterial isolates on wheat. Wheat seeds were first surface sterilized with 3% sodium hypochloride for 5 min and then washed seven times with sterilized water. Vernalization was done by short-term exposing the seeds to low temperatures (4 °C) for 7 d. Vernalized Seeds were germinated in a sterile 15-cm Petri plates lined with two layers of 50-mm diameter sterile filter paper saturated with sterile water. Petri dishes were then incubated at 20 °C till radicles and root hairs appeared. Bacterial isolates were grown in NFb medium. Germinated seed with radicles 2–3 cm long were inoculated with suspension of washed overnight bacterial culture (10^7–10^8 cells mL^{-1}). Germinated seeds were soaked in this prepared culture and kept shaking for 30 min at 25 °C. Autoclaved inocula were used as controls. Wheat seedlings were then transferred into 7 kg pots, filled with 6 kg sterilized soil. Each pot received equal amount of urea as source of nitrogen (6 g nitrogen pot^{-1}). Urea was broadcast and incorporated below the soil surface of the pot. Triple superphosphate as sources of P at a rate of 3 g pot^{-1} was incorporated into the soil before sowing the seeds. Each treatment (control, inoculated with Sp1, inoculated with Sp2) was replicated 4 times. Fifteen healthy germinated seeds were transplanted in each pot and were then thinned to leave 5 seedlings per pot. Pots were kept inside a greenhouse for 6 months. After 120 days of planting, each plant was harvested, at 5 cm from the above soil level, decapitated and the shoot systems were weighed. Total nitrogen (N) was determined according to method described by Page et al. (1982). Various agronomic traits such as total dry biomass, grain and straw weights, harvest index (%), tiller and spike numbers $plant^{-1}$, seed number $spike^{-1}$, plant and spike height and weight of thousand-seeds were recorded. Spike weight and dried biomass were recorded from five randomly chosen plants after oven-dried at 70 °C until no change in weight was observed.

Field experiment

A field experiment was carried out to evaluate the plant growth promoting effects of Sp2 on wheat. Experiment was done under a semi-arid region where precipitation was a limiting factor for wheat growth. The field experiment was established in agricultural lands in Dehgolan County, Kurdistan Province (35°17′N, 47°22E, West Iran), located in a cool temperate region. The soil contains approximately 2% organic matter and soil pH ranges from 7.2 to 7.6. Experimental design was a randomized complete block with three replications. The treatments consisted of the presence and absence of seed inoculation with Sp2. Fields were plowed in the fall and cultivated in the spring. Long time between

plowing and seed-bed preparation is usual in semiarid and arid areas of Iranian croplands. Seed-bed preparation was made at autumn when soil moisture was adequate for seed germination. Sowing was made following the first rain in autumn. The amount of rain must be sufficient to provide adequate moisture for seed to germinate. Therefore actual time of sowing during 2 years was not similar and dependent on the time of first adequate rainfall. The soil is classified as clay-loamy. Plot size was 3 m^2. Bacterial isolate, Sp2, was grown in NFb medium. Seeds were inoculated with 1 mL suspension of washed overnight bacterial culture (10^7–10^8 cells mL^{-1}). Plant density was 200 plants m^2. Yield components and grain yield were estimated at seed maturity. From each treatment, at grain maturity growth stage, a 1 m^2 of interior rows was cut and total grain yield was estimated. Agronomic components were estimated from 10 randomizely picked plants. Total spike per m^2 was calculated by means of spike per plant × plant density m^{-2}. All samples were cut from the middle rows to avoid border effects. Before threshing the bundles, numbers of spikes in each bundle were counted. Twenty stems were randomly selected from each bundle and each stem was threshed separately by hand. The seed number and kernel weight of each stem were measured. Nitrogen and phosphorus content in grains were measured at maturity growth stage. Grain nitrogen content was measured according to the method described by Van Schouwenberg and Walinge (1973). Ammonium vanadate-molybdate method described by Chapman and Pratt (1961) was followed to measure the phosphorus contents in grains.

Statistical analysis

Pot experiment was a randomized complete blocks with four replications of treatments. Field experiment was a randomized complete block design with four replications. Error variances for the 2 years were tested for homogeneity by Bartlett's test. Combined analysis of variance was done. Traits had homogeneous error variances for both years. Collected, pooled data were subjected to ANOVA. For both pot and field experiments treatment effects were considered significant at $P < 0.05$. Least significant differences (LSD) tests ($P < 0.05$) were used to compare means within treatments.

