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Enhancement of salinity stress tolerance in Cumin (*Cuminum cyminum* L.) as affected by plant growth promoting rhizobactria during germination stage

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Abstract

In order to investigate the effect of seed inoculation with Plant Growth Promoting Rhizobactria (PGPR) on germination and some biochemical and physiological indices of Cumin (*Cuminum cyminum* L.) under salinity stress, this experiment was conducted in laboratory of seed science and technology, Yasouj University in 2016. Seed inoculations with plant growth promoting bacteria at five levels (seed inoculation with three strains of *Pseudomonas fluorescens* such as of Pf2, Pf25 and CHA0 and one strain of *Bacillus subtilis* and hydration in distilled water (as control)) and three levels of salinity stress (0, -4 and -8 bar imposed by NaCl) comprised experimental factors. Salt stress reduced germination percentage, catalase (CAT) and ascorbate peroxidase (APX) activities and seedling potassium content but increased its sodium. Seed inoculations with plant growth promoting not only mitigated the inhibitory effect of salt stress on studied characteristics but also in some cases induced a stimulatory effect on seed physiological quality in both stress and non-stress condition . The highest germination percentage, APX activity and K^+ content were achieved in the seeds inoculated with CHA0 strain of *P. fluorescens*. The effect of this treatment was more obvious under salinity potentials of -4 and -8 bar. The results indicated that salinity can affect cumin seed germination and PGPR could be used to improve its salt tolerance.

Key words: Antioxidant Enzyme, *Pseudomonas fluorescens*, Salinity stress

Introduction

In arid and semi-arid regions of the world such as Iran, soil salinity is one of the major abiotic stresses affecting plant growth (Ravari *et al*., 2015). Salinity stress is the major constraint to seed germination in irrigated areas of Iran, with low rainfall (Kaya *et al*., 2003). Also, salinity is the most serious problems of crop production worldwide that limits plant growth. It can change the ingredients and medicinal properties of herbs (Muhammad and Hussain, 2010).

Cumin (*Cuminum cyminum*) is the second most popular spice in the world, after black pepper, and used as a medicinal plant. The small boat shaped seeds of cumin has been used for many medicinal and culinary purposes from the ancient time in the various countries from Latin America to Northern Africa and all over the Asia (Gohari and Saeidnia, 2011). The origin of this plant is attributed to Iran, Turkey, Egypt and western Mediterranean (Amini Dehaghi and Mollafilabi, 2011). It has good nutritional value as well as high consumer demand. Cumin yield per hectare is very low, and its productivity can be affected by abiotic stresses (Hassanzadeh deluei *et al*., 2013). Although, the cumin plant is relatively salt resistant during late vegetative and reproductive stages (Hassanzadehdeluei *et al*., 2013). Seed germination and seedling growth are the most sensitive stages to salinity stress (Ibrahim, 2016; Shoor *et al*., 2013). Salinity tension delays or prevents seed germination through water availability reduction, changes in the mobilization of stored reserves and affecting the structural proteins of organs and ionic stress (Ibrahim, 2016) and causes to reduce the seed germination percentage (Neamatollahi *et al*., 2009; Roodbari *et al*., 2013).

Salinity stress decreases seed water uptake during imbibition (osmotic stress). Also, salt stress may cause excessive uptake of ions (Murillo-Amador *et al*., 2002) such as Na^+, Ca^+ and Mg^{2+} and imbalance K^+/Na^+ ratio (Shoor *et al*., 2014). Almansouri *et al*. (2001) reported that salt induced inhibition in durum wheat seed germination was directly linked to Na^+ and Cl^- accumulation within embryonic axis. Potassium plays an important role in balancing cell turgor, activating enzymes, and regulating osmotic pressure in cells (Cherel, 2004). Also, salinity stress generates reactive oxygen species (ROS) that damage DNA, RNA, and proteins and cause to decrease seed germination activities. Antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), and ascorbate peroxidase (APX) scavenge the ROS and maintain them at low levels (Ibrahim, 2016; Habib *et al*., 2016).

