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CHARACTERIZATION OF BACTERIA ISOLATED FROM THE SAFFRON (*CROCUS SATIVUS* L.) RHIZOSPHERE

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ABSTRACT

One purpose of assessing the soil alive and active community is the identification of beneficial bacteria to use them as biological fertilizers, replacing or supplementing synthetic fertilizers. Such biofertilizers are predicted for the sustainability of agricultural production, especially for low input systems such as saffron fields. The aim of this work was to isolate and identify saffron rhizobacteria and to evaluate their possible effects on saffron growth. During 2013/14, some bacteria were isolated from the rhizosphere of the saffron plantations of different age in Gol village, Birjand, Iran. In total, 12 bacteria species were identified based on phenotypic traits and 16S rDNA sequences analysis. The strains were identified as *B. subtilis*, *B. anthracis*, *B. cereus*, *B. megaterium*, *Bacillus s*p., *Paenibacillus*, *Pseudomonas fluorescens*, *P. putida*, *Escherichia coli*, *Pectobacterium* sp. and *Pantoea* sp., with the dominant population belonging to the genus *Bacillus.* In the field study, inoculation of soil with these strains did not affect the leaf dry weight of the cultivated saffron, however, the strains of *P. fluorescens* increased the leaf area while *P. fluorescens, Paenibacillus, Pectobacterium and B. megaterium* increased the number of daughter corms and *Azotobacter, B. cereus, B. subtilis* and *B. megaterium* increased the corm weight. Our finding revealed that some bacteria present in the soil of perennial saffron plantations have a promising potential for developing as a plant growth promoting rhizobacteria.

INTRODUCTION

Saffron (*Crocus sativus* L.) flowers provide one of the most valuable spices of the world. More than 95 percent of this crop is produced in Iran (Kafi et al. 2006). Saffron is intrinsically adapted to low-input farming systems, and thereby shows a good response to biofertilizers (Ihsan et al. 2014). Identification of beneficial soil bacteria and using them as biofertilizer is a sustainable manner for promoting saffron growth and yield with minimum environmental risks (Singh & Kapoor 1999). It is proven that some bacteria can increase stigma fresh and dry weight (Aytekin & Acikgoz 2008), stigma length, leaf numbers, corm weight, and stigma yield of saffron (Rasouli et al. 2013). For example, inoculating saffron with *Bacillus subtilis* had led to higher quality and quantity of saffron flowers, probably as a result of bacterial siderophore production (Sharaf-Eldin et al. 2008). Some other reports have also emphasized on the positive effects of different biological fertilizers on saffron. In a study about integrated management of chemical and biological fertilizers, *Azotobacter* sp. could provide a supply of nitrogen to promote saffron growth, even at the lowest level of nitrogen consumption (Sofi et al. 2008). Omidi et al. (2009) reported that applying biological nitrogen fertilizers increased the stigma and style lengths, and they were as effective as those of synthetic nitrogenous fertilizers. The positive effects of biofertilizers on the number of leaves, corm weight, style and stigma weight, and contents of cytotoxic compounds crocin, safranal and picrocrocin have also been reported (Naghdi Badi et al. 2011). In a comparison of some bacteria and vermicompost types, the growth-promoting bacteria such as *Bacillus* spp. and *Pseudomonas* spp. strains had the greatest influence on the growth and physiological parameters

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of saffron, especially when they were applied together (Rasouli et al. 2013).

Very few studies have addressed the microbial community associated to saffron roots. Sharma et al. (2015) have isolated bacterial endophytes from saffron leaves and corms as possible growth-promoting bacteria. Among them *B. licheniformis* and *B. pumilus* were the dominant species. Because a large number of bacteria studied were able to produce siderophores and a wide range of enzymes, it seems that they can be employed as suitable growth promoting bacteria for saffron plants. It should be noted that not all bacterial species found in the saffron rhizosphere are beneficial, and *B. croci* and *Burkholderia gladioli* were recognized as potential saffron pathogens (Fiori et al. 2011).