Results

Isolation, characterization, and identification of strains

Root samples were collected from wild wheat roots, naturally growing in mountain region. Two distinct bacterial isolates were purified using NFb medium. Some biochemical and physiological characteristics of two isolates are listed in Table 2. Both bacterial isolates were able to grow on nitrogen-free medium. They did not grow properly when other carbohydrate compounds, glucose and sucrose, were served as sole carbon sources. Both bacterial isolates showed extremely slow growth on glucose as sole carbon source. Colonies of isolate Sp2 appeared to be longer in diameter than Sp1 in modified RC medium. Bacterial isolate Sp2 formed curved shaped colonies on modified RC medium in comparison to colonies of Sp1.

Indole-3-acetic acid (IAA) measured by colorimetric technique

The ability of both bacterial isolates to produce IAA was checked with use of Salkowski reagent. Isolates synthesized different IAA levels. Isolate Sp2 was more proficient producer of IAA in comparison to Sp1 (Table 2). Adding tryptophan to the NFb medium resulted in more IAA production compared with NFb without tryptophan. Sp2 produced 39.10 µg ml^{-1} IAA while Sp1 produced 36.97 µg ml^{-1} in NFb medium amended with 100 µg/ml DL-tryptophan. The photograph presented in Fig. 1 shows the phenotype of 1 and 4-day-old wheat plants inoculated with bacterial inoculum, Sp1 and Sp2.

16S rRNA Sequence analysis

Bacterial isolates were identified by using 16S rRNA. Figure 2 shows the phylogenetic tree based on 16S rRNA gene sequence, constructed by using the neighbour-joining method. Both bacterial isolates displayed homologies with other recognized species within the genus *Azospirillum*. Sp1 showed 99% homologies with the sequences in the GenBank database of *Azospirillum brasilense*. Homology of Sp2 with *Azospirillum zeae* strain Gr24 was 99%. The 16S rRNA gene sequences were deposited in the NCBI databank under the accession numbers MG282188 (Sp1) and MG384967 (Sp2).

Table 2 Indole-3-acetic acid (IAA) of two bacterial isolates grown in NFb liquid medium supplemented with (+) and without (−) L-tryptophan (100 µg ml^{-1}) (Means followed by same letters are not significantly different at 5% level)

Bacterial strain	IAA µg ml^{-1}	
	−	+
Sp1	29.25c	36.92b
Sp2	28.32c	39.11a

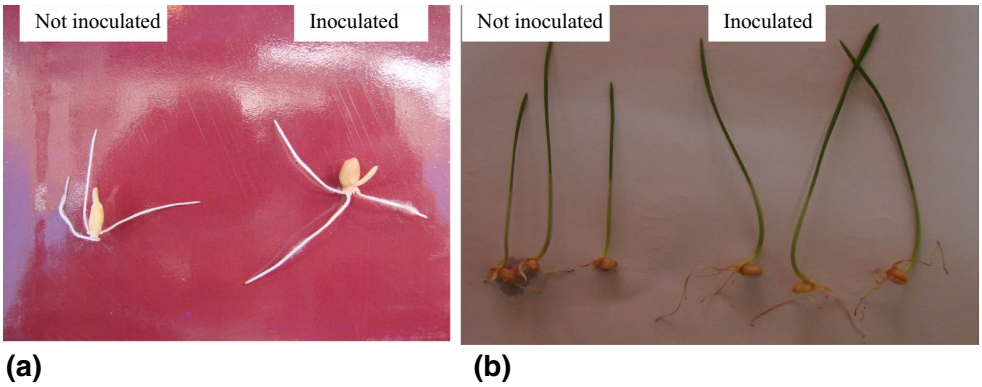

Fig. 1 Application of supernatants of Sp2 on early root growth (**a**) and in the seedling growth of bread wheat (**b**)

