TASOS1 AND *TATM20* GENES EXPRESSION AND NUTRIENT UPTAKE IN WHEAT SEEDLINGS MAY BE ALTERED VIA EXCESS CADMIUM EXPOSURE AND INOCULATION WITH *AZOSPIRILLUM BRASILENSE* SP7 UNDER SALINE CONDITION

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Abstract. Excess of salt and cadmium (Cd) damage the plant's growth; however, Cd toxicity is more severe under saline condition. *Triticum aestivum* transmembrane 20 (*TaTM20)* and salt overly sensitive *(SOS)* respond to salinity. Moreover, wheat-*Azospirillum* association system leads to an increase in wheat tolerance to abiotic stresses. Less information exists related to the effect of salinity and Cd under wheat-*Azospirillum* associated system. Therefore, this experiment was conducted to evaluate the effect of *Azospirillum*-wheat association under salinity and/or Cd stresses. Wheat seedlings (Sardari cultivar) inoculated with *Azospirillum brasilense* Sp7and grown for five days, and then transferred into hydroponic media for five more days with and without 200 mmol NaCl and/or 50 mg L^{-1} Cd, respectively. Root and shoot samples were separated and then dry weight, proline, photosynthetic pigments, catalase (CAT) and ascorbate peroxidase (APX) activities, Cd, Fe, Ca, Na, K were measured. Simultaneously, relative expression of *TaSOS1* and *TaTM20* in the root were measured. The results show salinity and/or Cd have increased root's *TaSOS1* expression and higher upregulation was seen in inoculated seedlings. Meanwhile *TaTM20* gene expression upregulated only under Cd and Cd plus salinity conditions. Salinity and/or Cd were increased root and shoot proline, CAT, APX, Na^+ and Cd whereas dry weight, pigments, Fe, Ca, K were decreased. *A. brasilense* could improve salinity and Cd adverse effects by more upregulation of *TaSOS1* transcript level, lower Na/K ratio and less Fe and Ca deficiency, higher pigments, proline and APX and CAT activities to produce more dry weight.
Keywords: *chlorophyll, carotenoid, Fe, Na, K, dry weight*

Introduction

A considerably large portion of arable land (estimated 20% of total cultivated and 33% of irrigated lands) in the world is affected by salinity (Nellemann, 2009). Furthermore, the salt affected lands are increasing 10% annually due to the low precipitation, high surface evaporation, weathering of rocks, irrigation with saline water, and poor cultivation. It has been estimated that more than 50% of the arable land would be salinized by the year 2050 (Jamil, 2011). Salinity has inhibitory effects on crop production (such as wheat) by altering phonological indexes through root growth (Neumann, 1995), root/shoot ratio (El-Hendawy, 2005), osmoticum components (Hamdia, 2004), ions imbalance (Wakeel, 2013) and total dry matter (Pessarakli and Huber, 1991).

Cadmium (Cd) is a heavy metal, considered harmful to plants, animals and also human beings (Yamaguchi et al., 2009). It occurs naturally in the earth's crust and can be added to the environment via water and soil through natural weathering and human

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activities such as adding fertilizers, pesticides and industrial and/or domestic effluents (Alloway and Steinnes, 1999; Nriagu and Sprague, 1987; Sheppard et al., 2009). Its values range 0.2-1.0 mg Kg^{-1} of dry soil to 50 mg Kg^{-1} of agricultural soil. The maximum permissible addition (MPA) of the heavy metal/metalloid content in the soil is the key idea upon the standardization of the soil contamination (Vodyanitskii, 2016).

Although Cd is not essential for plant and biological systems, different crops will take up and accumulate Cd differently depending on its availability in the environment (Grant and Sheppard, 2008). However, Cd induces oxidative stress, which damages cellular organelles in many plants (Yadav and Chandra, 2013). In addition, Cd could interfere with the uptake of some mineral nutrition such as iron and calcium (Astolfi, 2012; Roth, 2006) and causes mineral concentration imbalance (Chang et al., 2012; Dražić et al, 2004; Lux et al., 2011).

