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The effect of microbial inoculants on physiological responses of two wheat cultivars under salt stress

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ABSTRACT

Salinity is one of the most important abiotic stresses that limit crop growth and productivity. This study focuses on the effects of different strains of plant growth promoting rhizobacteria (PGPR) on the physiological responses of two wheat cultivars under normal and salt stress conditions. The wheat cultivars selected include one which is tolerant to salinity (Kavir) and one which is sensitive to salt-stress (Qods). The factors considered were four levels of PGPR (B1 to B4) and two levels of salinized culture solution (S1 to S2). Before planting, the wheat was inoculated with strains of PGPR. Results showed that salt stress reduced RWC, Leaf Chlorophyll index and photosynthesis characteristics. The application of PGPRs strains reduced the negative effects of saline stress by increasing the leaf's relative water content and enhancing photosynthetic pigment production in both stress and normal condition. The mechanism of PGPR elicitation of growth promotion may involve the enhancement of root hair development and therefore increased relative water content, chlorophyll pigments and water uptake. Single and dual inoculations of PGPR strains showed variations in their effect to enhance the wheat tolerance to salt. The bacterial consortium was effective for wheat plants as an acceptable and ecofriendly technology to improve plant performance and development.

Key words: *Azosprilliumc,* mixed inoculants, *Pseudomonas,* PGPR, salt stress, wheat,

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INTRODUCTION

Salt stress is an important growth-limiting factor for most non-halophytic plants. High levels of salt cannot be tolerated by most crops, a fact that severely limits the use of salt-affected soils for crop production (Tiwari *et al*, 2010). A considerable amount of land in the world is affected by salinity and this is increasing day by day. The United Nations Food and Agriculture Organization (FAO) and United Nations Environment Program (UNEP) estimate that there are currently 4 million square kilometers of salinized land worldwide with approximately 20%of agricultural and 50% of crop lands in the world being salt-stressed and therefore threatening agricultural productivity (Ravindran, 2007; Rozena and Flowers,2008). Estimates suggest that about 55.6 million ha, including 34% of the total area in Iran, are salt-affected (momeni, 2011). This is a world-wide problem. Salt accumulation may convert agricultural areas in unfavorable environments, reduce local biodiversity, limit growth and reproduction of plants, and may lead to toxicity in nonsalt- tolerant plants, known as glycophytes. Most of the cultivated plants are sensitive to salt-stress (Turan *et al*, 2009). To overcome the effects of salinity, scientists are also using several approaches to obtain salt tolerant plants. These approaches are time consuming and costly. The mechanisms of salt tolerance are not yet completely clear. Most plants possess several mechanisms to decrease the negative effects of salinity including regulation and compartmentalization of ions, synthesis of compatible solutes, induction of antioxidative enzymes, induction of plant hormones, and changes in photosynthetic pathways (Cheeseman, 1988; Parida and Das, 2005). Using rhizosphere microorganisms, particularly beneficial bacteria are an alternative strategy that can improve plant performance under stress environments and, consequently, enhance plant growth through different mechanisms (Dimkpa *et al*, 2009). Some plant growth-promoting rhizobacteria (PGPR) may cause a direct or indirect stimulation on plant growth and development by providing plants with fixed nitrogen, phytohormones and iron that has been sequestered by bacterial siderophores, and soluble phosphate (Hayat *et al*, 2010; Rodriguez & Fraga, 1999). Others do this indirectly by protecting the plant against soil-borne diseases, most of which are caused by pathogenic fungi (Lugtenberg & Kamolova, 2009). Bacterial IAA production also stimulates the activity of the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase involved in the degradation of the ethylene precursor ACC (Glick, 2005). ACC deaminase activity could be helpful in sustaining plant growth and development under stress conditions by reducing stress-induced ethylene production. Ethylene is an important hormone involved in plant growth and development. However, high concentrations of the hormone, especially when the plant is faced with salt and water stress, can create problems. The PGPR bacteria contain an enzyme called 1-aminocyclopropane-1-carboxylate (ACC) deaminase. This enzyme can regulate ethylene production by metabolizing ACC which is a precursor of ethylene biosynthesis in higher plants, into alpha-ketobutyrate and ammonia. This process reduces stress-induced ethylene production and helps maintain plant growth and development under stress conditions. Most of the studies have demonstrated the production of ACC deaminase gene in the plants treated with PGPR under environmental stress (Grichko & Glick, 2001). The accumulation of the amino acid proline is one of the most frequently reported modifications induced by water and salt stress as well as other stresses in plants (Hare & Gress, 1997; Kavi Kishor, 2005; Verbruggen & Hermans, 2008). It has been found that Medicago plants infected by IAA-overproducing PGPR strains are able to overcome different stressful environmental conditions and accumulate high levels of proline.