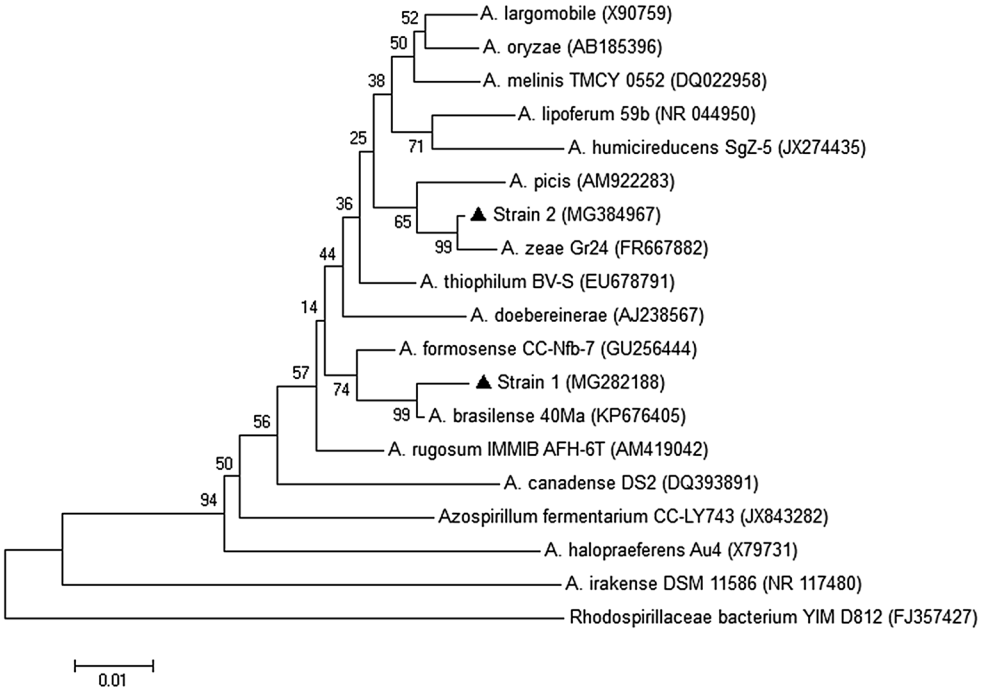

Fig. 2 Neighbor-joining tree phylogenetic analysis, with bootstrap values of 1000 replications at branch nodes, of isolates based on 16S rRNA gene sequences

Efficacy of isolated strains in growth promotion of wheat under pot culture

Bacterial isolates were screened for their plant growth promoting ability. Wheat straw, grain yield, harvest index, plant and spike height, spike weight, and grain yield components, tiller number, seed number, 1000-seed weight, were significantly improved by both bacterial isolates (Table 3). However, both the parameters were significantly higher in plants inoculated with Sp2 than in plants inoculated with Sp1 (Table 3). Plants inoculated with Sp2 produced higher rates of wheat straw (14.94%), grain yield (23.12%), harvest index (6.9%), plant height (4.1%), spike height (9.2%), spike weight (8.2%), tiller number (10.5%), seed number (2.6%) and 1000-seed weight (11.81%) over those of plants inoculated with Sp1. Nitrogen content of grains was significantly increased by both isolates (Table 3). Plants inoculated with Sp2 had higher concentration of N (11.8%) in their grains over those of plants inoculated with Sp1.

Field efficacy of isolate Sp2

Isolate Sp2 was used to evaluate wheat yield response in the field. Isolate Sp2 produced a significant increase in the grain yield of wheat under dryland farming conditions. This resulted to a 18% increase over the uninoculated control (Table 4). Isolate Sp2 increased the 1000-seed weight by 7.81% in comparison to those of control plants. The number of spikes per m^2 and seed number per spike of the inoculated plants were not significantly different from those of

Table 3 Effect of *Azospirillum* sp. isolated from wild wheat on some agronomic traits of bread wheat in 180-day-old plants under pot conditions

Treatment	Straw (g plant^{-1})	Grain yield (g plant^{-1})	Harvest index (%)	Plant height (cm)	Spike height (cm)	Spike weight (g plant^{-1})	Tiller (no plant-^{1})	Seed no (spike^{-1})	1000-Grain weight (g)	Grain N content (%)
Azospirillum Sp2	20.84a	8.36a	39.95a	88.26a	9.73a	1.84a	9.28a	51.1a	38.94a	8.71a
Azospirillum Sp1	18.13b	6.79b	37.36ab	84.78b	8.91b	1.70b	8.53b	46.24b	37.93b	7.79b
Control	16.1c	5.78c	35.969b	81.35c	8.55c	1.62c	8.13c	34.63c	36.47c	4.99c
LSD	0.96	0.62	3.44	1.51	0.26	0.078	0.37	2.21	0.64	0.24

Means followed by the same letter are not statistically different at 5% level according to least significant difference (LSD) test

the non-inoculated plants. Phosphorus contents in the grains of the inoculated plants were not found to be significantly different from those of the uninoculated control plants (Table 4). Nitrogen content of grains were positively influenced by isolate Sp2 (Table 4). Inoculation increased the N content of grains by about 5.8%.

