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Molecular identification of *Azotobacter* isolated from the rhizosphere of Karun variety of barley in desert and its comparison with standard *Azotobacter* and investigation of some of the vegetation indicators after inoculation

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ABSTRACT

This study was conducted in acompletely randomized block design with three replications using three concentrations of 10^7, 10^5, and 10^3 cfu/ml of *Azotobacter* isolated from the rhizosphere of Karun varieties of barley (*Az. salentris*) and standard *Az.chrococom* along with control (zero concentration of bacteria). The aim of this study was to study the reactions of barley plantin response to inoculation by mentioned bacteria in the form of infected separate inoculation and non-inoculated seeds as a control. For isolation the number of mentioned bacteria, Karun variety of barley in desert was cultivated in the mannitol broth environment, and macroscopic and microscopic characteristics of bacterial colonies were evaluated. Finally, using gene 16Sr DNA, isolated variety was identified molecularly. At the end of plant growth, important traits such as percent of germination, total protein level, and activity of antioxidant enzymes of catalase and peroxide were measured from the desired plant and they were analyzed statistically. After 18 days, results showed that the concentration of 10^7, 10^5, 10^3 cfu/ml of *Azotobacter* isolated from the rhizosphere Karun variety of barley plant in the desert (*Az. salentris*) increased the activity of antioxidant enzymes, total protein, and percentage of germination compared with control. In addition, different concentrations of *Azotobacter* isolated from the rhizosphere of barley plant (*Az. salentris*) showed higher and significant impact on catalase activity, total protein level, and percentage of Karun variety of barley germination compared with standard *Az.chrococom*.

1. Introduction

Indiscriminate and in appropriate use of pesticides and chemical fertilizers are very serious, and it can be investigated from some aspects that most important aspects includeenvironmental and health damage, high cost of their production, and disrupting the balance of ecosystems, causing that the production of bio-fertilizers to be considered seriously. Therefore, it is expected that biological fertilizers containing large amount of one or several types of useful soil microorganisms to be used to provide nutrients elements, creating favorable conditions for plant growth, and reduce theenvironmentalhazards (Kizilkaya et al., 2008). Plant growth promoting rhizobacteria (PGPR) are a diverse group of bacteria found in the rhizospherearea and surface of roots and they are able to improve the quality of plant growth directly or indirectly (Asma et al., 2012). These bacteria are often

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found near or even inside the roots of plants (Collavino et al., 2010). They can also improve the plant growth by nitrogen fixation, synthesis and production of iron complex siderophores, the production of plant hormones, antibiotics and deadly combination of various fungi (Onasanya et al., 2009). Some of the effects of these bacteria on plants are as follows: Positive effect on some physiological, biochemical and morphological factors (Ghamsari, 2007; Han and Lee, 2005): Germination is one of the important stages of plant growth that greater plant productivity is determined by the stage (Almansouri et al., 2001). Germination period depends on weather conditions, and soil optimum temperature for germination of barley is from 16 to 22°C (Koornneff et al., 2002). Seed proteins are mainly divided into two groups, Housekeeping proteins and storage proteins.The first group is responsible for the maintenance of normal cell metabolism. According to the latest classification, these proteins are divided to storage proteins, building proteins, and biologically active proteins. Biologically active proteins include lectins, enzymes and enzyme inhibitors (Mandal, 2000).

Martinetal (Martin et al., 2011) in field experiments that conducted by using *Azotobacter* inoculant on seed and transplanting plants such as wheat, rice, barley, tomatoes and corn in different climatic conditions, They concluded that promoting the performance in development and germination of all products was observed between 7-12%. Krishna et al (Krishna et al., 2008) noted that theuse of *Azospirillum* bio-fertilizers, phosphate solubilizing bacteria, *Azotobacter* and their combination the min the With an iasomniferumand Ocimumsanctu plants improved some traits of germination such as percentage, rate, and index of germination. Lus et al (Lus, 2003) reported that the plant growth promoting increased the yield ofantioxidant enzymes of corn by reducing plant diseases caused by the fungus Fusarium. The aim of this study is to investigate the effect of different concentrations of two varieties of *Azotobacter* on the germination percentage, total protein, and the level of antioxidant enzymes of Karun variety of barley plant in the desert in the controlled laboratory conditions.

