International Journal of Botany Studies
ISSN: 2455-541X; Impact Factor: RJIF 5.12
Received: 11-02-2019; Accepted: 09-03-2019
www.botanyjournals.com
Volume 4; Issue 3; May 2019; Page No. 35-42

Isolation and characterization of rhizobacteria from Dawadmi soil region of Saudi Arabia and their effect on *Triticum aestivum* Logaimi seedlings under drought stress

MM Alqahtani[1*], AS El-Enany[2]
[1] Department of Biology, Dawadmi Faculty of Science and Humanities, Shaqra University, Saudi Arabia
[2] Department of Biology, Sajer Faculty of Science and Arts, Shaqra University, Saudi Arabia

Abstract
Drought is the major abiotic stress of borderlands, which reduces the ability of plants to absorb water from the soil and reduces plant growth. The search for drought resistant bacteria from dry environments around the world to mitigate the negative effects of plant growth is our goal. So, we attend to isolate the characterize rhizospheric bacteria of legume and non-legume plants in the desert region of Dawadmi, Riyadh region, Saudi Arabia. Ten rhizosphere soil samples and plant roots were collected from Dawadmi region. In our laboratory study, it was observed that all rhizobacterial strains had the ability to produce auxin in varying amounts with different efficacy. Soaking wheat grains overnight in PGPB cultures showed an overcome on drought stress. The percentage germination, germination index and growth rate of wheat seedling were improved as compared with drought-stressed wheat seedlings.

Keywords: drought, rhizobacteria, 16S rRNA, phytohormones, *Triticum aestivum*

1. Introduction
Drought is a severe abiotic parameter that adversely affects plants growth and productivity worldwide. In the near future, the increase of global warming will increase the severity of drought leading to a significant decrease in world food production. It is expected that by 2050, the drought will cause severe plant growth problems for about 50% of the arable lands (Vinocur and Altman, 2005) [56]. Several agro-biotechnological strategies were used to improve the tolerance of plants to drought stress, such as the generation of novel transgenic plants with altered expression genes or by introducing novel genes (Liu *et al*., 2013) [29]. However, the generation of tolerant plant varieties to drought stress is very difficult due to the complexity of mechanisms, moreover, genetically altered plants are not accepted in some countries of the world (Wahid *et al*., 2007) [58]. Thus, the search for new applicable strategies for improvement of plants growth and productivity against the drought stress is very important.

Recently, there has been widespread interest in the inoculation of soil with plant growth promoting rhizobacteria (PGPR) for improving soil fertility and alleviating drought stress on plants as an alternative eco-friendly strategy (Dimkpa *et al*., 2009; Etesami and Beattie, 2018; Saghafi *et al*., 2018) [12, 14, 43]. Plant growth promoting rhizobacteria (PGPR) are about 2-5% of beneficial native soil bacteria that colonize plant roots and promotes plant growth, immunity, and productivity (Antoun and Kloepper, 2001; Yang *et al*., 2009) [6, 60]. Several previous studies indicated that the rhizobacteria isolated from plants grown under drought stress in arid and semi-arid regions are frequently adapted to adverse conditions and are able to stimulate plant growth under different environmental stresses (Ashraf *et al*., 2010; Dodd and Perez-Alfocea, 2012; Kavamura *et al*., 2013; Etesami and Beattie 2017a,b; Meena *et al*., 2017) [9, 13, 24, 14, 15, 36].

Several PGPR are reported to induce drought stress tolerance in some plants such as wheat, maize, sunflower, sugarcane and green gram (Sandhya *et al*., 2009, 2010; Moutia *et al*., 2010; Vardharajula *et al*., 2011; Saravanakumar *et al*., 2011; Kasim *et al*., 2013) [45, 46, 38, 55, 48, 23]. Additionally, combined inoculation of legumes with rhizobia and PGPB could increase the nodules number and thus may promote plant performance and crop yields under stress conditions (Etesami and Beattie, 2017; Shrivastava and Kumar, 2015) [14, 49]. The mechanisms by which PGPR could be beneficial to the plants are: (i) bioremediation of heavy metals from contaminated soil (Glick, 2010 a) [17]; (ii) synthesis of ACC (1-aminocyclopropane- 1-carboxylate) deaminase, an enzyme responsible for decreasing ethylene in the plant roots (Glick, 2004b) [19]; (iii) nitrogen fixation in plant roots; (iv) production of siderophores; (v) production of phytohormones such as indole-3-acetic acid (IAA); (vi) control of plant pathogens (Compant *et al*., 2005) [11]; (vii) phosphate solubilization; and (viii) improvement of abiotic stresses tolerance (Glick, 2014c) [20]. It has been stated that these rhizobacteria could be used instead of chemical fertilizers and pesticides (Adesemoye and Kloepper, 2009; Kim *et al*., 2011) [3, 25].