Birjand County, especially Gol village, is one of the most important provinces in Iran, where saffron is grown giving the most desirable product. Much of saffron in this area is produced by smallholders under low-input systems. Promoting the beneficial rhizobacterial community can lead to improve saffron production in an ecological way. This research aims to isolate the bacteria species from the rhizosphere of saffron roots and to study their effects on saffron growth.

MATERIALS AND METHODS

This study was conducted during 2013/2014 at Gol village, Birjand, Iran (32°41'12" N, 59°10' E). On average, soils of this region contain about 0.37% organic matter, with a pH of 8.19 and electrical conductivity of soil saturated extract (EC_e) 2.46 $dS \cdot m^{-1}$.

Isolation and characterization of bacteria

Ten saffron plantations of different ages (years after corm planting) were selected. Four to five soil samples were taken randomly from each plantation, both during and after the plant growth cycle based on the Luster et al. (2009) method. The samples from one field were mixed and then kept in a refrigerator at 4 °C until the isolation of bacteria from them. The same fields were subjected to sampling in two successive years.

Bacterial isolates were recovered with the streaking of soil suspension on nutrient agar (NA), nutrient glucose agar (NGA), and King's B medium (Schaad et a6l. 2001). The number of isolates from a single plantation was from 2 to 22, and the total number of isolates was 61.

Morphological, physiological, and biochemical characteristics of purified colony, such as gram stain reaction, tolerance to various pH and salt concentration, fluorescent pigments and extracellular lipopolysaccharides (LPS) production, growth on MacConkey and Luria Bertani media, catalase and oxidase activity, starch, cellulose and pectin degradation, siderophores production on King's B medium, carbohydrate (arabinose, dulcitol, dextrose, sucrose, sorbitol, adonitol, mannitol and citrate) utilization, endospore formation, and lecithinase production were identified by the Schaad et al. (2001) methods.

All isolates were tested for hypersensitive reactions (HR) on the leaves of *Pelargonium hortorum* L. and on the ability to cause soft rot on potato tuber slices to eliminate possible pathogenic ones. Corms planted in containers were also monitored during the growth season and the rotting symptoms were not recorded on saffron corms during the experiment.

From 61 isolates that were obtained from different fields, 24 was G^+, 11 isolates were able to produce siderophores. Eighteen isolates belonging to 12 bacterial species were selected for the field experiment based on the age of the farms from which were isolated, and their characteristics, such as the ability for siderophore production, salt tolerance, starch utilization and cellulose degradation (Table 3).

Molecular identification

Genomic DNA was extracted according to Dellaporta et al. (1983) protocol from bacteria grown in the nutrient broth (NB). Amplification of 16S rRNA regions was done with 27f (5-AGATTTGACMTGGCTAG-3) as forward and a 1492R (5-GGTTACCTTGTTACGCTT-3) as reverse primers (Lin et al. 1991). Each reaction mixture contained 10 ng per µl of each template DNA, 1 µM of each primer, 1 mM $dNTP_s$, 1X PCR buffer, 1.5 mM $MgCl_2$, 2.5 units per each reaction of Taq DNA polymerase (CinnaGen Co., Iran) and deionized sterile water.

The PCR conditions consisted of an initial denaturation step for 5 min at 95 °C, 35 cycles of

95 °C for 60 sec, 50 °C for 60 sec, 72 °C for 2 min and a final extension at 72 °C for 10 min in an Eppendorf Mastercycler®.

The PCR products were verified by electrophoresis on 1% agarose gel in TBE buffer (89 mM tris HCL, 89 mM Boric acid, 2 mM EDTA, pH = 3.8) for 1 h at 70-volt constant. After dying with 1% of DNA safe stain (CinnaGen Co., Iran), the image of the resulting gel was acquired using a Document Gel system (CS Cleaver Scientific Ltd). The purification and sequencing of PCR products was outsourced to a public biotechnology company (Macrogen, Seoul, South Korea). Sequence alignment was performed using the ClustalX and Mega6 software (Tamura et al. 2013). Neighbor-joining method was used to construct a phylogenetic tree using the Mega6 software. The reliability of phylogenetic tree was assessed with 1000 bootstrap replications. The bootstrap confidence values more than 50% appeared in the phylogenetic tree.