The present study investigated the response of wheat plants to normal and high NaCl treatment conditions at relative water content, Leaf Chlorophyll index, photosynthetic pigments (Chlorophyll a, Chlorophyll b, Total Chlorophyll, Cartenoid) and proline. In this study, inoculation especially co-inoculation with PGPR strains increased plant growth compared to the non-inoculated control treatment, and the inoculation with PGPR strains under soil salinity conditions improved plant growth compared to the non-inoculated control.

MATERIAL AND METHODS

The present study was carried out at Iran's soil and water research institute (SWRI) in 2011. The experiment was conducted as a completely randomized two-way factorial design with two levels for the first factor, and four levels for the second, and in 3 replications. The factors used were as follows: The first factor involved a salinized culture solution with two levels of salinity including S0 :(0.335 ds.m salinity (control or normal condition), S1: 14ds.m. salinity (stress condition), the second factor involved PGPR treatments as follows: B1: (without bacteria (control)), B2: (*Azosprillium lipoferum of*), B3: *(Pseudomonas fluorescens* 169), B4: (*Azosprillium lipoferum of +Pseudomonas fluorescens* 169). This study was carried out in greenhouse and sand culture conditions as two separate experiments. In the first experiment, Qods cultivar (sensitive to salinity) was used and in the second experiment the Kavir cultivar (tolerant to salinity) was used. In each cultivar the main effects and interactions of the above mentioned factors on the physiological parameters, relative water content (RWC), Leaf Chlorophyll index content, chlorophyll pigments (Chlorophyll a and Chlorophyll b carotenoids contents and prolin in wheat (*Triticum aestivum*) were studied.

Relative water content (RWC): Relative water content (RWC) was determined according to Turner (1981), based on the following equation: RWC = (FM – DM) / (SM – DM) × 100 Where FM is leaf fresh mass, DM is dry mass of leaves after drying them at 85 °C for 3 days, and SM is the turgid mass of leaves after soaking them in water for 4 h at room temperature (approximately 20 °C). Half of the third (from the top) fully expanded leaf was used.

Leaf Chlorophyll index content: Chlorophyll content was assessed using a chlorophyll meter (SPAD-502, Minolta). Measurements were taken at three points of each leaf (upper, middle and lower part). The average of these three readings were considered as SPAD value.

chlorophyll pigments (Chlorophyll a and Chlorophyll b carotenoids contents : Chlorophyl a, Chlorophyl b, Carotenoids and Total Chlorophyll were estimated by extracting the leaf content using 80% acetone as explained by Lichtenthaler et al. (1987). The chlorophyll content was calculated according to the following formulae:

Chl a (µg / ml) = 12.25 A663.2- 2.79 A646.8 **Chl b (µg / ml) = 21.50 A646.8- 5.10 A663.2**

Chl T (µg / ml) = Chl a + Chl b **CX+C = (1000 A470-1.8 Chl a-85.02 Chl b) / 198**

In this formula Chl a, Chl b, Chl T and CX+C respectively indicate the chlorophyll a, chlorophyll b, total chlorophyll and carotenoids concentrations.