Tolerance increment of crop plants to salinity stress is necessary in order to increase productivity under limited water supplies and high salinity condition (Azooz, 2009). The most used pretreatment to enhance seed germination is known as "seed inoculation with

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plant growth promoting". Seed treatment with microorganisms (inoculation) is an attractive proposition because of the combination of specific effect and the prohibition of negative effects of environmental factors (Chandra *et al*., 2008). The application of plant growth promoting bacteria strains (PGPR) in agriculture is a potential issue in international demand for food and improvement of environmental quality. PGPRs continuously increases plant growth, seedling emergence and overall crops performance used in different agricultural ecosystems (Bhattacharyya *et al*., 2012). Many studies have shown that beneficial microbes usage can resist plant against adverse environmental stress, such as drought (Gururani *et al.*, 2013; Jahanian *et al*., 2012; Piri *et al*., 2016) and salinity (Habib *et al*., 2016). In addition, ACC deaminase-containing PGPR can reduce the deleterious effects of environmental stress and can enhance stress tolerance of plants by a variety of mechanisms such as the synthesis of phytohormones, mineral solubilization, nutrient uptake and antioxidant enzyme activities (Dobbelaere *et al*., 2003).

Given the history of the use of medicinal plants as well as changing attitudes and increasing global demand for these plants in the remedy of diseases with regard to the harms caused by chemical drugs, comprehensive research on medicinal plants needs to be done (Oussalah *et al*., 2007). Rapid seed germination and stand establishment are critical factors affecting crop production under stress conditions. As our understanding of the processes involved in germination has expanded, methods to change those processes have been developed to grow seeds in agricultural applications. In this regards, applying new techniques to enhance crop performance under salt tolerance, especially during germination stage should always be considered. However, potential of seed inoculation with PGPR in direction to enhance the resistance of cumin against salt stress has never been explored. In this research, a laboratory experiment was conducted to evaluate the effect of seed inoculation with *Pseudomonas fluorescens* and *Bacillus subtilis* on seed germination, some biochemical activities and content of some elements in Cumin (*Cuminum cyminum* L.) under salinity stress.

Materials and Methods

In order to study the effect of seed inoculation with plant growth promoting rhizobactria (PGPR) on germination, some biochemical indices and content of some elements in cumin (*Cuminum cyminum* L.) under salinity stress, an experiment was conducted in laboratory of seed science and technology, Yasouj University in 2016 as factorial based on completely random design with four replications. Seed inoculation with plant growth promoting bacteria at five levels (seed inoculation with three strains of *Pseudomonas fluorescens* such as of Pf2, Pf25 and CHA0 and one strain of *Bacillus subtilis* and hydration in distilled water (as check)) and three levels of salinity stress (0, -4 and -8 bar imposed by NaCl) comprised experimental factors (All strains were prepared from Tehran University). In order to prepare the growth medium and suspension of bacteria NA (nutrient agar), culture medium was prepared with addition of 5 g NA and 25.1 g agar to 250 ml distilled water. The culture was then sterilized at temperature of 121 °C. After cooling, bacterial isolates were cultured on NA with loop test tube and in the zigzag shape and were incubated for 24-48 hours for growing. Bacterial population was adjusted to 108 colonies per ml of distilled water using spectrophotometer at a wavelength of 600 nm (Weller and Cook, 1983). Before inoculation, the seeds were disinfected with 2% sodium hypochlorite solution for five minutes. The sterilized seeds were then immersed for one hour in 20 ml of distilled water (control treatment) or bacterial suspension (for the inoculated treatments) at room temperature (20-25 °C).

The seeds were sown on top of a two-layer filter paper in 90 mm petri dishes and moistened with 3 ml of distilled water. The seeds were then incubated at 20-30 °C for 14 days (ISTA, 2010). During the experiment, germinated seed numbers were counted daily. At the end of the experiment, 10 seedlings were randomly selected from each petri-dish. To take the measure of catalase and ascorbate peroxidase activity, seeds were sampled after phase II of imbibition (germination *senso strico*) before root protrusion to be occured. For this purpose, the seeds were imbibed for 48 hours in specified osmotic stress (Van't Hoff, 1887), then 0.6 g of imbibed seeds were weighted and biochemical traits were measured. Protein was extracted in 2 mL of extraction buffer containing 100 mM KH_2P_4 and 100mM NaOH (pH 6.8). The homogenate was centrifuged at 12000 rpm for 30 mins at 4°C. The supernatant was used for enzymatic assays.