Open-field experiment in containers

The effect of 18 selected strains (Table 3) along with three commercial PGPRs (*Azotobacter* sp., *P. fluorescens*, and *Azospirillum* sp., provided by Iranian Soil and Water Research Institute, ISWRI, and recommended as PGPRS) and a control (no inoculation with bacteria) on saffron growth was tested in field conditions in a complete randomized block design with 3 replications.

Preparation of bacteria inoculum

The bacterial isolates were grown in the nutrient glucose broth (NG) medium in 250 ml flasks shaken at 125 rpm at 30 °C (Somasegaran & Hoben 1994). When the subculture reached the mid log phase, the inoculums were diluted in water and added to the soil at 10^6 cfu·g^{-1} before corm planting and also three times during the growth seasons.

Preparation of culture in containers

Plastic containers (50 × 30 × 25) were used for corm planting. The bottom of the boxes was porous, covered by a graph paper and some sand as a drainage. Because of the need of a soil with very low microbial population at this stage, a poor soil (Table 1) was used to fill the planting boxes after inoculation. The soil used for saffron growth was obtained from a non-cultivated area located near the crocus sampling plantations. Soil moisture at field capacity was 16%.

Saffron planting and inoculation

Three blocks (10 m in length, 50 cm in width and 25 cm in depth) were excavated as ditches in outdoor, then the planting containers were placed so that their surfaces were located 5 cm above the surrounding soil level to prevent the interference in case of rain. 4000 ml water was added to each container in two doses to get the status of field capacity in soil. On a 10-cm layer of the moist soil, 9 corms at 10 cm distance were planted in three rows with 20 cm row spacing and 5 cm apart from the container wall. Then, the containers were filled with the remaining soil and finally 2000 ml water with 84 ml of bacterial suspension was added to each container (106 cfu·g^{-1} soil). Uniformly sized corms (≈ 8 gr) were used for planting.

Growing and sampling

A total of four inoculations were done during the saffron growth period. The first application was given simultaneously with planting at September 24, 2014, the second application in about 120 days later, and the third and fourth applications with 20 days intervals. All the containers were hand-weeded during the growth period. On April 11, 2015, two plants from each were harvested to measure the leaf area using a leaf area meter (ΔT Devices, Burwell, England, UK). At the same time, the dry weight of vegetative parts was recorded after 48 h at 75 °C. In mid-June 2015 (end of the growing season), all the plants were harvested, and the number and weight of the newly formed daughter corms were measured.

Statistical analysis

The collected data were analyzed using SAS (v 9.2) software and means were compared using the Fisher's Protected Least Significance Difference (FLSD) procedure at 0.05 probability levels.

RESULTS AND DISCUSSION

Characteristics of bacterial isolates

It is worth noting that this is the first report on the isolation of bacteria from the saffron rhizosphere in this area. In total, 61 bacterial isolates belonging to 12 species were obtained, from which 18

strains (Table 1) were selected based on the morpho-physiological characterization and used for saffron inoculation. The isolate BA11, which was obtained from the 15 years-old plantations, was gram positive, produced endospores and water-soluble gray pigment, and was tolerant up to 10% NaCl. Based on the results of the sugar fermentation test and other biochemical properties, this strain was recognized as *B. atrophaeus* (Nakamura 1989). Shanmugam et al. (2013) stated that *B. atrophaeus* is one of the bacteria that colonize plant roots and its genome contains a large number of antibiotic-coding genes. Thus, it seems that *B. atrophaeus* can protect plants as a biocontrol agent for plant pathogens.

BA12 and BA15 were endospore forming gram positive bacteria and belonged to *B. subtilis*, which is one of the most common bacteria in the soil. These two strains could grow on media containing 7% NaCl (Table 1), but did not produce siderophores on King's B medium (Table 1). Some strains of this species are known as PGPR that can improve plant growth and performance (Tilak et al. 2005), and are especially known in terms of solubilization of insoluble phosphate and siderophores production (Rai 2006). However, the isolated strains in our study did not produce siderophores. *B. subtilis* can also act as a plant growth promoter under nitrogen deficiency conditions, leading to more vigorous plants (Compant et al. 2005).