2. Materials and Methods

2.1. Sampling

Sampling location included under cultivation crop soils of clover in some areas of the Isfahan province, because these soils have been infected to biological nitrogen fixing soil bacteria for long time. In addition, for enrichment of *Azetobacter* isolated from soil, considered bacteria were cultivated in cultivation environment of mannitol broth at 28 to 30°C. After 7-4 days, by forming slimy and layer on the surface of the tube indicated the presence of aerobic *Azetobacter*. In order to isolate bacteria from slime layer on culture environment of, it was cultured on the culture environment of mannitol agar. After 4-3 days, at 28 to 30°C, colonies with slimy and shiny, cream-colored, spherical medium-sized and brown macroscopic characteristics were created. Inthenextstages, macroscopic and biological characteristics of bacteria were identified, referring to the tables of identifying the bacteria, genus and variety of samples.

2.2. Molecular analysis of samples

Azotobacter strains isolated from clover under cultivation of crop soils was purified after identifying with numerous passages on mannitol agar culture and after the appearance of colonies PCR was performed using PCR-colony method. In addition, universal primers (oF BUN and oR BUN) and gene 16S rDNA was used. To determine the sequence of DNA, samples were sequenced and then the results were analyzed by Chromas 2/1 software, and they were identified in NCBI site by BLAST server. Finally, based on found homology, the considered variety was identified.

2.3. Preparation of different concentrations of Azotobacter varieties

To prepare the concentrations (10^3,10^5 and 10^7 cfu/ml) of the considered bacteria, series of dilutions were prepared, firstly. For this purpose, in the first stage, 6 sterile tubes were picked and 1 ml sterile saline was poured into first tube to create McFarland turbidity. In every 5 other tubes, 4.5 ml of saline was added, and 0.5 ml was transferred to next tube, then it was centrifuged, that in this series, we prepared

10^7cfu/ml of bacteria, and 10^5 and 10^3 cfu/ml dilutes were also prepared in the next series (Ahmad et al., 2008; Sharma et al., 2009).

2.4. Sterilization, rising, and sowing seeds

After preparation the plant seeds from agricultural research center in Isfahan, the seeds were placed for 10 minutes in a solution of sodium hypochlorite 1% for disinfection. Then, they were rinsed by distilled water three times. Then, in each plate of concentrations of 10^3,10^5 and 10^7cfu/ml, 1 ml of distilled water was added to consider bacteria, and 1 ml of it was added to control samples, then they were transferred to germinator at 25°C for 20 days and the period of 8 hours of darkness and 16 hours of light.

2.5. Measuring the germination of Karun variety of barley leaf in the desert

Daily counting of germinated seeds was carried out for 10 days at a specified time and germination percentage was calculated by following equation. 100 × (number of seeds / number of germinated seeds until the last day) = the percentage of germination.

Measuring total protein level in Karun variety of barley in desert using Lowry method

The base of work in this method according to reference (Lowry. et al., 1951) is based on the hydrolysis of proteins and amino acids reaction with the reagent of Folan and creating colored complex.

2.6. Measuring the peroxidase and catalase activity Karun variety of barley in desert

A) Preparation of extraction solution: Mixture of 1.2 g of Tris, 0.1 g of ascorbic acid, 17.2 g sucrose, 0.1 g of cysteine chloride, and 26.8 ml of hydrochloric acid reached to pH of 7.5 by 100 ml of distilled water.

B) An enzyme extract extraction: 1 g of fresh tissue of plant was pulverized with 5 ml of extraction solution and it was placed for 10 minutes at a standstill. Then, it was centrifuged for 30 minutes with 10000 g round.

C) Measuring enzyme activity: To measure peroxidase activity, following solutions were used:

The solutions of 2 ml of acetate buffer 2.0 M with pH=4.8; 2 –0.2 ml of hydrogen peroxide 3%; 0.1-3 ml of benzidine (0/02 M -solution in methanol 50%) were mixed with 0.1 ml of extraction solution (containing peroxidase) and read in a spectrophotometer device at 530 nm wavelength absorption rate.

The amount of enzyme activity in terms of absorbance unitper minute per each milligram of protein in fresh weight of the plant was calculated (Koroi, 1989).

2.7. Measuring the activity of catalase, following solutions were used

The solutions of 2.5 ml of acetate buffer with pH =7; 2-0.3 ml of 3% hydrogen peroxide were mixed with 0.2 ml of extraction solution, and they were placed in a spectrophotometer at the wavelength of 530 nm and its absorbance was read (Chance and Maehly, 1995).