The total CAT activity in the seed was measured based on the rate of H_2O_2 consumption at 240 nm (Cacmak and Horst, 1991). The assay mixture of 3 mL contained 25 mM phosphate buffer (pH 6.8), 0.1% H_2O_2, and 20 μL enzyme extract. After the addition of enzyme extract to the reaction mixture, decrement of H_2O_2 levels was determined by measuring the absorbance at 240 nm with a spectrophotometer (UV/VIS Shimaozo 54a) and CAT activity quantified by using the extinction coefficient (0.394 $mMol^{-1}cm^{-1}$).

Total seed APX activity was estimated at 290 nm by the method described by Nakano and Asada. (1978). 3 mL of APX assay mixture contained 50mM phosphate potassium buffer (pH 7.0), 0.3 mM H_2O_2, 0.1 μM EDTA, ascorbate 0.5 mM, and 20 μL of enzyme extract. The amount of ascorbate oxidized was calculated using extinction coefficient (2.8 mM^{-1} cm^{-1}).

Sodium (K^+) and potassium (Na^+) content of germinated seed (seedling) was calculated with Peng *et al*. (2004) method. HCl was used to hydrolyze 0.5 g dried powder of seed samples. (Seeds dried using electric furnace at the 500 ^oC temperature). Then, the

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supernatants of the K^+ and Na^+ extracts were analyzed by atomic absorption spectrometry.

Germination percentage (GP) was calculated using the following equation:

GP = (total number of germinated seeds / total number of seeds) *100 (Agrawal, 2005).

Data were analyzed using SAS (ver. 9.1) and with the significance of the experimental factors interaction, the comparison of the mean was done using L.S.Means test at the probability level of 5%.

Results

Analysis of variance showed that simple and double interaction of the studied factors includes bio-inoculation and salinity were significant on CAT and APX activity, Na^+, K^+ content and Na^+/K^+ ratio and germination percentage (Table, 1).

Catalase (CAT) activity: CAT enzyme activity decreased with increment of salinity stress. Seed inoculation significantly increased CAT activity under both stress conditions. Under control condition (salinity 0 bar), the highest CAT activity (73.34 U mg^{-1} Protein) was obtained from the *B. subtilis* inoculated seeds that was approximately 1.92 times more than non-inoculated seeds. The CAT activity significantly increased in CHA0 inoculated salinized seed that was approximately 2.5 times higher than non-inoculated salinized seed that had no significant difference with *B. subtilis* strain (Figure 1).

Ascorbate peroxidase (APX) activity: The results showed that APX activity was significantly ($p \leq 0.01$) affected by the salinity stress (Table 1). In the non-inoculated group, seed APX activity was 0.2 (U mg^{-1} Protein) at zero bar concentration while the lowest APX activity (0.08 U mg^{-1} Protein) was recorded at -8 bar NaCl potential (Figure 2). PGPR inoculated seeds showed APX activity 1.5 to 2.5 times higher than non-inoculated salinized seeds. In all of the salinity stress levels, the highest APX activities were obtained in *Pseudomonas* sp. CHA0 treated seeds compared to others. Also, the same pattern was observed in primed seeds with PF25.

Sodium (Na^+) content: The Na^+ content of seedling was significantly enhanced by increasing of salinity stress in both inoculated and non-inoculated seeds. The presence of high amounts of Na^+ caused excessive absorption of Na^+ in stressed seeds but inoculation of seeds with PGPR reduced this absorption. Under osmotic potentials of -4 and -8 bar, the highest seed Na content was obtained from non-inoculated seeds. However, inoculation of seeds with all studied bacteria strains, decreased the Na^+ content of cumin seeds. Under stress levels of -4 and -8 bar, the lowest Na^+ content was observed in CHA0 and *Bacillus subtilis* treated seeds, respectively (Figure 3).

Potassium (K^+) content and Na^+/ K^+ ratio: Unlike Na^+, the results showed that the K^+ content was decreased by increasing salinity level in germinated seeds (Figure 4). The K^+ content was decreased by 0.78 and 0.57 %, respectively, at -4 and -8 bar in non-inoculated seeds, while inoculation was ameliorated this effect. However, seed inoculation had moreover significant impact on mineral balance under both salinized and non-salinizes condition. In non-stress seeds, the highest potassium content (0.16 %) was related to CHA0 inoculated seeds. The highest value for this element was obtained from the CHA0 inoculated seeds (Figure 4). Under -4 and -8 bar stress. According to the results, the Na^+/K^+ ratio increased with salinity stress (Figure 5). In inoculated seeds, the Na^+/K^+ ratio was lower than in non-inoculated ones especially at salinity levels of -4 and -8 bar. Under these conditions, the lower Na^+/K^+ ratio was observed in CHA0 inoculated seeds.