BA9 strain, from a 4 years-old plantation, belongs to *B. anthracis* and was facultative anaerobic, and salt tolerant up to 10% (Table 1). *B. anthracis* can cause both human and animal diseases, colonizes the soil, and is known as one of the saprophyte agents in the soil (Govindasamy et al. 2010). BA6, from the 1-year-old saffron plantation, belongs to *B. cereus* and is resistant to high salt concentration (Table 1). Some strains of *B. cereus* are known as PGPRs and can activate pathways of salicylic and jasmonic acids in plants, leading to an improved plant growth and resistance against plant pathogens (Niu et al. 2011).

Table 1. Some biochemical characteristics of soil bacteria isolated from saffron plantations of different age in Gol village, Birjand, Iran

Isolate code	Species	Plantat. age	Siderophore production	% salt resistance	Starch utilization	Cellulose degradation	Ability to cause soft rot
BA1	*Pantoea sp.*	2	-	6	+	-	-
BA2	*Bacillus sp.*	2	-	10	-	-	+
BA3	*B. megaterium*	7	-	10	+	+	-
BA4	*B. megaterium*	10	-	10	-	-	-
BA5	*Paenibacillus sp.*	7	-	10	+	+	-
BA6	*B. cereus*	1	-	10	+	-	-
BA7	*Pseudomonas fluorescens*	7	+	6	-	-	-
BA8	*P. putida*	5	+	4	-	-	-
BA9	*B. anthracis*	4	-	10	+	-	-
BA10	*B. subtilis*	7	-	6	+	-	-
BA11	*B. atrophaeus*	15	-	10	+	-	-
BA12	*B. subtilis*	7	-	6	+	-	-
BA13	*P. fluorescens*	6	+	4	+	-	-
BA14	*Pectobacterium sp.*	10	-	6	-	+	+
BA15	*B. subtilis*	6	-	6	+	-	-
BA16	*Escherichia coli*	2	-	4	+	-	-
BA17	*Paenibacillus sp.*	1	+	6	+	-	-
BA18	*B. megaterium*	2	-	10	+	-	+
Azt	*Azotobacter sp.*		-	-			
Ps	*P. fluorescens*		+	-			
Azo	*Azospirillum sp.*		-	-			

Table 2. Result of 16S rRNA gene sequence homology between the saffron isolates and GenBank sequences

Isolate code	Accession No. of the 16S rDNA sequence	Best closest match	Similarity (%)
BA2	KY357306	*Bacillus megaterium* (KY007586)	99
BA3	KY363590	*B. megaterium* BCRh8 (KT153604)	99
BA5	KY363584	*Paenibacillus apiarius* DSM 5581(NR_040890)	98
BA8	KY399977	*Pseudomonas putida* B33 (KT767698)	100
BA12	KY400654	*B. subtilis* RS2 (KF844069)	100
BA4	KY363592	*B. megaterium* IAM 13418 (NR_043401)	100

BA4, BA3, BA10 and BA18, which were isolated from 2, 10, and 7-years-old plantations respectively, belong to *B. megaterium* species. All these strains could tolerate 6–10% NaCl concentration and produced pectolytic enzymes (Table 1). *B. megaterium*, like other *Bacillus* spp., can produce large amounts of endospores and easily withstand adverse climatic conditions. It is a halophilic species able to grow at up to 15% salt concentration (Alderton et al. 1964), produces indole-acetic acid (IAA) and thereby can increase plant biomass and yield (Chakraborty et al. 2006).

BA5 and BA17 strains, which were isolated from the plantations of the 7 years-old and 1 year-old respectively, belong to *Paenibacillus* spp. (Table 1). They tolerated 6% and 10% of salt concentrations, and had pectolytic activity as well. BA17 was able to produce siderophores on King's B medium. *Paenibacillus* spp. is a facultative anaerobic, endospore-forming bacteria (Ash et al. 1993), and thus, is able to survive under harsh conditions for a long time (Mandic-Mulec & Prosser 2011). The isolates were able to solubilize phosphates, and produce exo-polysaccharides, hydrolytic enzymes (glucanase, cellulase, chitinase) and enhance soil porosity. They can also produce auxin and cytokinin. *Paenibacillus* spp. can use different carbohydrate sources and also produce various antibacterial compounds including antibiotics, bacteriocins, and antifungal compounds (Govindasamy et al. 2010).