The present study was carried out under natural (non-sterile) conditions how the single or combined inoculation of drought-isolated plant growth promoting bacteria mitigate the plants grwon under drought stress. As drought stress markers, morphological, physiological and biochemical plant parameters such as the growth parameters and shoot enzymatic antioxidant activities [superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR)] will be measured. All these values are involved in the plant responses for survival in drought stressed soil. Additionally, the production of proline, MAD and and IAA-like molecules by bacteria growing in axenic culture under increasing osmotic stress levels [induced by polyethylene glycol (PEG)] was also evaluated over time to determine the ability of this strain to

thrive under drought conditions.

The research aims to minimize the negative impact of the water deficit (drought) using biological methods by inoculating the soil with isolated growth promoting bacteria as an alternative to costly chemicals, which have a negative impact on the environment and human health.

2. Material and Methods

2.1 Collection of Samples

Ten samples were collected from rhizospheric soil of Mdicago sativum and Cyperus sp plants growing in a drought affected area of Dawadmi, Saudi Araia. The rhizospheric soil sample was collected by removing the plant root and the attached soil was collected into a sterile plastic bag and immediately transferred to the laboratory for isolation of bacteria.

2.2 Isolation of Bacteria

The bacterial strains were isolated from the rhizospheric soil by pour plate method using the serial dilution plating technique on nutrient agar medium contained (g/l): Beef extract, 10.0; peptone 10.0; NaCl, 5.0; agar 15.0 and supplemented with griseoflavin (50µg/ml) and adjusted to pH 7. The plates were incubated at 37°C for 4 days and the developed individual colonies were picked up, purified and stored at 4°C.

2.3 Phenotypic and genotypic identification of the isolated bacteria

2.3.1 Morphological and biochemical characterization

Morphological and biochemical characteristics of the isolated bacteria and actinomycetes were determined based on the standard methods described in Bergey's Manual of Determinative Bacteriology (Holt, 1994) [22] and Bergey's Manual of Systematic Bacteriology (Logan and De Vos, 2009) [30]. The Gram stain was performed on 24 h culture for bacteria and 4 days for actinomycetes and the shape of cells was recorded under the oil immersion of light microscope. The surface and shape of the spores of the isolated actinomycetes were examined by scanning electron microscope. Biochemical tests such s catalase, methyl red, Voges-Proskauer, citrate, urease, H_2S production, starch hydrolysis, glucose, and lactose fermentation and motility were studied for bacteria. On the other hand, starch hydrolysis, casein hydrolysis, milk coagulation, milk peptonization, gelatine liquefaction, utilization of D-glucose, D-arabinose, D-xylose, raffinose, meso-inositol, sucrose and L-rhamnose were detected for actinomycetes.

2.3.2 Identification of bacterial isolates using 16S rRNA

Isolated DNA of bacterial samples were extracted using kit of bacterial DNA preparation (Jena Bioscience) as follows: Pellets of the cell were mixed to 300 µl lysis buffer and 2 µl RNAase A and vigorously vortexed for 20-60 sec. 8 µl proteinase K was added, incubated at 60°C for 10 min. Then add 300 µl binding buffer and place on ice for 5 min to centrifuge for 5 min at 10,000 g. 500 µl washing buffer was added into spin column to the mixture, centrifuge for 30 sec at 10,000 g. Finally, 40-50 µl elution buffer was added into the column, incubate at room temperature for 2 min. Then, centrifuge at 10,000 g for 2 min and store DNA at -20°C till used.

The amplification of 16S rRNA gene using PCR was done by Qiagen Proof-start Tag Polymerase kit (Qiagen, Hilden, Germany) using the two primers 16SF: 5′-GAGTTTGA TCCTGGCTTAG-3′ and 16SR: 5′-GGTTACCTTGT TACGACTT-3′. 25 µl of reaction mixture,2 µl of template DNA (20 ng /µl), 12.5 µl PCR master mix, 20 pmol of each forward and reverse primers and DNAase free water (8.5 µl) was incubated at automated thermo cycler TC3000. The condition of reactions were: an initial denaturation at 94°C for 5 mins, 37 cycles of denaturation at 94 °C for 30 sec, annealing at 51°C for 30 sec, and extension at 72° C for 30 sec. The final extension was done at 72 ° C for 5 minutes. PCR products were electrophoresis on 1.5 % (w/v) agarose in 1X TAE buffer and then analyzed by Gel Docu advanced ver.2 software.