Germination Percentage: The results of this experiment showed that the germination of cumin seeds was significantly ($p \leq 0.05$) influenced by the PGPR isolates under different salt concentrations (Table1). Seed germination declined by 22 and 44 % with -4 bar and -8 bar salinity, respectively. Seed inoculation significantly enhanced the germination percentage of cumin seeds under absence (0 bar) and the presence of NaCl at -4 and -8 bar. Application of the PGPR enhanced seed germination by 16-32 percentage at -8 bar salinity. Among the studied isolates, CHA0 inoculated seeds had the highest germination which had no significant difference with *Bacillus subtilis* treated seeds (Figure 6).

Discussion

The study revealed that the increment of salt stress caused to decrease the germination percentage of cumin seeds, whereas seed inoculation with PGPR reduced the adverse effects of salinity. Decreasing of cumin seed germination with salinity levels enhancement were also observed by other researchers (Roodbari *et al*., 2013; Mohammadizad *et al*., 2014; Shoor *et al*., 2014; Piri *et al*., 2016). Inoculation with bacteria strains of PGPR enhanced germination percentage under salt stress conditions. In this experiment, seed inoculation with *Bacillus subtilis* and CHA0 had significant effect on seed germination of cumin under optimal and salt stress conditions.

The previous research findings showed that inoculated seeds increased the compatible solutes such as proline, maintaining ions balance (Shoor *et al*., 2014; Habib *et al*., 2016) total sugars (Ghezal *et al*., 2016) and α-amylase activity, soluble carbohydrate and free amino acids (Metwali *et al*., 2015) and exhibited earlier initiation of protein, RNA, and DNA synthesis. Consequently, when the seeds are out for germination, cellular events are much activated. These results are also supported by the findings of Habib *et al*. (2016) who demonstrated that the germination of okra seeds inoculated with *P. fluorescens* was significantly higher under salinity stress.

Increase in various free radical scavenging enzymes, such as superoxide dismutase, catalase, and peroxidase,

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Table 1. Analysis of variance (mean square) for the effect of seed inoculation on some characteristics of cumin seed under salinity stress

S. O. V	df	Catalase activity	Ascorbate peroxidase activity	Na^+	K^+	Na^+/K^+	Germination percentage
Bio-inoculation	4	1685**	0.0214**	0.000127**	0.00139**	0.09195**	943.06**
Salinity	2	1846**	0.0716**	0.001813**	0.00365**	0.39100**	0.12035**
Bio-inoculation × Salinity	8	63.99**	0.0014**	0.000016**	0.00009**	0.01835**	73.46*
Error	-	10.17	0.0003	0.00001	0.00003	0.00046	21.77
C.V. (%)	–	6.47	9.32	3.41	2.90	3.58	6.81