Discussion

In the present study, we isolated two bacterial strains of the genus *Azospirillum* from root interior of wild wheat naturally grown in the mountain region. Both bacterial strains showed excellent growth in the NFb medium, which is a putative selective medium for *Azospirillum* (Okon et al. 1977). Bacterial isolates were examined and characterized through molecular approach. Isolate Sp1 showed 99% similarity with *A. brasilense* which has been previously reported as a plant associated bacterium. The isolate Sp2 was identified as *A. zeae* having 99% homology with the reported gene sequence which has been reported as a plant growth promoting bacterium (Mehnaz et al. 2010). *A. brasilense* has been isolated from roots of diverse plants such as finger millet (Rai 1991), *Triticum durum* (Boyko et al. 2011) and strawberry (Fontana et al. 2018). *A. zeae* have been isolated from root of wheat (Venieraki et al. 2011) and rhizosphere soil of corn (Mehnaz et al. 2007a) and wheat (Ayyaz et al. 2016). Our findings showed that the wild wheat root is colonized by *A. brasilense* as well as *A. zeae*. This is the first report revealing the existence of *A. zeae* in the root of wild wheat in Iran. *A. zeae* was first isolated from roots of maize in Canada (Mehnaz et al. 2007a) and was reported as *A. lipoferum* (Mehnaz and Lazarovits 2006) but was later redescribed on the basis of a polyphasic taxonomic technique. Both bacterial strains failed to grow in NFb medium amended with sucrose or glucose. Previous studies indicated that *Azospirillum* cannot utilize disaccharides and prefer organic acids, such as malic over disaccharides sources (Westby et al. 1983).

Quantitative analysis revealed that both bacterial isolates have the ability to produce IAA. However, higher production was observed by Sp2. The present findings seem to be consistent with other researches which report that the ability to produce IAA is a major property of species of *Azospirillum.* The ability to form plant hormones has been postulated as a major property of PGPR in general and specifically, by the species of *Azospirillum* (Tsavkelova et al. 2006). Verma et al. (2011) showed that 88% of the evaluated *Azospirillum* strains (50 isolates) from maize soils were poor in IAA production indicating a substantial variability among *Azospirillum* strains for IAA production. The variation in the ability of plant growth promoting bacteria to produce IAA had also been observed earlier (Zahir et al. 2000; Majeed et al. 2015). Several factors have been assumed to be involved in IAA

Table 4 Effect of Sp2 on wheat yield and nitrogen and phosphorus content of grains under dry land farming

Treatment	Fertile spike (no per m^2)	Seed (no per $spike^2$)	1000-Grain weight (g)	Total grain yield	Nitrogen contents (g kg^{-1})	Phosphorus contents (g kg^{-1})
+*Azospirillum*	230.33a	20.73a	29.54a	1411.13a	28.83a	2.99a
−*Azospirillum*	221.50a	19.69a	27.40b	1191.52b	27.24b	2.92a
LSD	9.695	2.2634	0.69	177.72	0.66	0.18

Means followed by the same letter are not statistically different at 5% level according to least significant difference (LSD) test

biosynthesis including genes involved and the presence of enzymes responsible for converting free IAA into conjugated forms (Patten and Glick, 1996). Both bacterial isolates preferred tryptophan for IAA production. L-Tryptophan is generally considered as an IAA precursor and its addition into culture medium results in enhancing of IAA biosynthesis by the IAA producing bacteria (Costacurta and Vanderleyden 1995). In the present study, the quantity of IAA produced by Sp2 in NFb supplemented with tryptophan was 39.10 µg ml^{-1}. Venieraki et al. (2011) reported IAA production ranging from 29.8 and 194.8 mg L^{-1} by two *A. zeae* strains.

In order to evaluate the association between wheat and bacterial isolates, Sp1 and Sp2, a pot experiment was done. Based on the obtained result of pot trial, isolate Sp2 was used to assess wheat yield response in the field. Sp2 increased the yield of wheat under field conditions. The beneficial effect of *Azospirillum* as PGPR on increasing the yield of wheat under non-stress and stress conditions have been previously reported (Zarea et al. 2012; Jafarian and Zarea 2016; Zarea 2017). The plant growth promotion could be attributed to the result of plant growth hormone production, nitrogen fixation, and P solubilization. However Sp2 succeeded to increase the nitrogen content of the produced grains, suggesting the biological nitrogen fixation activity of this strain, but failed to increase the phosphorus content of grains, indicating no activity of phosphate solubilization under filed conditions. Phosphate-solubilizing activity of the Sp2 was assessed in vitro and it could solubilize insoluble inorganic phosphate in the modified Pikovskaya medium (data not shown). Ayyaz et al. (2016) have also reported failures of inoculations with three strains of *Azospirillum* to increase phosphorus contents in wheat grains and shoots. In the present study, Sp2 increased grain weight and total yield of wheat under field condition. Previous study indicated that total yield of wheat was significantly correlated ($P < 0.01$) with initial seed weights (Austen-Son and Walton 1970). Pervious experiments also showed that inoculation of wheat with *Azospirillum* increases the productivity of wheat (Santa et al. 2004; Piccinin et al. 2011, 2013; Ayyaz et al. 2016).