PCR products of about 1000 bp were purified with kit of QIA quick gel extraction (Qiagen, Hilden, Germany) and sequenced by cycle sequencing with didesoxy mediated chain-termination (Sanger *et al*., 1977) [41]. DNA sequencing was done by 3500 Genetic Analyzer, Applied Biosystems (Biotechnology Research Center, Suez Canal University, Egypt). The resulted sequences of 16S rRNA genes of the bacterial isolates were investigated on the advanced BLAST search program on the NCBI website: http:// www.ncbi.nlm.nih.gov/BLAST/ in order to determine similarity of DNA. Multiple sequence alignment and the phylogenetic tree were carried out using MEGA6 program. Phylogenetic tree derived from 16S rRNA gene sequence was generated in comparison to 16S rRNA gene sequences of different bacterial strains in Gen Bank database.

2.3.3 Screening for IAA Production

IAA production by the isolated bacteria was assayed based on the method of Abd-Alla *et al*., (2013) [2]. One ml inoculum of each bacterial isolate grown on nutrient broth and incubated at 37°C for 48 hrs, was transferred to 50 ml tryptone yeast extract broth containing 2 mg/ml L-trytophan. These cultures were incubated at 37°C with shaking at 125 rpm for 48 hrs and then harvested by centrifugation at 11,000xg for 15 min. One milliliter of the bacterial supernatant was mixed with 2 ml of Salkowski reagent and left for 10min at room temperature. The reagent was prepared by dissolving 2.03 g $FeCl_3.6H_2O$ in 500 ml water and 300 ml concentrated H_2SO_4 (Salkowski, 1885) [44]. The appearance of a pink color indicated IAA production and its optical density (OD) was read at 535 nm. The concentration of IAA produced was determined from the IAA standard curve.

2.4 PGPB Treatments

The plastic pots containing 5 Kg soil will divided into three groups, group 1 serve as control, group 2 plants were inoculated with Rhizobium, and group 3 plants were inoculated with Streptomyces. The three groups were grown under different regime of drought stress (0, 25, 50 and 75% as low, moderate and hight drought, respectively). Each treatment was triplicated.Experiment was carried out Climatic greenhouse of Biology department, Dawadmi Faculty of Science, Shaqraa University Saudi Arabia

II. Measurements of Growth Parameters: Germination percentage and the fresh and dry wight of seedlingswere determined according to Ashref *et al*., (1990) [9].

3. Results and Discussion

3.1 Isolation of rhizobacteria

The major concern of the current study amied to isolate and

select the most drought tolerant bacterial isolates to be used as inoculants to enhance the plants growth and productivity. In the current study, A total of 63 (1419 x10^4 cfu/g soil) bacterial isolates were recovered from the ten collected rhizospheric soil samples, 37 isolates (578.5 x10^4 cfu/g soil) out of them were isolated from the rhizospheric soil of Medicago sativeum (legume plant), whereas the other 26 (840.5 x10^4 cfu/g soil) were isolated from Cyperus sp rhizospheric soil (non-legume plant) Figure (1). Drought is a major abiotic stress may cause severe productivity losses in arid and semiarid regions where the agriculture mainly depends on rains (Minakshi *et al*., 2013) [37]. Overcoming abiotic stress gaining importance through using rhizobacteria (Kavamura *et al*., 2013; Goswamia *et al*., 2014; Vurukonda *et al*., 2016) [24, 20, 57]. The rhizopshere is a region of soil highly loaded by rhizobacteria because it is the zone of the plant and microbes interaction. It is a vital source of biomolecules in soil which contributes to soil fertility by the release and decomposition of root debris and also through root exudation (Lynch, 1990) [31].