***, ** significant at 5 and 1 % probability levels, respectively.**

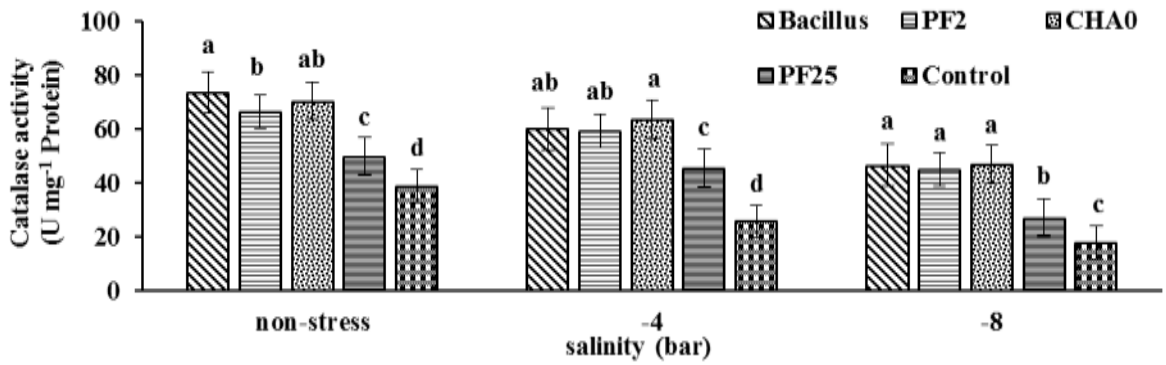

Figure 1. Mean comparison of catalase activity of cumin seed as affected by inoculation with plant growth promoting rhizobacteria at different levels of salinity. Seed germination values are means from four replications of 25 seeds. PF2, CHA0 and PF25 are *Pseudomonas fluorescens* isolates. At each salinity level, means with the same letter are not significantly different according to LSD test at *P* = 0.05.

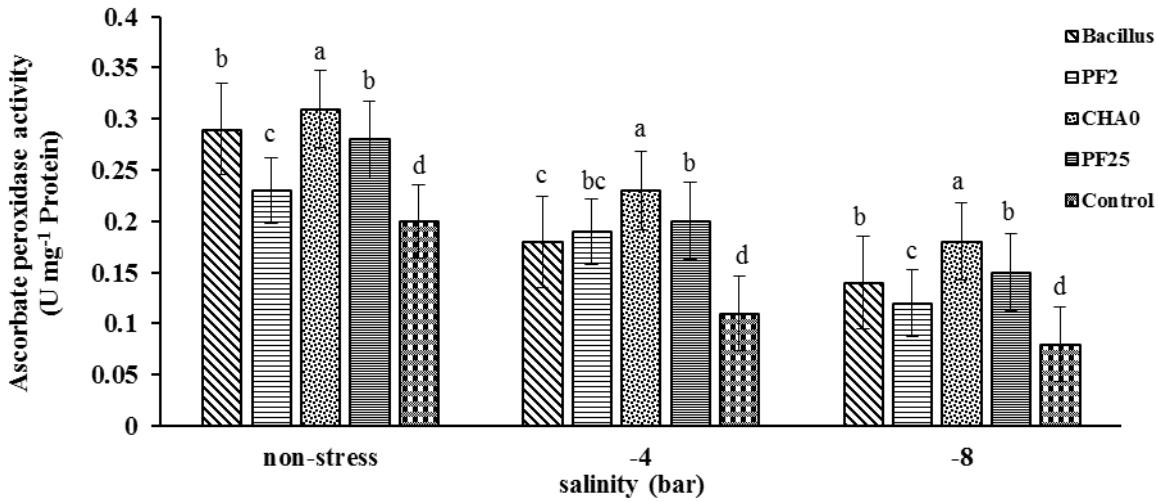

Figure 2. Mean comparison of ascorbate peroxidase activity of cumin seed as affected by inoculation with plant growth promoting rhizobacteria at different levels of salinity. Seed germination values are means from four replications of 25 seeds. At each salinity level, means with the same letter are not significantly different according to LSD test at *P* = 0.05. PF2, CHA0 and PF25 are *Pseudomonas fluorescens* isolates.

have also been demonstrated to influence the germination (Azooz, 2009). CAT and APX activities were significantly higher in cumin seeds receiving bacterial suspension compared with the control plant. Recent reports have confirmed that oxygen species (ROS) had a detrimental effect on the percentage of seed germination under salinity stress (Azooz, 2009). Thus, increase in APX activity confirming that PGPR inoculated seeds were adapted to saline conditions by eliminating ROS through antioxidant enzyme activities (Habib *et al*., 2016). These findings are in agreement with Han and Lee (2005) who reported maximum values of APX activity with PGPR treatments. Achieved results of this experiment are also supported by the findings of Nidhi *et al*. (2014) who reported the enhancement activity of CAT and ROS scavenging enzymes in PGPR inoculated *Senate meadow* seeds under salinity stress. Thus, it could be concluded that