BA13 and BA7 were isolated from 6 and 7 years-old plantations respectively and identified as *P. fluorescens*. Both strains were salt tolerant up to 6% NaCl concentration and produced siderophores (Table 1). These bacteria can protect plants against potentially harmful microorganisms, and contribute to better plant growth through the production of various compounds such as hydrogen cyanide, siderophores, antifungal metabolites, and antibiotics (Kloepper et al. 1980; Kumar et al. 2002).

BA8, from 5 years-old plantation, which produces siderophores but only tolerates 4% salt concentration, was identified as *P. putida* (Table 1). It is a species that can biologically control the plant pathogens and increase the efficiency of iron absorption in saline-sodic soils, and thereby promote plant growth (Meziane et al. 2005). *E. coli* (BA16) was isolated from the soil of the 2 years-old saffron plantations (Table 1). These bacteria have not yet been proposed as plant pathogens, and were characterized as providing suitable conditions for plant growth by root colonization (Cooley et al. 2003).

BA14 and BA1 strains were isolated from 10 and 2 years-old plantations, and were identified as *Pectobacterium* spp. and *Pantoea* spp., respectively. Both can tolerate salt up to 6% concentration (Table 1).

Molecular identification

The sequence of 16S rRNA of BA4 (KY363592) isolate showed more than 99% similarity with the sequence of *B. megaterium* species in the gene bank (Table 2). In the phylogenic tree design based on the sequences of gene bank and our results, this strain was placed in the same group with *B. megaterium*, *B. flexus*, *B. aryabhattai* and *B. simplex* (Fig. 1). The BA4 biochemical characteristics were identical with BA18 strain, and the difference was only for starch degradation. Both tolerated 10% salt concentration and produced pectolytic enzymes (Table 1); thus, these two strains may belong to the same species, *B. megaterium*. Based on the similarity of 16S rRNA sequences and some biochemical

properties, it is suggested that BA2 (KY357306) and BA3 (KY363590) isolates also belong to *B. megaterium* (Fig. 1, Table 2). This species is one of the most abundant species of *Bacillus* in terms of distribution. Sequences of ITS regions showed that BA5 (KY363584), BA8 (KY399977) and BA12 (KY400654) isolates belong to the *Paenibacillus* sp., *P. putida* and *B. subtilis* species respectively (Table 2).

Based on ANOVA results, only plant dry weight did not differ significantly among plants inoculated with different strains, whereas there were significant differences in the leaf area, number of daughter corms and corms weight (Table 3).