Auxin production has been assumed to have a major effect on attaining the early growth promotion in wheat (Khalid et al. 2004). Auxin is involved in root initiation and development and increases root surface area (Ivanchenko et al. 2010) and consequently assist the plant in increasing nutrient absorption by increased formation and development of better root systems (Dey et al. 2004; Gray and Smith 2005; Sukumar et al. 2013). Enhanced grain yield and N content of grains may also be related to the developed root system due to isolate application.

The field experiment was conducted under dryland farming. Drought is the most prevalent environmental stress and considered as one of the most important factor limiting wheat production under semi-arid and arid areas of Iran. Root system is the major organ of plants responsible for water and nutrient absorption. Root system influences plant growth and grain productivity (Palta and Yang 2014). Several PGPR (*Azospirillum*, *Azoarcus*, *Azotobacter*, *Bacillus polymyxa*, *Burkholderia*, *Gluconoacetobacter* or *Herbaspirillum*) have been known to fertilize several important crops such as wheat (Boddey et al. 1986). Inoculation of these PGPR with plants usually results in increase in plant's dry weight, flowering and grain production (Pérez-Montaño et al. 2014). Improvement of yield caused by these PGPR could often be attributed to an increase in root development (Okon et al. 1998; Bashan et al. 2004; Pérez-Montaño et al. 2014). Developed root system allows plant to uptake water and minerals in a better way (Okon et al. 1998; Bashan et al. 2004; Pérez-Montaño et al. 2014). In the present study, bacterial isolates showed ability to produce IAA in vitro. Khalid et al. (2004) showed that those PGPR strains that produce high quantity of auxins in non-sterilized soil, cause maximum improvement in growth and yield of the wheat crop. In the present study, plants inoculated with Sp2 had higher amount of N content in their grains as well. Nitrogen nutrition has been elucidated to mitigate drought stress in crops. Nitrogen nutrition maintains metabolic activities of plant even at low tissue water potential (Zlatev and Lidon 2012; Abid et al. 2016). Pervious study demonstrated that photosynthetic rate under drought stress is closely related to the chlorophyll and N contents of the leaf (Park and Lee 2003). Previous studies have also reported that low N is the major constraint to wheat yield under drought stress (Madani et al. 2010; Abid et al. 2016). Nawaz et al. (2012) reported that early and late drought stress significantly decreases the N uptake by the wheat.

In the present study we report two strains of *Azospirillum* species associated with wild wheat, growing in mountainous regions of Iran. Both isolated bacterial strains have capability of IAA-production. Isolate *A. zea* Sp2 that produced higher level of auxins in vitro, and also caused higher N content in the grains under pot experiment, also enhanced growth and yield of the wheat under field conditions. Crop productivity in semiarid regions is mostly restricted by water availability. The results of this study show that under semiarid area conditions of Iran the production of wheat could be improved by using *A. zea* Sp2 leading to sustainable agriculture in these regions.

Acknowledgements The authors would like to thank to two anonymous reviewers for their constructive and valuable comments which helped us to improve the manuscript.

References

Abid M, Tian Z, Ata-Ul-Karim ST, Cui Y, Liu Y, Zahoor R, Jiang D, Dai T (2016) Nitrogen nutrition improves the potential of wheat (*Triticum aestivum* L.) to alleviate the effects of drought stress during vegetative growth periods. Front. Plant Sci 7:981

Austen-Son HM, Walton PD (1970) Relationships between initial seed weight and mature plant characters in spring wheat. Can J Plant Sci 50:53–58

Ayyaz K, Zaheer A, Rasul G, Mirza MS (2016) Isolation and identification by 16S rRNA sequence analysis of plant growth-promoting *Azospirilla* from the rhizosphere of wheat. Braz J Microbiol 47:542–550

Bashan Y, Holguin G, de Bashan LE (2004) *Azospirillum*–plant relationship: physiological, molecular, agriculture, and environmental advances (1997–2003). Can J Microb 50:521–577

Beche E, Benin G, da Silva CL, Munaro LB, Marchese JA (2014) Genetic gain in yield and changes associated with physiological traits in Brazilian wheat during the 20th century. Eur J Agron 61:49–59

Boddey RM, Baldani VLD, Baldani JI, Dobereiner J (1986) Effect of inoculation of *Azospirillum* spp. on nitrogen accumulation by field grown wheat. Plant Soil 95:109–121

Boyko AS, Konnova SA, Fedonenko YP, Zdoroven-ko EL, Smol'kina ON, Kachala VV, Ignatov VV (2011) Structural and functional peculiarities of the lipopolysaccharide of *Azospirillum brasilense* SR55, isolated from the roots of *Triticum durum*. Microbiol Res 166:585–593

Caceres EAR (1982) Improved medium for isolation of *Azospirillum* spp. Appl Environ Microbiol 44:990–991