Proline content

The proline content was quantified by using the acid-ninhydrin procedure of Bates et al. (15). The leaf tissues (0.5 g) were ground with 3% sulphosalicylic acid (10 ml) and clarified by centrifugation. Supernatant (2 ml) was mixed with the same volume of acidninhydrin and acetic acid, the mixture was oven incubated at 100 oC for 1 h, and the reaction was finished in an ice bath. The reaction mixture was extracted with 4 ml toluene using vortex mixer for 15-20 s and the absorbance was read at 520 nm.

Statistical analysis

The recorded data was analyzed using SAS 9.1.3 (SAS Institute Inc., Cary, NC, USA) and the treatment means and standard error (S.E) were differentiated by a Tukey's honest significant difference (HSD) at $P = 0.05$ using PROC-GLM.

RESULTS AND DISCUSSION

The results of the analyses of variances indicated highly significant differences among salinity stress conditions and PGPR treatments for all traits and significant pgpr × salinity interaction for Prolin and RWC in both cultivars QODS and KAVIR. (Tables 1–2).

Table 1. Analysis of variances of measured parameters under different treatments of bacterial inoculation under normal and stress conditions in the sensitive genotype (QODS)

		Mean Square Error						
S.O.V	df	RWC (%)	Leaf Chlorophyll index	Chlorophyll a	Chlorophyll b	Total Chlorophyll	Carteniod	prolin
Salinity	1	2561.63**	4.15*	16.67**	83.85**	175.28**	43.82**	8022.73**
Bacteria(PGPR)	3	237.65**	63.33*	3.72*	21.38*	18.90	4.72*	3994.42**

Salinity* bacteria	3	95.16**	90.73 ns	0.03 ns	0.70 ns	0.87 ns	0.22 ns	1127.41**
Error	16	3.78	12.98	0.66	2.24	2.02	0.50	5.05
C.V.(%)		4.03	18.11	15.26	16.77	11.69	14.01	3.36

Table 2. Analysis of variances of measured parameters at under different treatments of bacterial Inoculation in normal and stress condition in sensitive genotype

S.O.V	df	RWC (%)	Leaf Chlorophyll index	Chlorophyll a	Chlorophyll b	Total Chlorophyll	Carteniod	prolin
Salinity	1	2155.56**	178.54*	57.20**	82.47**	277.30**	28.62**	1818.56**
Bacteria(PGPR)	3	408.29**	10.05*	4.28**	13.68**	22.35**	13.05**	519.34**
Salinity* bacteria	3	110.00**	119.2 ns	0.11 ns	3.35 ns	4.55 ns	0.10 ns	239.91**
Error	16	4.13	55.41	0.67	1.32	1.03	0.85	2.63
C.V.(%)		4.01	17.86	18.38	11.03	6.80	13.57	6.68

*, **, ns indicate statistical significance at P ≤0.01 , P ≤0.05, nonsignificant

Table 3. Mean comparison of the all traits under different levels of salinity and PGPR treatments, in both cultivars (QODS & KAVIR)

Treatments	RWC (%)		Leaf Chloro phyll index		Chloro phyll a (mg/g.fw)		Chloro phyll b (mg/g.fw)		Total Chloro phyll (mg/g.fw)		Carte noid (mg/g. fw)		Prolin (µmol/ g.fw)	
S0	58.60^{a}	60.21^{a}	26.06^{a}	29.45^{a}	4.06^{a}	6.01^{a}	10.79^{a}	12.28^{a}	14.85^{a}	18.29^{a}	6.42^{a}	7.87^{a}	97.06^{b}	107.23^{b}
S1	37.94^{b}	41.25^{b}	22.8^{b}	24.0^{b}	2.39^{b}	2.92^{b}	7.05^{b}	8.57^{b}	9.44^{b}	11.49^{b}	3.72^{b}	5.69^{b}	133.63^{a}	132.97^{a}
B1	39.07^{c}	39.11^{d}	23.16^{a}	25.2^{a}	2.67^{b}	3.80^{a}	7.25^{a}	8.51	9.93^{b}	12.58^{b}	3.97^{b}	4.62^{b}	143.95^{a}	129.32^{a}
B2	51.40^{ab}	51.14^{c}	25.27^{a}	26.29^{a}	3.19^{ab}	4.07^{b}	8.82^{b}	10.85	12.0^{ab}	14.02^{ab}	5.00^{ab}	7.94^{a}	128.75^{b}	119.92^{b}
B3	49.58^{b}	54.56^{b}	26.6^{a}	27.17^{a}	2.69^{b}	5.70^{b}	8.04^{b}	12.14	12.3^{ab}	16.42^{a}	5.19^{a}	7.21^{a}	102.05^{c}	127.92^{c}
B4	53.03^{a}	58.10^{a}	30.8^{a}	28.24^{a}	4.35^{a}	4.28^{b}	11.58^{b}	10.22	14.26^{a}	16.55^{a}	6.13^{a}	7.34^{a}	86.65^{d}	113.47^{d}
S0B1	444.39	443.45	222.78	226.21	33.43	55.50	88.93	110.10	112.36	15.60	55.18	55.70	1114.83	1118.60