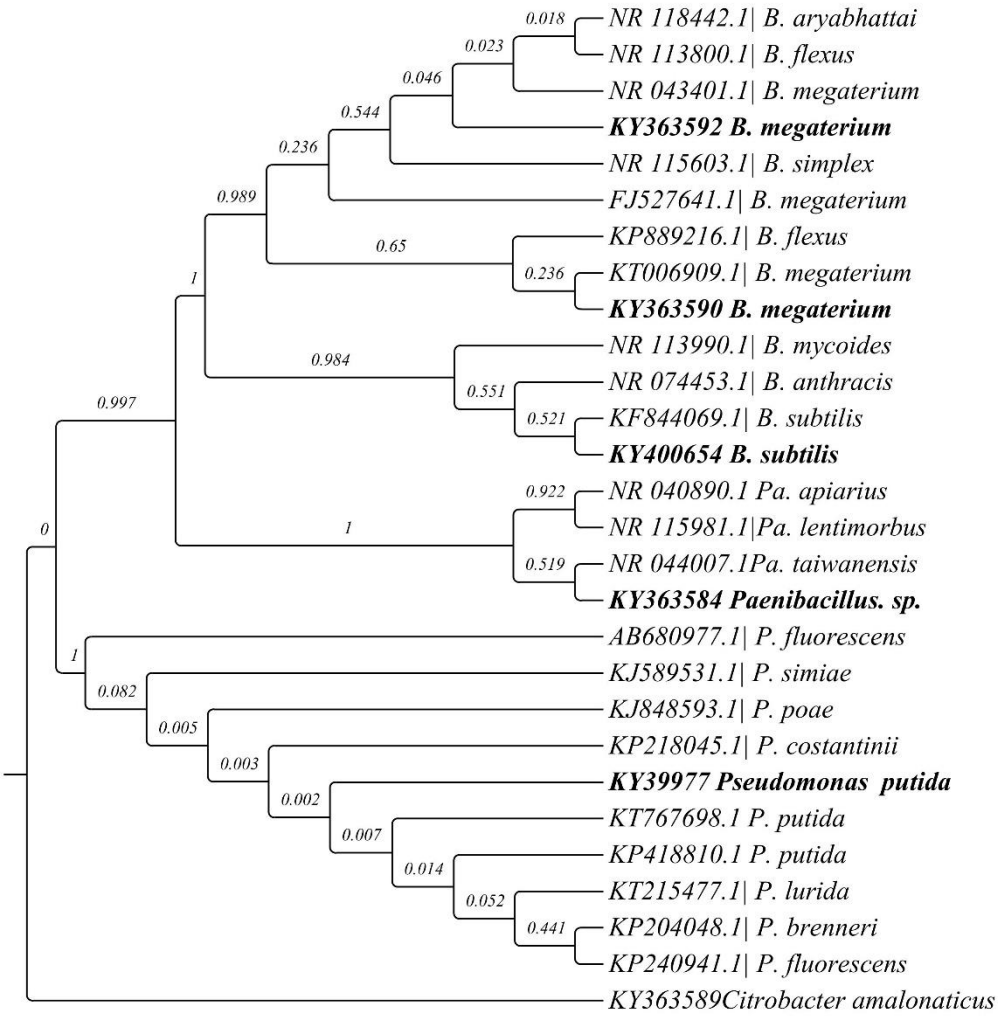

Fig. 1. Neighbor-joining phylogenetic tree showing the relationships among bacterial strains based on partial 16S rRNA gene sequences (bold). Neighbor-joining distance tree was constructed with bootstrap values (% of 1,000 replicates)

Table 3. Mean squares of measured traits of saffron inoculated with selected strain.

S.O.V	d.f	Plant dry weight	Leaf area	Daughter corms	
				No.	weight
Block	2	$18.37^{n.s}$	$0.029^{n.s}$	$2^{n.s}$	$13.19^{n.s}$
Isolates	21	$16.86^{n.s}$	0.126^{**}	21^{**}	16.79^{**}

$^{n.s}$ – means non-significant; ** – is significant difference at 0.01 probability level.

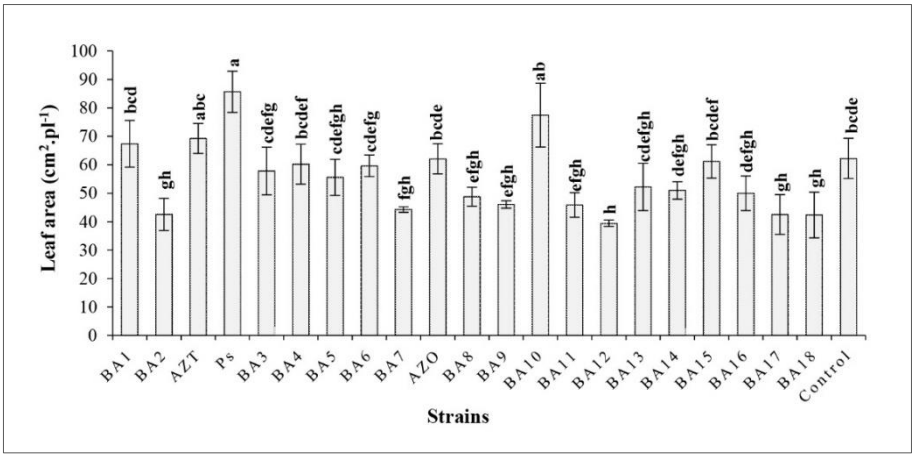

Fig. 2. Leaf area of saffron plants. Saffron corms were planted outdoor on September 24, 2014 in soil inoculated with different bacteria strains. The measurements were done 200 days after planting. For more information about strains, please refer to text and Table 2. Vertical bars on the columns show SEM and different letters above the columns indicate significant differences at the 0.05 level according to the LSD test.