Chapman HD, Pratt PF (1961) Ammonium vanadate–molybdate method for determination of phosphorus. In: Chapman HD, Pratt PF (eds) Methods of analysis for soils. Plants and Water University of California, Berkeley, pp 184–203

Chen J, Zhang Y, Du GC, Hua ZZ, Zhu Y (2007) Biodegradation of polyvinyl alcohol by a mixed microbial culture. Enzyme Microb Technol 40:1686–1691

Cheng HR, Jiang N (2006) Extremely rapid extraction of DNA from bacteria and yeasts. Biotechnol Lett 28:55–59

Costacurta A, Vanderleyden J (1995) Synthesis of phytohormones by plant associated bacteria. Crit Rev Microbiol 21:1–18

Dey R, Pal KK, Bhatt DM, Chauhan SM (2004) Growth promotion and yield enhancement of peanut (*Arachishypogaea* L.) by application of plant growth promoting rhizobacteria. Microbiol Res 159:371–394

Díaz-Zorita M, Micucci FG, Fernandez-Canigia MV (2012) Field performance of a seed treatment with *Azospirillum brasilense* on corn productivity. In: Perez JC, Soler Arango J, Posada Uribe LF (eds) PGPR. 9th International and 1st Latinamerican PGPR workshop. "Returning to Our Roots". Quirama, Medellín (Colombia), June 3rd–8th 2012

Döbereiner R, Day JM (1976) Associative symbioses in tropical grasses: characterization of microorganisms and dinitrogen fixing sites. In: Newton WE, Nyman CJ (eds) Proceedings of the 1st International symposium on nitrogen fixation, Pullman, Washington, U.S.A.: Washington State University Press, Pullman, pp. 518–538

Ehmann A (1977) The Van Urk-Salkowski reagent—a sensitive and specific chromogenic reagent for silica gel thin-layer chromatographic detection and identification of indole derivatives. J Chromatogr 132:267–276

Feng F, Han Y, Wang S, Yin S, Peng Z, Zhou M, Gao W, Wen X, Qin X, Siddique KHM (2018) The effect of grain position on genetic improvement of grain number and thousand grain weight in winter wheat in North China. Front Plant Sci 9:129

Fontana CA, Salazar SM, Bassi D, Puglisi E, Lovaisa N, Toffoli LM, Pedraza R, Cocconcelli PS (2018) Genome sequence of *Azospirillum brasilense* REC3, isolated from strawberry plants. Genome Announc 6:1–2

Fujita M, Hashimoto S (1985) Isolation of a bacterium requiring three amino acid for polyvinyl alcohol degradation. J Ferment Technol 63:471–4745

García de Salamone IE, Döbereiner J, Urquiaga S, Boddey RM (1996) Biological nitrogen fixation in *Azospirillum strainmaize* genotype associations as evaluated by 15 N isotope dilution technique. Biol Fertil Soil 23:249–256

Gordon SA, Weber RP (1951) Colorimetric estimation of indole acetic acid. Plant Physiol 26:192–195

Gray EJ, Smith DL (2005) Intracelluler and extracellular PGRP: commonalities and distinctions in the plant–bacterium signaling processes. Soil Biol Biochem 37:395–412

Hartmann A, Singh M, Klingmuller W (1983) Isolation and characterization of *Azospirillum* mutants excreting high amounts of indole acetic acid. Can J Microbiol 29:916–923

Ivanchenko MG, Napsucialy-Mendivil S, Dubrovsky JG (2010) Auxin-induced inhibition of lateral root initiation contributes to root system shaping in *Arabidopsis thaliana*. Plant J 64:740–752

Jafarian T, Zarea MJ (2016) Hydrogen peroxide affects plant growth promoting effects of *Azospirillum*. Crop Sci Biotech 19:167–175

Khalid A, Arshad M, Zahir ZA (2004) Screening plant growth-promoting rhizobacteria for improving growth and yield of wheat. J Applied Microbiol 96:473–480

Khammas KM, Ageron E, Grimont PAD, Kaiser P (1989) *Azospirillum irakense* sp. nov., a nitrogen-fixing bacterium associated with rice roots and rhizosphere soil. Res Microbiol 140:679–693

Kopylov IP, Spyrydonov VH, Patyka VP (2009) Identification of *Azospirillum* genus bacteria isolated from the spring wheat root zone. Mikrobiol Z 71:13–19

Li Y, Cui ZY, Ni YL, Zheng MJ, Yang DQ, Jin M, Chen J, Wang Z, Yin Y (2016) Plant density effect on grain number and weight of two winter wheat cultivars at different spikelet and grain positions. PloS One 11(5):e0155351