Salinity and heavy metals impose adverse effects on plant growth and its productivity especially in arid and semi-arid regions (Leblebici et al., 2011). Several researchers have reported higher toxic effects of Cd in saline condition (Khoshgoftar et al., 2004). Although the addition of NaCl causes higher sodium absorption by a plant which decreases plant production (Dražić et al., 2004). Chloride of NaCl forms $CdCl_2$ in the present of Cd and therefore leads to more Cd absorption due to the much higher solubility of $CdCl_2$ among its Cd components. $CdCl_2$ has solubility limit of 1,680,000 mg L^{-1} at 20°C in water (National Research Council, 1997). This condition causes more Cd toxicity for plant growth (Smolders et al., 1998).

Plant growth promotion rhizobacteria (PGPRs) help the plant to grow better by different mechanisms. It has been reported that PGPRs act in favor of plant mineral uptake (Askary et al., 2009), more phytohormones production (Kang et al., 2014) as well as modification in some gene expression (Vargas-Garcia et al., 2012). Creus et al. (1997) also confirmed the reduction of adverse effects of salinity and osmotic stresses on the length and dry weight of wheat plants when inoculated by *Azospirillum brasilense*.

Concerning inoculation and heavy metal, there are two consequences of using PGPRs in environments polluted by heavy metals. Some researchers believe that PGPRs can absorb and accumulate heavy metals and reduce soil and water pollution level (Belimov et al., 2005; Ma et al., 2009; Prapagdee and Khonsue, 2015), while some others believe PGPRs are able to reduce the availability of heavy metals in the soil and water which in turn lead to more growth and yield production (Belimov et al., 2005; Belimov and Dietz, 2000; Dell'Amico et al., 2008; Gao et al., 2012). In any way, one of the safe approaches to revert the detrimental effects of Cd and salinity on crop production is establishing an associated system between crops and soil microorganisms such as *Azospirillum* species with wheat cultivars. *Azospirillum* spp. not only can improve wheat productivity through higher root and shoot development (Amooaghaie et al., 2002), more nutrient uptake (Askary et al., 2009) and improvement of phytohormones but also can reduce adverse effects of salinity and Cd under saline (Upadhyay et al., 2011) and Cd-stressed conditions (Belimov and Dietz, 2000).

Kim et al. (2008) showed *Triticum aestivum* transmembrane 20 (*TaTM20*) gene expression in wheat plant upregulated due to the excess of Cd. They also showed that this gene confers Cd tolerance to yeast transgenic containing *TaTM20* gene. Ramezani et al. (2013) and Kim et al., (2008) The upregulation of salt overly sensitive 1 (*TaSOS1*) and *TaTM20* under saline condition (Ramezani et al., 2013) and *TaTM20* upregulation under Cd polluted conditions (Kim et al., 2008) are also reported. Xu et al. (2008) demonstrated *SOS1* upregulation at both roots and shoots of wheat plants under saline condition. They

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also showed that yeast transgenic containing *TaSOS1* gene has higher salt tolerance as compared to non-transgenic ones. In addition, Taherinia et al. (2015) revealed that salinization was affected *SOS1* transcript level positively in kallar grass (*Leptochloa fusca* L.) a halophyte plant which is highly tolerant to saline and sodic soil and water.

Accordingly, it can be proposed that *Azospirillum* species may contribute to the upregulation of *TaSOS1* and *TaTM20* gene expression in wheat plants under saline and Cd polluted condition and improve salinity and Cd tolerance. Not only that, *Azospirillum* may help to limit Cd absorption by plant root. Therefore, this research was conducted to evaluate *TaSOS1* and *TaTM20* gene expression in the roots and also chlorophyll (a and b) and carotenoid of the shoots. Simultaneously other indexes such as Fe, Ca, K , Na, Cd, proline, antioxidant enzymes (CAT and APX) of the roots and shoots of wheat seedlings (Sardari cultivar) were measured under excess of salinity, Cd and inoculated conditions.

Materials and methods

Preparation of inoculants and seeds

Azospirillum brasilense Sp7 (standard strain) was obtained from NCIMB Ltd, Germany. Then cultured in an NFb liquid medium supplemented with NH_4Cl (0.25 g L^{-1}) at 30°C (Brenner et al., 2005) in Erlenmeyer flasks for 48 h and used a rotary shaker at 200 rpm (logarithmic phase). The growth was harvested by centrifuging (1000 g, 10 min), washed with sterile saline phosphate buffer and then re-suspended in phosphate buffer at concentration of 10^7 CFU ml^{-1} of *A. brasilense* Sp7 (Askary et al., 2009).