2.8. Statistical analysis

In this study, the considered treatments were studied in a completely randomized design in factorial form, and the results were analyzed using Spss 19 software and the comparison of means was conducted by Duncan's test. GLM test was used to draw the charts.

3. Results

As mentioned in the methodology section, *Azotobacter* isolated from the environmentwas identified by using molecular colony - PCR *Az. salentris* method, and universal primers designed from gene 16SrDNA were used that their sequences are shown in the table below. In the following table, instead of using the word of *Azotobacter* isolated from the environment, the name of the genus and variety of these bacteria was used. The results of electrophoresis of PCR of samples are shown with universal primers in Fig1.

Based on the results of sequencing and BLAST of final sequence of 360bp encoded as 1c1 | 28251 in the NCBI, it can be said that the mentioned variety selected for sequencing, is one of the varieties genus *Azotobacter*.

Table 1. The sequences of selected universal primers

Primer	Sequence
OF BUN	5'-CGCATTTCACCGCTACAC -3'
OR BUN	5'-TATGTACACACCGCCCGT -3'
IF BUN	5'-TAAACCACATGCTCCACC -3'
IR BUN	5'-ACACACGTGCTACAATGG -3'

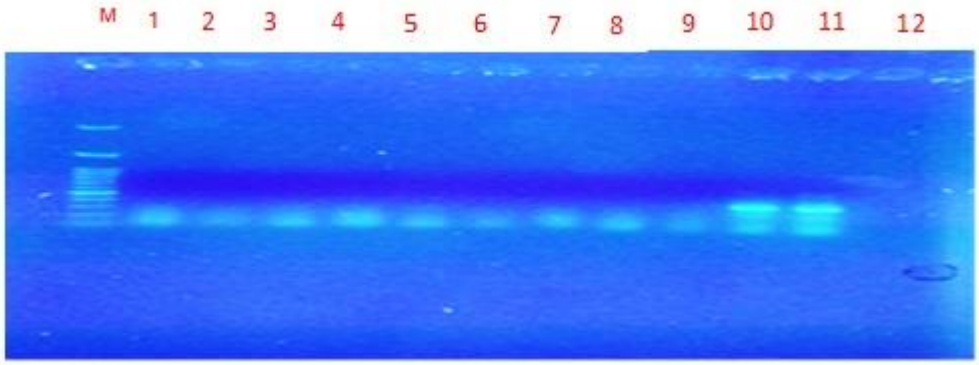

Figure 1. The results of the electrophoresis of PCR product, the *Azotobacter* strains isolated from the environment with universal primer on 1% agarose gel. **Line M**: the ladder with 100 bp, **line 10**: positive control or standard *Az.chrococomstrains* with 360 bp, **line 11**: *Azotobacter* strains isolated from the environment (*Az. salentris*), **line 12**: negative control (distilled water).Sequence considered in the NCBI site was compared with bacteria of *Azotobacter* family and the results showed that the obtained sequence showed high homology with the strains of *Az. salentris*.

Max ident: 99%Quary coverage: 99%

In the following sections, we refer to findings of the impact of *Azotobacter* isolated from the environment on some physiological and biochemical traits in Karun variety of barley plants in the desert:

1. Investigation of changes of germination percentage in Karun barley plant in the desert

A) The results of effect of different concentrations of *Az. salentris* on the germination of Karun barley in the desert

As Figure 1 shows, the treatment of plant with a concentration of 10^3,10^5 and 10^7cfu/ml of *Az. salentris*increased the germination of Karun barley in the desert, respectively, compared with control, that this increase was not statistically significant at 1% level.

B) The results of effect of different concentrations of *Az.chrococom* on germination percentage in the Karun barley plant in the desert:

Based on Chart (2), results showed that the treatment of plant with a control concentration of 10^3,10^5 and 10^7cfu/ml of standard *Az. chrococom* increased germination percentage compared with the control. As it is seen, there is no statistically difference between the concentrations of 10^3 and10^5 cfu/ml. However, this increase was statistically significant at the 1% level.

2. The effect of different concentrations of *Azotobacter* on total protein content in Karun barley plant in desert.

A) The effect of different concentrations of *Az. salentrison* the protein content of the Karun barley plant in desert.

Based on Chart (3), results showed that the treatment of plant with concentration of 10^3,10^5 and 10^7cfu/ml of *Az. salentris*increased total protein level compared with control. This increase was statistically significant at the 1% level.

B) The effect of different concentrations of standard *Az.chrococom* on total protein content of Karun barley plant in desert.