S0B2	663.30	665.84	226.33	229.09	44.10	55.54	111.08	112.77	115.18	18.60	66.59	88.48	999.37	1117.32
S0B3	660.34	663.18	225.91	227.52	33.53	55.75	110.07	113.20	115.24	18.95	66.62	88.30	888.23	1114.44
S0B4	636.37	668.36	229.21	334.97	55.17	77.24	113.08	113.06	116.61	20.01	77.31	99.01	885.80	1111.92
S1B1	333.74	334.78	220.41	221.51	11.82	22.07	55.56	65.91	77.50	9.56	22.75	33.54	1173.07	1149.37
S0B2	339.68	435.94	223.99	224.18	11.93	22.64	66.55	86.93	88.84	13.08	33.42	66.87	1158.07	1142.12
S0B3	335.86	336.45	227.77	223.48	22.29	22.81	66.00	76.38	99.53	13.88	33.77	55.94	1115.87	1125.41
S0B4	442.47	447.84	335.38	226.81	33.53	44.15	110.08	110.08	111.90	10.23	44.95	66.39	887.50	1115.01

QODS(sensitive to salinity cultivar), KAVIR (tolerance to salinity cultivar), S0:(0.335 ds.m normal condition), S1:14 ds.m. stress condition), B1:(without Bacteri(control)), B2:(*Azosprillium lipoferum of*), B3:*(Pseudomonas fluorescens* 169), B4:(*Azosprillium lipoferum of +Pseudomonas fluorescens* 169).

Relative Water Content (RWC)

Salt stress, PGPR and salinity × PGPR had significant effects on relative water content in both QODS (Table 1) & KAVIR cultivars (Table 2). Mean comparison showed that an increase in salt stress caused a significant reduction in RWC (Table 3). RWC was reduced by 54 (%) in the QODS cultivar and 45(%) in the KAVIR cultivar under salt stress. This reduction was caused by an increase in of evapotranspiration in plants. Santos *et al*, (2002) reported that RWC decreased under stress conditions in comparison with non-stress conditions. Therefore osmotic regulation will help cell development and plant growth in water stress. It is believed that a decrease of RWC will reduce the rate of photosynthesis (Cornic, 2000). It is reported that high RWC is a preferential mechanism for stress tolerance (Merah, 2001). Siddique *et al*,

(2000) reported that RWC decreased from 88 - 45 % when under drought stress conditions. Mationn *et al*, (1989) reported a similar result for RWC in tolerant and sensitive cultivars of barley. Under salinity stress all the inoculated treatments showed significantly enhanced RWC. The inoculation treatment with PGPR strains B2, B3 and B4 showed an increase in relative water content by 12-22 % in the QODS cultivar and 5-25% in the KAVIR cultivar under normal conditions and by 15.2-25.6% in QODS and 8-20% in KAVIR under salt stress conditions. The highest content for RWC observed was in the application of dual inoculations of PGPR strains under normal and stress conditions as compared to the un-inoculated control and single strain inoculations. Shaharoona *et al*, (2006) reported that the inoculation treatment with PGPR isolates increased RWC from 5 -16% under normal and 21.7-28.4% under stress conditions as compared to the un-inoculated control. Moslemi et al., (2011) reported a similar result for RWC in maize.