3.2 Identification of the isolated rhizobacteria

The collected 63 bacterial isolates were divided into 10 groups according to their morphological characteristics; the groups A, B, C, D, E, F, and G were assigned as eubacteria whereas the groups H, I and J were assigned as actinomycetes. The morphological and biochemical characteristics of the seven groups of the isolated bacteria were shown in table (1). Based on the morphological and biochemical characteristics of the isolated bacteria, the groups A, B, C, D, E, F, G were identified on the species level as Bacillus cereus, Bacillus megaterium, Bacillus firmus, Bacillus timonensis, Enterobacter cloacae, Pseudomonas alcaligenes and Ochrobactrum intermedium, respectively. However, the characteristics of the others three groups (H, I and J) of the isolated actinomyctes were identified as Streptomyces griseoflavus, Streptomyces roseolus, Streptomyces flavoviridis, respectively as shown in table 2. The captured images by scanning electron microscope of spores chains of the isolated actinomycetes were shown in figure (2).

One representative bacterial isolate from each group was subjected to partial for further identification by 16S rRNA gene sequene analysis. The 16S rRNA gene sequences of the selected isolates were compared to 16S rRNA gene sequences in Gen Bank database using BLAST search analysis, and the phylogenetic tree of their positions between the most related bacterial species was shown in figure (3). The selected ten bacterial isolates; Streptomyces roseolus SAU30, Streptomyces griseoflavus SAU3, Bacillus megaterium SAU4, Ochrobactrum intermedium SAU7, Bacillus cereus SAU19, Pseudomonas alcaligenes SAU22, Bacillus firmus SAU24, Enterobacter cloacae SAU34, Bacillus timonensis SAU46 and Streptomyces flavoviridis SAU12 were deposited under the accession numbers MH917244, MH917245, MH917246, MH917247, MH917248, MH917249, MH917250, MH917251, MH917252 and MH917253, respectively, in GenBank database.

3.3 Occurrence and frequency of the isolated rhizobacterial species

The total colony forming units of the isolated ten bacterial species from the collected ten rhizospheric soil samples was 1419 x10^4 cfu/ g soil with the highest frequency of Bacillus timonesis (8 t out of 10 samples) Table (3). Each of Streptomyces flavoviridis, Streptomyces griseoflavus, Streptomyces roseolus, Bacillus cereus, Bacillus firmus, Bacillus timonensis and Pseudomonas alcaligenes were isolated from both of the rhizosphere of Medicago satevium and Cyperus sp. However, the others three bacterial species Bacillus megaterium, Ochrobactrum intermedium and Enterobacter cloacae were isolated only from the rhizosphere of Medicago satevium. The highest bacterial count (129 X 10^4 cfu/g) in the rhizosphere of Medicago satevium was obtained with B. cereus, while the highest bacterial count (280 x 10^4 cfu/g) in the rhizosphere of Cyperus was obtained with Streptomyces flavoviridis. The high frequency of Bacillus spp. in the arid land might be attributed to their ability to form endospores, which enable bacteria to survive unfavorable environmental conditions such as drought stress (Nicholson *et al*., 2000; Marasco *et al*., 2012) [39, 34]. Some Bacillus spp. have also been isolated from desert regions like the Sinai desert (Othman *et al*., 2003) [40], semi-arid regions (Puente *et al*., 2009) [41]. Hanna *et al*., (2012) [21] isolated bacteria associated to several plants from a desert in Egypt, similar to those obtained in the present study such as B. megaterium and Enterobacter cloacae.

3.4 Screening of IAA production by the isolated rhizobacteria

All the isolated bacterial strains in the present study were screened for their ability to produce indole acetic acid (IAA) from tryptophan, it was indicated that, most of the isolated rhizobacterial strains were able to produce IAA but with varied efficiency. Among the tested 33 bacterial isolates, 22 isolates were able to produce IAA, while only 8 isolates out of 30 of actinomycetes have the ability to produce it. The most highly IAA producer isolate of the recovered actinomycetes was Streptomyces roseolus SAU60 with IAA of 32.76 µg/ml (Figure, 4). On the other hand, the most highly IAA producer isolate of the recovered bacteria was Enterobacter clocae SAU34 with IAA of 79.15 µg/ml, followed by Bacillus firmus SAU24 with IAA of 32.76 µg/ml (Figure, 5).