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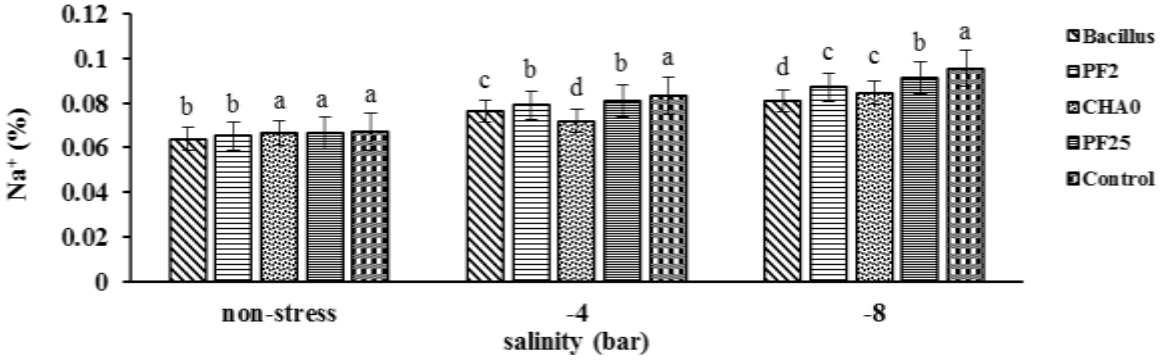

Figure 3. Mean comparison of the seed Na content in cumin seeds as affected by inoculation with plant growth promoting rhizobacteria inoculation at different levels of salinity. Seed germination values are means from four replications of 25 seeds. At each salinity level, means with the same letter are not significantly different according to LSD test at *P* = 0.05. PF2, CHA0 and PF25 are *Pseudomonas fluorescens* isolates.

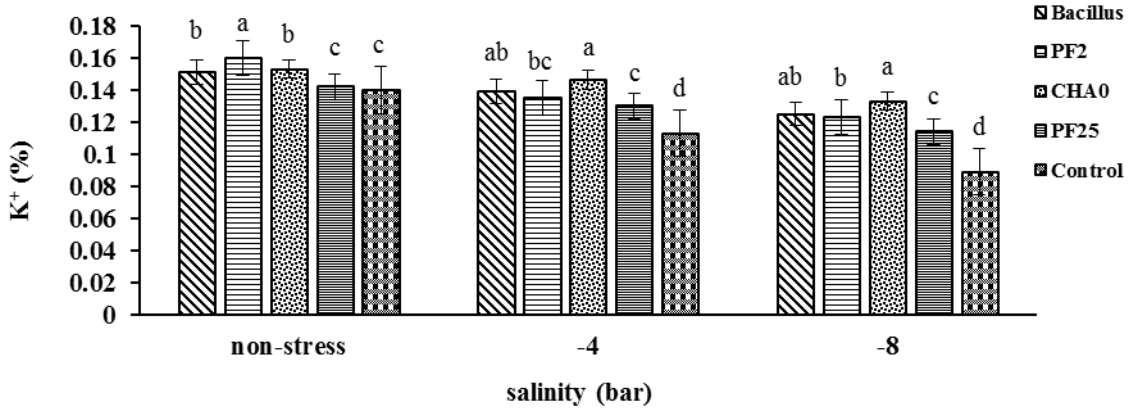

Figure 4. Mean comparison of seed K^+ of cumin seed affected by inoculation with plant growth promoting rhizobacteria (PGPR) at different levels of salinity. Seed germination values are means from four replications of 25 seeds. At each salinity level, means with the same letter are not significantly different according to LSD test at *P* = 0.05. PF2, CHA0 and PF25 are *Pseudomonas fluorescens* isolates.