Madani A, Rad AS, Pazoki A, Nourmohammadi G (2010) Wheat (*Triticum aestivum* L.) grain filling and dry matter partitioning responses to source:sink modifications under postanthesis water and nitrogen deficiency. Acta Sci Agron 32:145–151

Maddonni GA, Ruiz RA, Vilarinõ P, García de Salamone IE (2004) Fertilizacio´n en los cultivos para grano. In: Satorre E, Benech Arnold R, Slafer GA, de la Fuente EB, Miralles DJ, Otegui

ME, Savin R (eds) Produccio´n de Granos, Bases funcionales para su manejo. Universidad de Buenos Aires, Buenos Aires, pp 499–557 **(ISBN 950-29-0713-2)**

Majeed A, Abbasi MK, Hameed S, Imran A, Rahim N (2015) Isolation and characterization of plant growth-promoting rhizobacteria from wheat rhizosphere and their effect on plant growth promotion. Front Microbiol 6:1–10

Mehnaz S, Lazarovits G (2006) Inoculation effects of *Pseudomonas putida*, *Gluconacetobacter azotocaptans*, and *Azospirillum lipoferum* on corn plant growth under greenhouse conditions. Microb Ecol 51:326–335

Mehnaz S, Mirza MS, Haurat J, Bally R, Normand P, Bano A, Malik KA (2001) Isolation and 16S rRNA sequence analysis of the beneficial bacteria from the rhizosphere of rice. Can J Microbiol 47:110–117

Mehnaz S, Weselowski B, Lazarovits G (2007a) *Azospirillum zeae* sp. nov., diazotrophic bacteria isolated from rhizosphere soil of *Zea mays*. Int J Syst Evol Microbiol 57:2805–2809

Mehnaz S, Weselowski B, Lazarovits G (2007b) *Azospirillum canadense* nov., a nitrogen fixing bacterium isolated from corn rhizosphere. Int J Syst Evol Microbiol 57:620–624

Mehnaz S, Kowalik T, Reynolds B, Lazarovits G (2010) Growth promoting effects of corn (*Zea mays*) bacterial isolates under greenhouse and field conditions. Soil Biol Biochem 42:1848–1856

Naiman AD, Latrónico A, García de Salamone IE (2009) Inoculation of wheat with *Azospirillum brasilense* and *Pseudomonas fluorescens*: Impact on the production and culturable rhizosphere microflora. Eur J Soil Biol 45:44–51

Nawaz F, Ahmad R, Waraich EA, Naeem MS, Shabbir RN (2012) Nutrient uptake, physiological responses, and yield attributes of wheat (*Triticum aestivum* L.) exposed to early and late drought stress. J Plant Nutr 35:961–974

Niranjana M (2017) Gametocidal genes of *Aegilops*: segregation distorters in wheat–*Aegilops* wide hybridization. Genome 60:639–647

Okon Y (1994) *Azospirillum*/plant association. CRC Press, Boca Raton

Okon Y, Albrecht SL, Burris R (1977) Methods for growing *Spirillum lipoferum* and for counting it in pure culture and in association with plants. Appl Environ Microbiol 33:85–88

Okon Y, Bloemberg GV, Lugtenberg BJJ (1998) Biotechnology of biofertilization and phytostimulation. In: Altman A (ed) Agricultural biotechnology. Marcel Dekker, New York, pp 327–349

Page AL, Miller RH, Keeney DR (eds) (1982) Methods of soil analysis, Part 2: Chemical and microbiological properties. American Society of Agronomy Inc, Madison

Palta JA, Yang JC (2014) Crop root system behaviour and yield preface. Field Crops Res 165:1–4

Park JH, Lee BW (2003) Photosynthetic characteristics of rice cultivars with depending on leaf senescence during grain filling. J Crop Sci 48:216–223

Patten C, Glick BR (1996) Bacterial biosenthesis of indole-3-acetic acid. Can J Microbiol 42:207–220

Pérez-Montaño F, Alías-Villegas C, Bellogín RA, del Cerro P, Espuny MR, Jiménez-Guerrero I, López-Baena FJ, Ollero FJ, Cubo T (2014) Plant growth promotion in cereal and leguminous agricultural important plants: from microorganism capacities to crop production. Microbiol Res 169:325–336

Piccinin GG, Dan LGM, Braccini AL, Mariano DC, Okumura RS, Bazo G, Ricci TT (2011) Agronomic efficiency of *Azospirillum brasilense* in physiological parameters and yield components in wheat crop. J Agron 10:132–135

Piccinin GG, Braccini AL, Dan LGM, Scapim CA, Ricci TT, Bazo GL (2013) Efficiency of seed inoculation with *Azospirillum brasilense* on agronomic characteristics and yield of wheat. Ind Crop Prod 43:393–397