Wheat (*Triticum aestivum* L., Sardari cv.) seeds obtained from Institute of Agricultural and Research, Isfahan, Iran. The seeds were surface sterilized by dipping in 95% ethanol for 2 min and then in 1% sodium hypochlorite (NaOCl) for 1 min followed by six washes in sterile distilled water (Sauer and Burroughs, 1986). The sterilized wheat seeds were vernalized at 4°C for one night.

For germination, sterilized seeds were kept in dark on water agar in autoclaved petridishes held at 25 °C temperature. After 24 hours, uniform seedlings were divided into two groups. The first group was inoculated by 10^7 CFU ml^{-1} *A. brasilense* Sp7 and the second group transferred into free bacteria phosphate buffer (Bashan, 1990) as control (non-inoculated). After 3 hours, all plants (inoculated and none inoculated) were transferred into pots containing sterile perlite and irrigated with 1/4 strength of Hoagland's nutrient solution (Hoagland and Arnon, 1950). The plants kept in a glasshouse under 16/8 h (Light/Dark) photoperiod using white light (photon density 650 µmol m^{-2} S^{-1}) at 25±2 °C for 5 days. Then, besides control pots, both groups were treated with 200 mM of NaCl and/or 50 mg L^{-1} of cadmium as $CdCl_2$ as treatment for five more days. This experiment conducted in a completely randomized design with three replicates. At day-10 of the experiment (five days after salt and Cd stresses applied), roots and shoots of plant samples were separated and washed with distilled water. Some of the separated plant samples immediately frozen in liquid nitrogen for real-time quantitative PCR and the remaining samples were used for different analysis.

Real-time quantitative PCR

Real-time PCR was performed using RB SYBR master mix (RNA Biotech, Iran). The total RNA was isolated from frozen roots using Iraizol reagent (RNA biotech, Iran)

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and the first strand cDNA was synthesized using the M-MLV reverse transcriptase (Fermatas). Gene-specific primers were used for *TaSOS1* (Gen Bank Accession No. AY326952), *TaTM20* (Gen Bank Accession No. DQ323065) and *G3PDH* (Gen Bank Accession No. EU022331). The sequences of the primers designed to amplify *TaTM20* were as follows: 5'-CCGATCCTCTTGCACAACTA-3' and 5'-ATGGACAGCATGAAGCTCAC-3'; (Kim et al., 2008) the primers for *G3PDH* were as follows: 5-TCACCACCGAGTACATGACC-3' and 5'-TCGTCCTTGAGCTTGATGT-3' (Kim et al., 2008); and the primers for *TaSOS1* were as follows: 5'-GGGATGATGAGGAACTTGGG-3' in sense direction and 5'-CTTGTCAGGAACATCGTGGG-3' in anti-sense direction (Xu et al., 2008). The PCR conditions were 94 °C for 4 min followed by 40 cycles of 94 °C for 10 s, 62 °C for 40 s, 72 °C for 60 s, followed by 7 min at 72 °C. Serial dilutions of cDNA were used to obtain optimized standard curve amplification efficiency and the best cDNA concentration for real-time PCR. The relative expression of each gene as the fold expression was calculated through Livak and Schmittgen's method (2001).

Plant pigment determination

Chlorophyll a and b and carotenoid were performed according to Arnon (1967) with some modifications. 100 mg fresh leaves were homogenized in 2 ml 80% acetone with mortar. Homogenates were centrifuged at 4°C for 10 min (3500 rpm). Supernatants were used for the analysis of pigments. Absorbance of plant sample was determined at 645, 663 and 470 nm, respectively and then the following equations (Eq. 1-3) were used for calculation of different pigments.

$$Chlorophyll\ a = \frac{(19.3 \times A_{663} - 0.86 \times A_{645})V}{100W} \quad \text{(Eq.1)}$$

$$Chlorophyll\ b = \frac{(19.3 \times A_{645} - 3.6 \times A_{663})V}{100W} \quad \text{(Eq.2)}$$

$$Carotenoids = \frac{100(A_{470}) - 3.27(mg Chl\ a) - 104(mg\ Chl\ b)}{227} \quad \text{(Eq.3)}$$

Determination of sodium and potassium content

Sodium and potassium content of shoot and root were measured based on the method described by Skoog et al. (2005). To do so, 100 mg powder of dry sample (70 °C for 72 hours) was digested with 10 ml 3 % (w/v) aqueous sulfosalicylic acid for 24 h at 4 °C, then sample extract was purified using Whatman No. 1 filter paper. Na^+ and K^+ concentration were measured using flame photometry method (Gallenkamp flame analyzer, England).