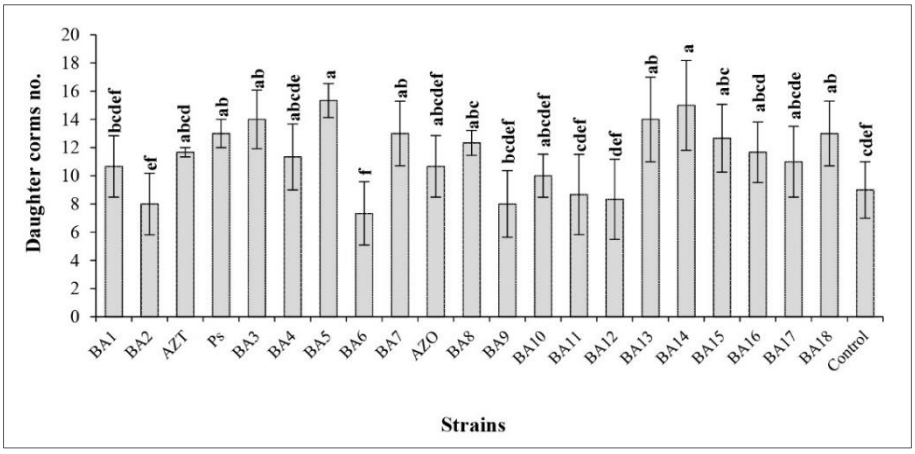

Fig. 3. The number of newly formed daughter corms. Saffron corms were planted outdoor on September 24, 2014 in soil inoculated with different bacteria strains, and the number of newly formed daughter corms was counted in mid-June 2015 (at the end of the growth season). More comments see Fig. 2.

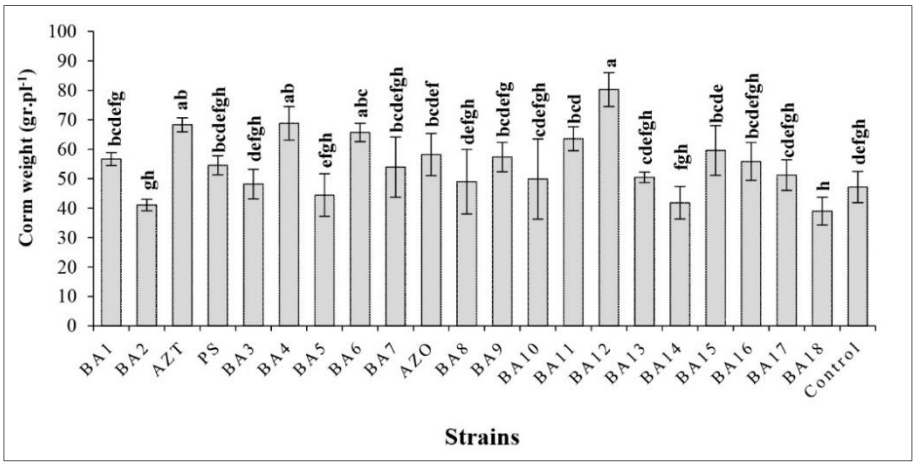

Fig. 4. The weight of newly formed daughter corms. Saffron corms were planted outdoor at September 24, 2014 in soils inoculated with different bacteria strains, and daughter corms were harvested and weighed in mid-June 2015 (at the end of the growth season). More comments see Fig. 2.