Indole acetic acid is produced by many microbes including PGPR which is an important plant growth regulator (Spaepen *et al*., 2007) [45]. Therefore, there is a close interaction between auxin producing PGPR and plants (Malhotra and Srivastava, 2006; Anjum *et al*., 2011; Abd-Alla *et al*., 2014) [32, 5, 1]. IAA production is an important feature because it is responsible for the regulation of several cellular and plant growth processes (Lambrecht *et al*., 2000) [27]. Several bacterial species have capacity to produce IAA, like Pseudomonas sp., Bacillus sp., Azotobacter sp. and others (Ahmad *et al*., 2008) [4]. Manulis *et al*., (1994) [33] have reported various Streptomyces sp. that produce IAA when provided with tryptophan while (Swain *et al*., 2007)

[52] have reported IAA producing Bacillus subtilis. In Enterobacter cloacae, IAA was synthesized via indole-3 pyruvic acid (Koga *et al*. 1991) [26]. It was possible to observe a high IAA production by members of the Enterobacteriaceae family and this has also been reported.

Table 1: Phenotypic characteristics of the isolated bacteria

Test	Bacterial groups						
	A	B	C	D	E	F	G
Gram staining	+	+	+	+	-	-	-
Cells shape	rod	rod	rod	rod	rod	rod	rod
Spore formation	+	-	-	-	-	-	-
Catalase	+	+	+	+	+	+	+
MR	+	+	+	+	+	+	-
Vp	+	-	-	-	-	-	-
Citrate	-	+	+	-	+	+	+
Glucose ferm.	-	+	+	-	+	-	-
Lactose ferm.	-	+	-	-	+	-	-
Urease	+	-	-	-	-	-	+
H_2S production	-	-	-	-	-	-	+
Motility	-	-	-	-	-	-	+
Starch hydrolysis	+	+	+	+	-	-	-

Table 2: Phenotypic characteristics of the isolated Actinomycetes

Test	Bacterial groups		
	H	I	J
Colony colour	gray	light pink	white
Spore chain morphology	spiral, long	Straight	spiral
Spore surface	spiny	smooth	smooth
Production of diffusible pigment	none	Pale yellow	yellow
Melanin production	negative	negative	negative
Starch hydrolysis	positive	positive	positive
Casein hydrolysis	positive	positive	negative
Milk coagulation	negative	negative	positive
Milk peptenoization	negative	negative	negative
Gelatin liquefaction	positive	negative	positive
Utilization of			
D-glucose	positive	positive	positive
D-galactose	positive	positive	positive
D-arabinose	positive	positive	positive
D-xylose	positive	positive	negative
raffinose	negative	negative	negative
Meso-inositol	positive	negative	negative
Sucrose	positive	negative	negative
L-rhamnose	positive	positive	Positive

Table 3: The colony forming units (CFU/g) and frequency of the isolated bacterial species

Bacterial species	Medicago Satevium		Cyperus sp.		Total		
	X 10^4 CFU/g	Frequency (out of 5)	X 10^4 CFU/g	Frequency (out of 5)	X 10^4 CFU/g	Frequency (out of 10)	Frequency (%)
Streptomyces flavoviridis	111.5	4	280	2	391.5	6	60
Streptomyces griseoflavus	119	4	177	2	296	6	60
Streptomyces roseolus	15	1	71.5	2	86.5	3	30
Bacillus cereus	129	3	20	1	149	4	40
Bacillus firmus	16.5	2	50	1	66.5	3	30
Bacillus timonensis	111.5	5	157	3	268.5	8	80
Bacillus megaterium	9	1	0	0	9	1	10
Ochrobactrum intermedium	25	2	0	0	25	2	20
Pseudomonas alcaligenes	40	3	85	1	125	4	40
Enterobacter cloacae	2	2	0	0	2	2	20

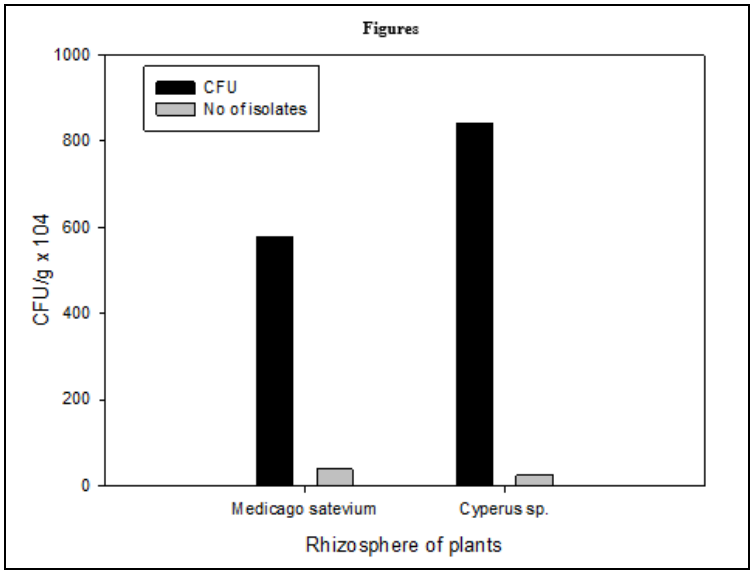

Fig 1: Colony forming units (CFU/g soil) and number of isolates of the recovered bacteria from the two studied plants.