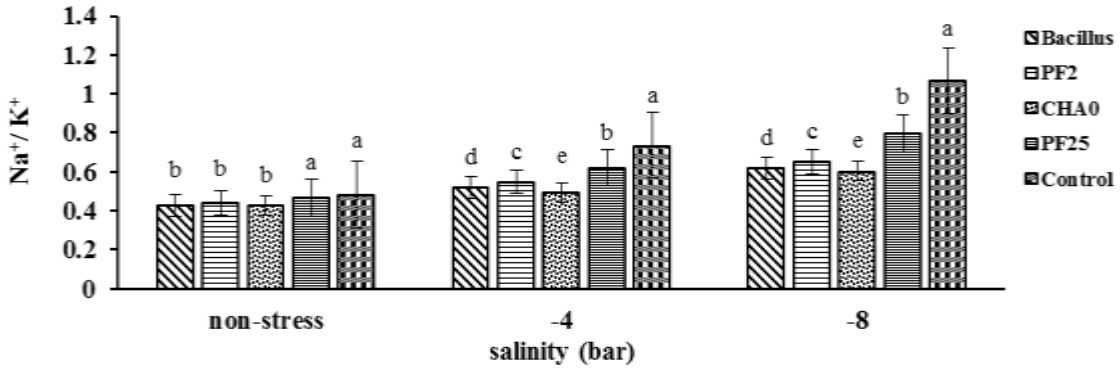

Figure 5. Mean comparison of seed Na^+/K^+ of cumin seed affected by inoculation with plant growth promoting rhizobacteria at different levels of salinity. Seed germination values are means from four replications of 25 seeds. At each salinity level, means with the same letter are not significantly different according to LSD test at *P* = 0.05. PF2, CHA0 and PF25 are *Pseudomonas fluorescens* isolates.

there is a strong correlation between salt tolerance and APX activity.

Moreover, salinity stress and seed inoculation with PGPR had significant impact on mineral balance in cumin. In this study, seed Na^+ content and Na^+/K^+ ratio increased whereas decreased seed K^+ concentration at the presence of NaCl. Reducing the percentage of germination under the influence of rising salinity levels can be attributed to the increase of ions around the seeds (Safarnejad and Hamidi, 2006).The results of this research are in agreement with the findings of Shoor *et al*. (2014), who investigated the cumin seeds submitted to salt stress and recorded a significant decrease in K^+ contents. The ability of plant to limit Na^+ transport into

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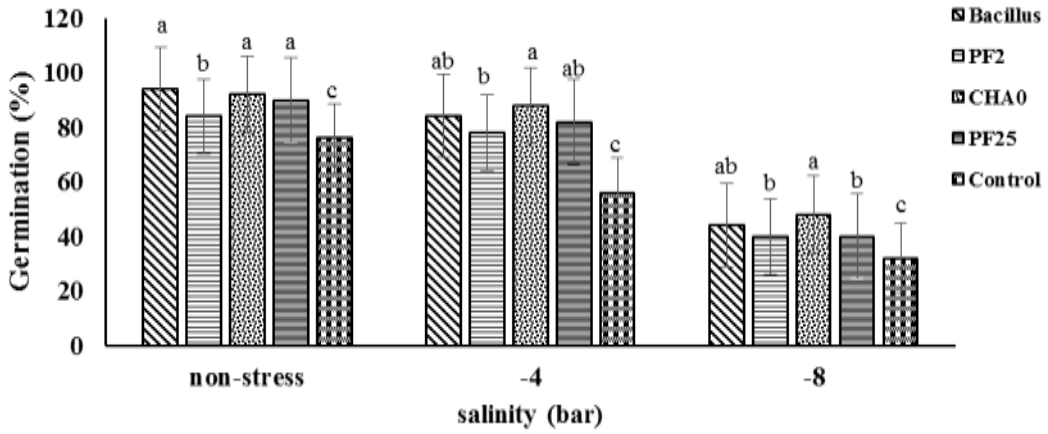

Figure 6. Mean comparison of germination percentage of cumin seed affected by inoculation with plant growth promoting rhizobacteria at different levels of salinity. Seed germination values are means from four replications of 25 seeds each. At each salinity level, means with the same letter are not significantly different according to LSD test at *P* = 0.05. PF2, CHA0 and PF25 are *Pseudomonas fluorescens* isolates.

the plant body is critically importance for the maintenance of high growth rates and protection of the metabolic processes in elongation cells from the toxic effects of Na^+ (Razmjoo *et al*., 2008). The decline of K^+ content as affected by NaCl in seeds might be attributed to the non-specific membrane damage of NaCl and loss of membrane stability (Demidchik *et al*., 2014), that leads to leakage K^+ ions from seed. Also, the NaCl-induced K^+ efflux has been observed in different species (Demidchik *et al*., 2010). Seed priming alleviated the inhibitory effect of salt stress of pea germinated seeds and aerial parts and all of them positively responded to seed priming. Decreasing seedling Na^+ content by PGPR might be due to the fact that the PGPR reduce the excess absorption of Na^+ ions through binding Na^+ with surface polysaccharides and make it less available to plants and could restrict Na^+ influx in roots (Ashraf *et al.*, 2004). The reduction of ion leakage might be related to the inductive responses of antioxidant enzymes that protect plants from oxidative damage (Azooz, 2009). These results confirm with that of Ghezal *et al*. (2016) that observed seeds bio-priming were significantly improved the resistance against salt stress in pea by modulating membrane stability, and ionic homeostasis. It was reported that NaCl stress prevented water absorption by seeds and decreased significantly total germination percentage (Ghezal *et al*., 2016).