Based on Chart (3), results showed that the treatment of plant with concentration of 10^3,10^5

and 10^7cfu/ml of standard *Az.chrococom* increased total protein level compared with control. This increase was statistically significant at the 1% level.

The results of effect of different concentrations *Azotobacter* isolated from environment (*Az. salentris*) on the activity level of antioxidant enzymes (catalase and peroxidase) in Karun barley in the desert showed that the treatment of plant with a concentration of 10^3,10^5 and 10^7 of *Az. salentris* respectively increased activity of antioxidant enzymes compared with control. These changes were statistically significant at the 1% level.

A) The results of effect of different concentrations of standard *Azotobacter* isolated from environment on catalase activity in Karun barley:

Based on Chart (4), the results showed that the treatment of plant with concentrations of10^3,10^5 and 10^7 of standard *Az.chrococom* and *Azotobacter* isolated from environment (*Az. salentris*) respectively increased significantly the peroxidase activity, compared with control. This increase was statistically significant at the 1% level.

B) The effect of different concentrations *Az. salentris*on the peroxidase activity in Karun barley Karun in the desert:

Based on Chart (4), the results showed that the treatment of plant with concentrations of 10^3, 10^5 and 10^7 of *Azotobacter* isolated from environment respectively increased significantly the peroxidase activity, compared with control. No significant difference was found between 10^3 and 10^5 cfu/ml concentrations of inoculant of *Az. salentris*. This increase was statistically significant at the 1% level.

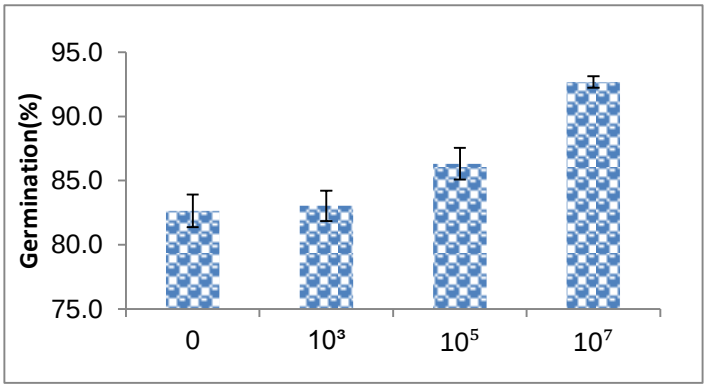

Chart 1. The effect of different concentrations of *Azotobacter* isolated from environment on germination percentage in the Karun barley plant in the desert.

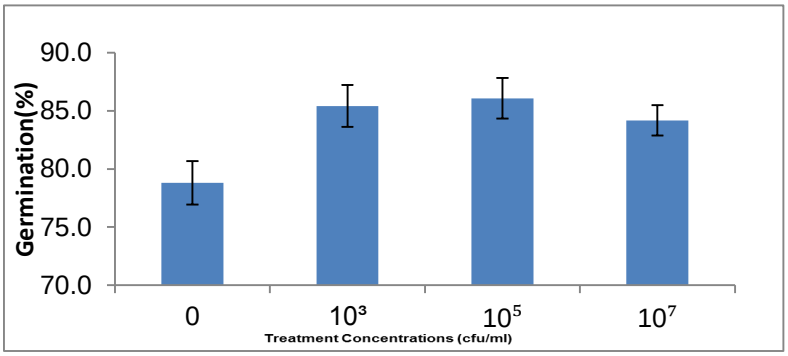

Chart 2. The effect of different concentrations of *Az.chrococom* on germination percentage in the Karun barley plant in the desert