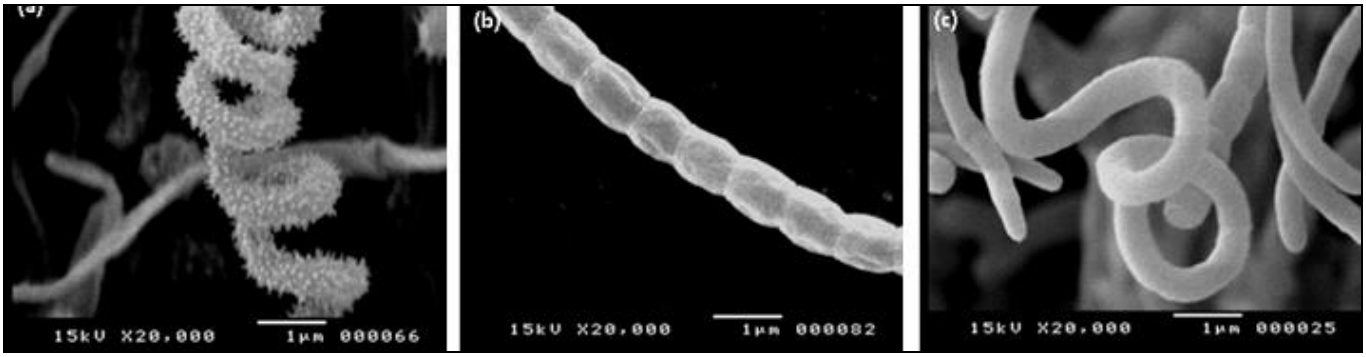

Fig 2: Shapes of spore chains of (a) bacterial group H, (b) bacterial grou
p I and (c) bacterial group J.