High K^+/Na^+ ratio is more important for many species than simply maintaining a low Na^+ concentration (Cuin *et al*., 2003). The increment of Na^+ content in the tissue disturbs the normal cellular function of plants. Chinnusamy *et al*. (2005) reported that a low Na^+/K^+ ratio in the cytosol is essential for normal cell functions. These results indicated that seed priming induced a reduction of Na^+ absorption and toxicity. Amelioration of Na^+/K^+ ratio by priming has been also reported in Cumin (Shoor *et al*, 2014), maize (Zaman *et al*., 2012) and wheat (*Triticum aestivum* L.) (Salama *et al*., 2011). Further, the antagonistic relation between Na^+ and K^+ as a result of priming indicates that priming could play a role in modifying K^+/Na^+ selectivity under salt stress, which is reflected in lowering membrane damage and higher water content in cumin especially under salinity stress (Ghezal *et al*., 2016).

The study revealed that increasing salt stress leads to decrease in germination of cumin seeds, but seed priming with PGPR reduced the adverse effects. It was reported that NaCl stress prevents water absorption by seeds and decreases significantly total germination percentage (Keshavarzi, 2011). The ameliorative effects of priming on germination percentage in cumin seeds under salinity stress have been also reported in previous studies (Neamatollahi *et al*., 2009). Concerning the effect of the seed priming on germination, results demonstrated that this treatment limited the negative impact of salinity because plants developed from PGPR inoculated seeds recorded better germination and growth than plants developed from not inoculated seeds and they showed significant amelioration in the absence and in the presence of NaCl (Ghezal *et al*., 2016). It has been reported that the lower reduction in germination parameters in inoculated seeds with PGPR under the present of salinity stress may be due to the ability of PGPR to limit Na^+ and Cl^- transport into the seed (Metwali *et al*., 2015). Also, it seems that enhancement in germination percentage is due to the improvement of some hormones production particularly GA and Cytokenin as effected by PGPR (Demidchik Grosskinsky *et al*., 2016). GA by activating of some enzymes such as α-amylase that are involved in starch metabolism affectes the germination (Kaymak *et al*., 2009). The classic growth-stimulating phytohormone family of cytokinins (CKs) comprises important regulators of many physiological and developmental plant processes such as cell division, nutrient mobilization and seed germination (Dominik Grosskinsky *et al.*, 2016). Inoculation of seed with

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PGPR by induction of indole acetic acid and various germination-induced amino acids increased germination percentages. Also, ethylene, as a plant growth regulator, is involved in various physiological responses under abiotic stress such as salinity (Stearns and Glick, 2003; Asaduzzaman Siddikee *et al*., 2011). Ethylene stress decreases seed germination and eventually hinders plant growth. It has been reported that the ACC- deaminase enzyme synthesizing microorganisms such as *Bacillus* sp and *Pseudomonas* sp can cleave ACC to *α*-ketobutyrate and ammonia, thereby decrease ethylene stress in plants (Sun *et al.*, 2009; Asaduzzaman Siddikee *et al*., 2011).

Conclusion

This study investigated PGPR effects on cumin during germination of seeds. Seed Inoculation is one of the new methods for improving and enhancing the medicinal plants. Little is known about the enhancement of salinity tolerance in cumin seeds due to PGPR. In this study, seed inoculation with *Pseudomonas fluorescens*. CHA0 showed priority for germination percentage of cumin seeds under salinity stress to other treatments. This can be related to the reduction of growth inhibitory effect of salt on cumin seed through enhancing activity of antioxidant enzymes, modifying ion hemostasis induced by the PGPR. Therefore, CHA0 strain of *Pseudomonas fluorescens* could be recommended for cumin cultivation in areas with salinity tension. However, further research is essential to evaluate the effectiveness of the studied PGPR strains under field conditions.

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