Puente ML, Gualpa GL, Lopez GA, Molina RM, Carletti SM, Cassán FD (2017) The benefits of foliar inoculation with *Azospirillum brasilense* in soybean are explained by an auxin signaling model. Symbiosis. https://doi.org/10.1007/s13199-017-0536-x

Rai R (1991) Isolation, characterization and associative N-fixation of acid-tolerant *Azospirillum brasilense* strains associated with *Eleusine coracana* in low pH-Al-rich acid soil. In: Wright RJ, Baligar VC, Murrmann RP (eds) Plant–soil interactions at low pH. Developments in plant and soil sciences, vol 45. Springer, Dordrecht, pp 663–671

Reis VM, Baldani VLD, Baldani JI (2015) Isolation, identification and biochemical characterization of *Azospirillum* spp. and other nitrogen-fixing bacteria. In: Cassán FD, Okon Y, Creus CM (eds) Handbook for *Azospirillum*. Springer, Switzerland, pp 3–26

Robe P, Nalin R, Capellano C, Vogel T, Simonet P (2003) Extraction of DNA from soil. Eur J Soil Biol 39:183–190

Santa ORD, Hernadez RF, Alvarez GLM, Ronzelli P, Soccol CR (2004) *Azospirillum* sp. inoculation in wheat, barley and oats seeds greenhouse experiments. Braz Arch Biol Technol 47:843–850

Sharma E, Sharma R, Borah P, Jain M, Khurana JP (2015) Emerging roles of auxin in abiotic stress responses. In: Pandey GK (ed) Elucidation of abiotic stress signaling in plants. Springer, New York, pp 299–328

Shime-Hattori A, Kobayashi S, Ikeda S, Asano R, Shime H, Shinano T (2011) A rapid and simple PCR method for identifying isolates of the genus *Azospirillum* within populations of rhizosphere bacteria. J Appl Microbiology 111:915–924

Steenhoudt O, Vanderleyden J (2000) *Azospirillum*, a free-living nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects. FEMS Microbiol 24:487–506

Sukumar P, Legué V, Vayssières A, Martin F, Tuskan GA, Kalluri UC (2013) Involvement of auxin pathways in modulating root architecture during beneficial plant–microorganism interactions. Plant Cell Environ 36:909–919

Tamura K, Stecher G, Peterson D, Filipski A, Kumar S (2013) MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol 30:2725–2729

Tien TM, Gaskinsa MH, Hubbell NDDH (1979) Plant growth substances produced by *Azospirillurn brasilense* and their effect on the growth of pearl millet (*Pennisetum americanum* L.). Appl Environ Microbiol 37:1016–1024

Tsavkelova EA, Klimova SY, Cherdyntseva TA, Netrusov AI (2006) Microbial producers of plant growth stimulators and their practical use: a review. Appl Biochem Micro 42:117–126

Van Schouwenberg J, Walinge I (1973) Methods of analysis for plant material. Agricultural University, Wageningen

Venieraki A, Dimou M, Pergalis P, Kefalogianni I, Chatzipavlidis I, Katinakis P (2011) The genetic diversity of culturable nitrogen-fixing bacteria in the rhizosphere of wheat. Microb Ecol 61:277–285

Verma R, Chourasia SK, Jha MN (2011) Population dynamics and identification of efficient strains of *Azospirillum* in maize ecosystems of Bihar (India). Biotech 1:247–253

Westby C, Cutshall D, Vigil G (1983) Metabolism of various carbon sources by *Azospirillum brasilense*. J Bacteriol 156:1369–1372

Zahid M, Abbasi MK, Hameed S, Rahim N (2015) Isolation and identification of indigenous plant growth promoting rhizobacteria from Himalayan region of Kashmir and their effect on improving growth and nutrient contents of maize (*Zea mays* L.). Front Microbiol 6:207

Zahir A, Abbas SA, Khalid M, Arshad M (2000) Structure dependent microbially derived plant hormones by improving growth of maize seedlings. Pak J Biol Sci 3:289–291

Zarea MJ (2017) *Azospirillum* and wheat production. In: Kumar V, Kumar M, Sharma SH (eds) Probiotics in agroecosystem. Springer, Singapore, pp 329–348

Zarea MJ, Hajinia S, Karimi N, Mohammadi Goltapeh E, Rejali F, Varma A (2012) Effect of *Piriformospora indica* and *Azospirillum* strains from saline or non-saline soil on mitigation of the effects of NaCl. Soil Biol Biochem 45:139–146

Zlatev Z, Lidon FC (2012) An overview on drought induced changes in plant growth, water relations and photosynthesis. Emir J Food Agric 24:57–72