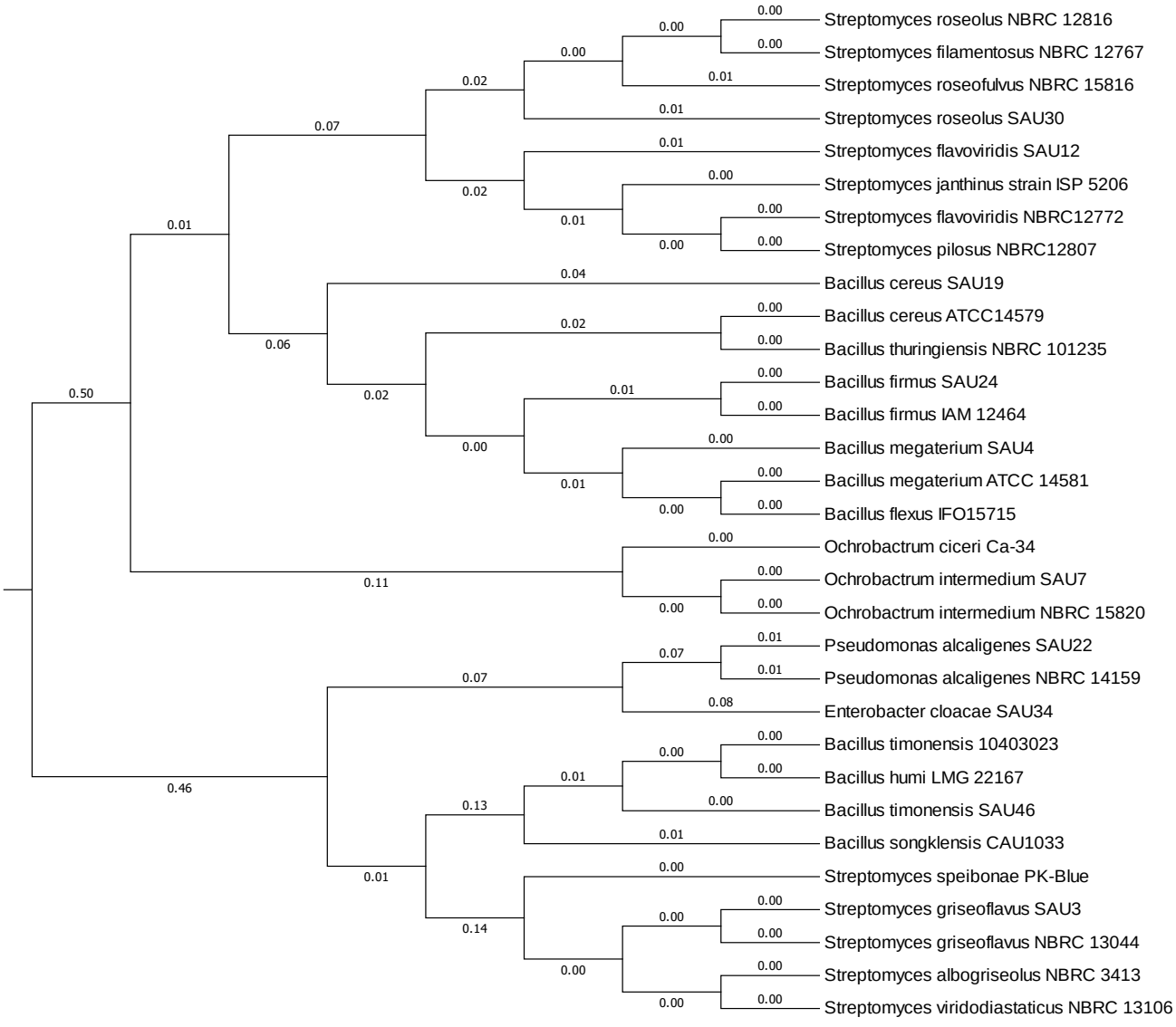

Fig 3: Evolutionary relationships of the isolated taxa and the related species in the GenBank database.

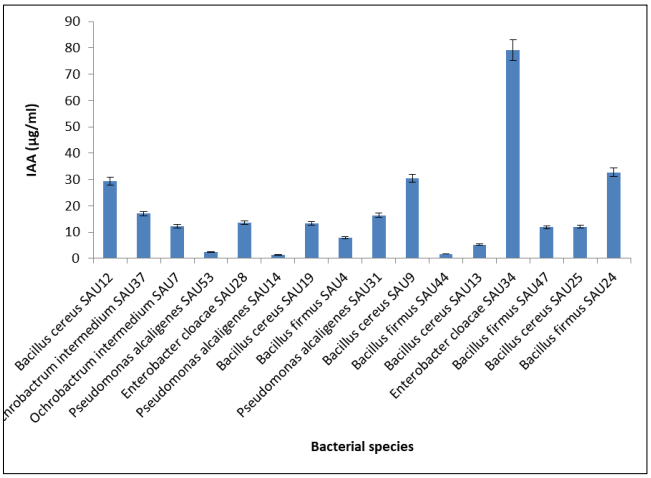

Fig 4: Indole acetic acid production by the isolated bacterial species

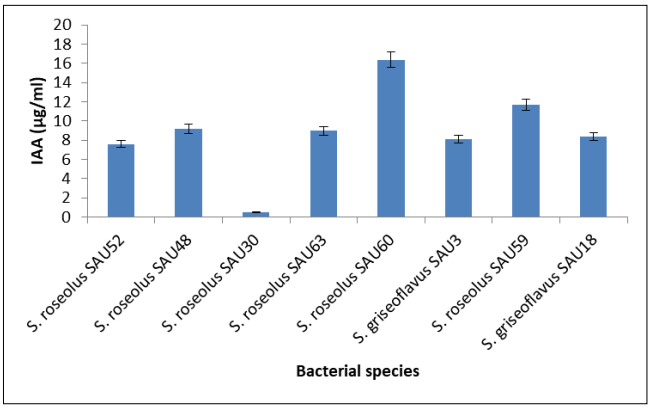

Fig 5: Indole acetic acid production by the isolated Streptomyces species

Inoculation of drought stress seedlings with Bacillus or Streptomyces significantly (P<0.05) enhanced seedling growth parameters as compared with control (Fig. 5). Each of germination percentage of grains, length and fresh weight of seedlings were increased significantly (Figure 5 a, b).

PGPR can directly facilitates the growth and development of plants through mechanisms such as nutrient uptake or increases nutrient availability by nitrogen fixation, mineralization of organic compounds, solubilization of mineral nutrients, and production of phytohormones. These mechanisms affect plant growth activity directly and vary according to the microbial strain and the plant species. Direct enrichment of mineral uptake occurs due to increases in individual ion fluxes at the root surface in the presence of PGPR.