Leaf Chlorophyll index

The leaf's chlorophyll content was significantly influenced by salinity treatments and PGPR applications in both cultivars QODS (Table 1) and KAVIR (Table 2). Mean comparison showed that increasing stress conditions caused a significant reduction in the chlorophyll content (Table 3). Chlorophyll content has also been known as a proper index for the evaluation of stress intensity (Ommen *et al*,1999). Under salt stress conditions, tolerance genotypes showed high content of chlorophyll (Sairam and Siravastava, 2002). The reduction of chlorophyll content in abiotic stress conditions is mainly caused by chloroplast damages that are caused by active oxygen species (Manivannan *et al*, 2007). Under salt stress all the inoculated treatments showed a significant enhancement in Leaf Chlorophyll index. The inoculation treatments with PGPR strains B2, B3 and B4 showed an increase in Leaf Chlorophyll index by 26-33 in the QODS cultivar and 31-36% in the KAVIR cultivar under normal conditions and by 40.2-49.6% in QODS and 5-27% in KAVIR under salt stress conditions as compared to those that were un-inoculated. Dual inoculations of PGPR strains showed a higher increase in Leaf Chlorophyll index as compared to the un-inoculated control.

Chlorophyll pigments (Chlorophyll a and Chlorophyll b carotenoids contents)

The results showed that salt stress and PGPR treatments had significant effects on chlorophyll contents (chlorophyll a, b and carotenoid) **in** QODS (Table 1) and KAVIR cultivars (Table 2). The highest values of chla and Chlb and carotenoids, in both cultivar under both normal and stress conditions, was observed during co-inoculation of PGPR strains (*Azosprillium lipoferum of +Pseudomonas fluorescens)* respectively (Table 2 and Table 3). The lowest content of chlorophyll pigments in both normal and stress conditions was observed in strains that had not been inoculated with PGPR strains (B1) and this was seen in both cultivars (Table 3). The application of different strains of PGPR treatments showed that the highest value for chlorophyll a, b and carotenoid was observed during co-inoculation with *Azosprillium +Pseudomonas* in normal and stress conditions (Table 3). *Nadeem* et al., (2006) reported that salt stress decreased chlorophyll pigments (a, b and carotenoids contents) of maize, but inoculation with strains also increased the chlorophyll pigments. This may be the result of increased photosynthetic leaf area of plants even when under high levels of salt stress by PGPR inoculation compared to the control where leaf area reduced due to stress (Marcelis and

Van Hooijdonk, 1999). Similar results were also reported by Han and Lee (2005) that inoculation increased the chlorophyll content in lettuce. Antolin *et al*, (1995) reported that dehydration stress increased the oxidative process, disturbed the chloroplast structure and caused a reduction in photosynthesis. Shaharoona *et al*, (2006) reported that inoculation with PGPR containing ACC-deaminase activity significantly affected the pigments under salinity stress.

Prolin content

Salt stress, PGPR and salinity × PGPR had significant effects on prolin content in both QODS (Table 1) & KAVIR cultivars(Table 2). Mean comparison showed that an increase in salt stress caused a significant reduction on prolin (Table 3). The results showed that the un-inoculated plants compared to the inoculated ones, under soil salinity conditions had an increased in their prolin concentration. The results suggested that inoculation of salt-stressed plants with PGPR strains could alleviate salinity stress. Bacterial exopolysaccharides (EPSs) in the soil ecology system play an important role in soil aggregation and soil adhesion. PGPR strains, especially EPS-producing bacteria, can induce soil salinity tolerance and growth promotion in crops under greenhouse conditions. Prolin accumulation is a very common response in plants exposed to abiotic stress, which contribute to osmotic adjustment and protect the structure of macromolecules and cell membranes during stress (Prado et al, 2000). Meloni *et al*, (2001) suggest that amino acids such as prolin act as organic nitrogen reserves in plant metabolism, as a readily available source of energy, and as possible precursors for alkaloid formation. This could explain why the plants inoculated with PGPR strains produced higher contents of tropane alkaloids.

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