Open-field container experiment

At the end of growth season, only *P. fluorescens* significantly increased (by 8.3%) the leaf area compared to the non-inoculated control. Five isolates influenced negatively on this trait (Fig. 2). According to Mayak et al. (2004), *Pseudomonas* sp. can enhance plant photosynthesis through producing phytohormones, thus increasing phosphorus absorption by plant, nitrogen fixation and synthesis of enzymes modifying ethylene level in plants. BA2 (*Bacillus* spp.), BA7 (*P. fluorescens*), *B. subtilis* (BA12), BA17 (*Paenibacillus* spp.) and BA18 (*B. megaterium*) reduced the saffron leaf area significantly, compared to the control.

Data from literature provide information that isolates belonging to the species tested here also stimulate the growth of plants (Kaymak et al. 2008; Sharaf-Eldin et al. 2008; Rasouli et al. 2013) but isolates obtained from saffron rhizosphere did not have such properties or were improperly applied.

Based on ANOVA results, applied inoculants had a significant effect on the number of saffron daughter corms (Table 3). At the end of the growing season, the largest number of corms resulted from inoculation with BA5 (*Paenibacillus* spp.) and BA14 (*Pectobacterium* spp.), which caused 70.3 and 66.6% increase in the number of daughter corms, respectively, compared to the control (Fig. 3). These bacterial species, with a possibility of enzymatic degradation of starch to fructose and glucose, provide plants with more energy and stimulate root expansion by producing growth regulators that improve absorption of water and nutrients by plants (Kumar et al. 2011). A significant increase (44 to 55%) in the bulb number was also caused in *P. fluorescens*, and *B. megaterium*, whereas inoculation with BA6 (*B. cereus*) led to the lowest daughter corms number (Fig. 3).

In addition to nitrogen fixation and modification of the uptake of macro- and micronutrients by plants, these species were characterized as synthesizing and secreting some plant growth regulators and various amino acids, antibiotic, and so on (Mayak et al. 2004; van Loon 2007); and in this way, they have been able to stimulate the growth and development of saffron roots and shoots.

The weight of saffron daughter corms was significantly affected by applying inoculants (Table 3). The greatest daughter corm weight was recorded with the BA12 (*B. subtilis*) treatment (70.25% more weight than the control) (Fig. 4). Phosphate solubilization, siderophore production, and degrading starch to glucose (Huang et al. 1998; Rai 2006) are among the assumed mechanisms by which *B. subtilis* can improve plant growth. BA4 strain (*B. megaterium*) and AZT (*Azotobacter* spp.) increased the weight of daughter corms by about 45% over control plants (Fig. 4). These species are known as nitrogen fixation bacteria in rhizosphere that are able to synthesize and release some compounds including B vitamins, nicotinic acids, pantothenic acid, biotin, auxins and gibberellins, that have useful and effective roles in augmenting the root uptake efficiency (Lugtenberg & Kamilova 2009). BA6 (*B. cereus*) also increased the weight of daughter corms by 39.37% over the control. This positive effect may be attributed to its ability to degrade starch to fructose and glucose, supplying plants with more energy sources and thereby promoting the growth of corms and plant. In contrast, four strains: BA2, BA5, BA14 and BA18 decreased the weight of the produced daughter corms (Fig. 4). It should be mentioned that BA5, BA14 and BA18 are between isolates that caused the production of significantly more daughter corms then control plants. Moreover, plants grown in the soil inoculated with BA18 strain also had one of the lowest leaf areas (Fig. 2) that was accompanied with 17.4% lower weight of daughter corms than the control (Fig. 4). A possible reason is that this strain caused soft rot of plants, which is expressed by an excessive corms proliferation.

CONCLUSION

In this study, 61 bacterial isolates were obtained from the soils of 1 to 15 years-old saffron plantations. The results of biochemical and molecular tests indicated that the dominant species was genus *Bacillus*. This may be caused by the special growth behavior of saffron plants, which is in a quiescent state over the summer period when plantations are not irrigated and in hot and dry soil only

species forming endospores, as *Bacillus* are able to survive. Most of the isolates selected in this study for the evaluation of their ability to improve saffron growth were able to tolerate a higher level of salt in in vitro tests, probably due to the adaptation of these bacteria to the saline soils that are prevalent in this area. Some bacteria can improve the saffron agronomic traits as compared to the control. To be sure that they can be recommended for use on commercial plantations as PGPRs, further field experiments should be undertaken.

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