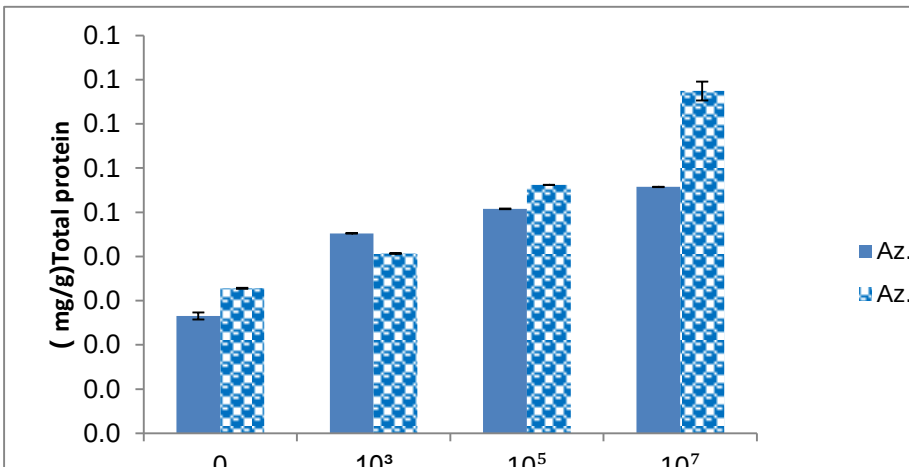

Chart 3. comparison of the effect of different concentrations of *Azotobacter* isolated from environment on total protein of Karun barley plant in desert.

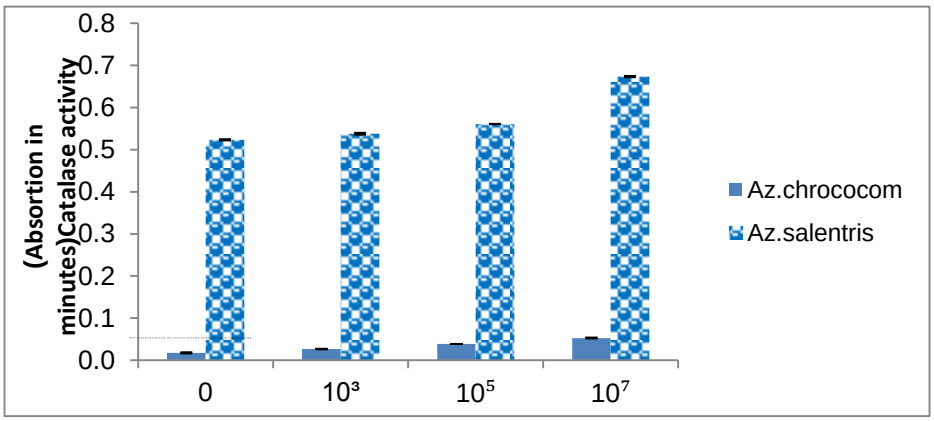

Chart 4. comparison of the effect of different concentrations *Azotobacter* isolated from the environment and standard *Az. chrococom* on catalase activity in Karun barley plant in the desert.

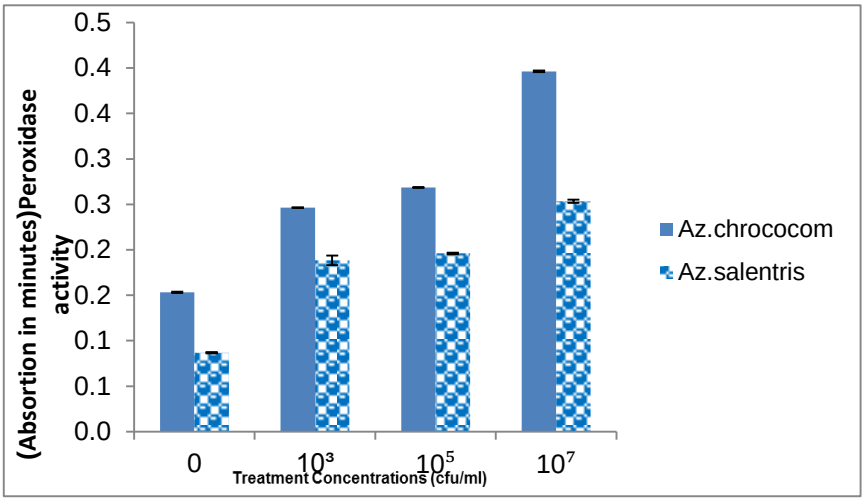

Chart 5: Comparison of the effect of different concentrations of *Azotobacter* isolated from the environment and *Az. chrococom* on peroxidase activity in Karun barley plant in the desert.