Inoculation of Pseudomonas putida or Bacillus megaterium and AM fungi resulted in alleviation the harmful effect caused by drought stress (Marulanda *et al*., 2009) [35]. Application of Pseudomonades sp. under water stress improved the antioxidant and photosynthetic pigments in basil plants. Interestingly, combination of three bacterial species caused the highest CAT, GPX and APX activity and chlorophyll content in leaves under water stress. Pseudomonas spp. was found to cause positive affect on the seedling growth and seed germination of A. officinalis L. under water stress (Liddycoat *et al*., 2009) [28]. Photosynthetic efficiency and the antioxidative response of rice plants subjected to drought stress were found to increase after inoculation with arbuscular mycorrhiza (Aroca *et al*., 2013) [8]. The Liddycoat beneficial effects of mycorrhizae have also been reported under both the drought and saline conditions (Gill *et al*., 2012) [16].

Araújo and Guerreiro, (2010) [7] observed that bacteria that promoted maize growth were not necessarily those that produced more IAA. Increase in growth, yield and nutrient absorption by plants may occur through the expression of one or more plant growth-promoting characteristics. In this way, the in vitro selection of PGP bacteria should be achieved with multiple traits and their greenhouse evaluation under controlled conditions is important (Yang *et al*., 2009; Rana *et al*., 2011) [59, 42].

Figure 5: Percentage germination (A) and Fresh weight (g/ seedlings) (B) of wheat grains grown under different levels of drought (D0, D1, D2 and D3) supplemented with Rhizobia sp and Actinomycetes (Significance level = 0.05).

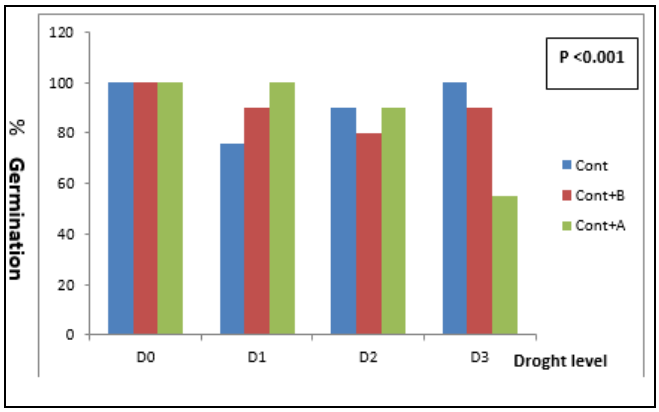

Fig 6

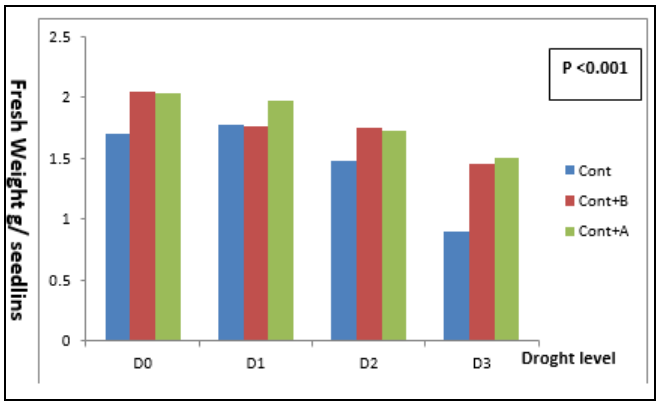

Fig 7

Our results demonstrated that Rhizobia and Actinomycetes isolated from the rhizosphere of Medicago and Cyperus roots grown on desert lands could be used for alleviating drought stress in some crop plants. The inoculation of seeds with these isolates and amendments is promising technological alternative for seed treatment.

4. Conclusion

The result of the current study suggests that bacterial strains recovered from the rhizosphere of plants grown under the harsh condition in Saudi Arabia have the capacity to produce phytohormones and antioxidant enzymes. Inoculation of drought-stressed plants with these strains significantly support the plant growth and alleviate the adverse effect of drought stress. These inoculants can be applied directly to seeds or to furrows in soils during

planting. Such inoculants will undoubtedly be used in arid regions, especially in Sudi Arabia.

5. Acknowledgment
The authors gratefully acknowledge the financial support of this work by the Deanship of the Scientific Research, Shaqra University, Shaqra, Saudi Arabia (Grant No.1/1/2015)**.**

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