4. Discussion

A) The percentage of germination: the process of seed germination is one of the critical steps in plant growth that plant yieldis determined by the stage (Boisnic, 2005). Based on Charts (1 to 2), results of this study showed that the percentage of germination of Karun variety of barley plant in the desert under the concentrations of10^3, 10^5 and 10^7 of *Azetobacter* isolated from environment and *Az. Chrococom* respectively increased the germination percentage compared with control. The highest impact was seen in the concentration 10^7cfu/ml, and the results were significant at the 1% level. Similar to our results, Kader (Kader, 2002) stated that *Azotobacter* increase the germination percentage of seeds through growth promoting hormones such as indole, acetic acid, and cytokinins. Similar to our results, Shaukat et al (Shaukat et al., 2006) reported that strains of *Azospirillum, Azotobacter* and *Pseudomonas* had significant positive effects on germination and seedling growth of corn. Turan et al. (Turan et al., 2006) stated that plant growth promoting bacteria improves seed germination, seedling growth and leaf surface of plant that is the main cause of accelerating germination in the presence of this species of bacteria related to the production of growth hormones that is similar to our results. Yasminet al (Yasmin et al., 2004) reported that Quito lipo-oligosaccharides is one the main symptoms of specific bacteria of host plant for successful symbiotic of legume Rhizobium, creating physiological changes in lower concentrations.

They showed that bacterium of *Rhizobium japonicum* Brady increases the germination of various plants such as rice, corn, beet, soya, beans and cotton in the laboratory, greenhouse and field conditions. Since the plant of barley germination is somewhat compatible with a number of these plants, it seems that these factors increase the germination of this plant in response to concentrations of *Azotobacter* inoculation *Az.salentris* isolated from rhizosphere of this plant.

B) The total protein: Essentially, plants, unlike other organisms, can build amino acid by basic elements of carbon, oxygen, hydrogen, nitrogen and photosynthesis. Amino acids are forming units of proteinand vegetable-based proteins are consist of 20 amino acids and two amides (Van et al., 2001).

Based on figures (4 and 3) the results of our research showed that the protein content of the leaves of barley plant under concentrationsof 10^3,10^5 and 10^7 cfu/ml of *Azotobacter* isolated from environment and *Az. chrococom* was increased respectively compared with the control. As we see, the greatest impact is seen in the concentration of 10^7cfu/ml and results were significant at 1% level. Similar to our results, Souzar et al (Zouzar et al., 2013) reported that the percentage of protein and weight of each seed in wheat increased by Rhizobium bacteria. Given that *Azotobacter* and *Azospirillum* are nitrogen-fixing bacteria, and this element is raw material for forming protein, they are probably one of the reasons for increasing the protein content using these bacteria. Similar to our results, Diaz Diaz-Zorita et al (Diaz Diaz- Zorita et al., 2008) reported that consumption of inoculant of Azetobacter brasiliensis leads to an increase of 8% wheat protein compared with that cause of this increase is likely due to nitrogen fixation by these bacteria that is largely consistent with our results. Similar to the results of our study, Ajam Norouziet al (2011) reported that using fertilizer of nitroxin increased significantly the seed protein and number of seed in the wheat cluster compared with control (non-inoculated).

C) The activity level of antioxidant enzymes: measuring the change in the activity of antioxidant enzymes can be used as an indirect method to estimate the amount of production of reactive oxygen species (Tripathi et al., 2006).

Based on Chart (5), the results of our research showed that the activity of antioxidant enzymes of barley leaf increased underconcentrations of 10^3,10^5 and 10^7cfu/ml. The results were significant at the 1% level.

Similar to our results, Das and Saha (Dos and Saha, 2000) investigated the effect of *Azotobacter* and *Azospirillum* with nitrogen at a rate of 50 kg per hectare on wheat plants. They found that using these bacteria had a positive impact on increasing seed yield, and especially *Azotobacter* had higher effect onascorbate peroxidase and catalase enzymes activity.

Similar to our results, Rajeawari (Rajeawari, 2011) studied the beneficial effects of inoculation with *Az. chrococomon* antioxidant enzymes yieldof cereals, legumes, oilseeds,

fruits and vegetables. They found that theimpact of various species of *Azetobacter* on growth and different plants is through molecular nitrogen fixation ability, production of substances regulating the plant growth such as auxin, gibberellin, cytokinin and similar compounds and release of these substances into root environment and surrounding environment of seed.Unlike our results, Davoudi Fard et al (Davoudi Fard et al., 2010), in a study on wheat, stated that the level of catalase activity in control treatment was higher than other studied treatments by symbiotic bacteria with wheat plant. Lower activity of enzyme bacteria-containing treatments indicate that bacteria have provided inappropriate condition for plant. As a result, plant has produced lower level of catalase.

Conclusion

Finally, it can be concluded that various concentrations of two species of *Azotobacter* (standard *Az. chrococom* and *Az. salentris* isolated from the environment) had positive impact on some physiological characteristics of Karun variety of barley in the desert, while they has higher impact on species isolated from environment that these effects were statistically significant at the 1% level.

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