Determination of Ca, Cd, and Fe

100 mg dry weight of each sample (roots and shoots separately) was digested in 3 ml of a 1- 4 (v/v) mixture of 37% (v/v) HCl and 65% (v/v) HNO_3 in Teflon cylinders for 7 h at 140 °C. After adjustment of volume to 10 ml with deionized water, Ca, Cd, and Fe was determined using an atomic absorption spectrophotometer (AAS, Shimadzu model 6200).

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Determination of proline content

Proline content of roots and shoots were determined using Bates et al. (1973) method. The fresh plant samples (100 mg) was homogenized with 4 mL sulfosalicylic acid (3.0%) in a mortar. The suspension was centrifuged at room temperature at 3000 rpm for 5 min. The supernatant was mixed well with 4 mL acidic ninhydrin reagent. The reaction mixture was vortexed and the content of the tubes was placed in a boiling water bath for 60 min. Then, the content was cooled in the ice bath and the mixture was extracted with 4 mL of toluene. The absorbance of toluene layer was recorded at 520 nm using Shimadzu spectrophotometer (Shimadzu UV-160, Japan). The concentration of the unknown samples was calculated using standard curve.

Enzyme extract and antioxidant assay

Plant's shoot samples were extracted for enzymes assay by homogenizing in an ice-cold 50-mM sodium phosphate buffer of pH 7.4 along with 1% (w/v) polyvinyl poly pyrrolidone (PVP) by a pre-chilled mortar and pestle. Homogenized mixture was centrifuged at 13000 g for 20 min at 4°C. The supernatant was directly used for various enzymatic assay as crude enzyme extract. The quantification of soluble protein present in the extract was done by Bradford's method (Bradford, 1976) using bovine serum albumin (BSA) as standard. Catalase (CAT) activity (EC.1.11.1.6) was measured by estimating the breakdown of H_2O_2, which was determined at 240 nm as described by Beers and Sizer (1952). The enzyme activity was expressed as μmol decomposition of H_2O_2 min^{-1} mg^{-1} protein. Ascorbate peroxidase (EC.1.11.1.11) activity was done by using the method described by Nakano and Asada (1981) and the enzyme activity was expressed as μmol decomposition of ascorbate min^{-1} mg^{-1} protein.

Statistical analysis

MSTAT-C software was used for ANOVA calculation and Duncan's multiple range tests were used to compare the mean values using 95% confidence interval. Excel was used to draw the necessary graphs. The experiment was a completely randomized design.

Results

Root and shoot dry weight

The maximum amount of root (7.44 mg $plant^{-1}$) and shoot (21.91 mg $plant^{-1}$) dry weight was observed in inoculated plants not exposed to any salinity and/or Cd. At non-inoculated condition, adding Cd to the nutrient media has caused a significant reduction in dry weight of root (24.1) and shoot (18.1%) as compared to their control ($P \leq 0.05$), while dry weight of shoot and root wheat seedlings unaffected under 200 mmol NaCl (*Fig. 1*). The dual stresses of salinity and Cd lead to more reduction of roots (14.8%) and shoots (9.0%) dry weight when compared with the Cd stress alone.

In inoculated plants not exposed to Cd and/or salinity, root and shoot dry weight significantly were increased by 22.97 and 9.7%, respectively in comparison to their control. At Cd stress condition, inoculation significantly alleviated harmful effects of Cd on shoot dry weight as compared to non-inoculated plants. Root and shoot dry weight of inoculated seedlings and also the seedling plants were grown under saline condition showed significant difference when compared with control seedlings.

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Although in dual stresses a reduction in root and shoot dry weight of inoculated plants were observed, but inoculation significantly improved the root and shoot dry weight by 12.8 and 16.67%, respectively when compared to non-inoculated ones.

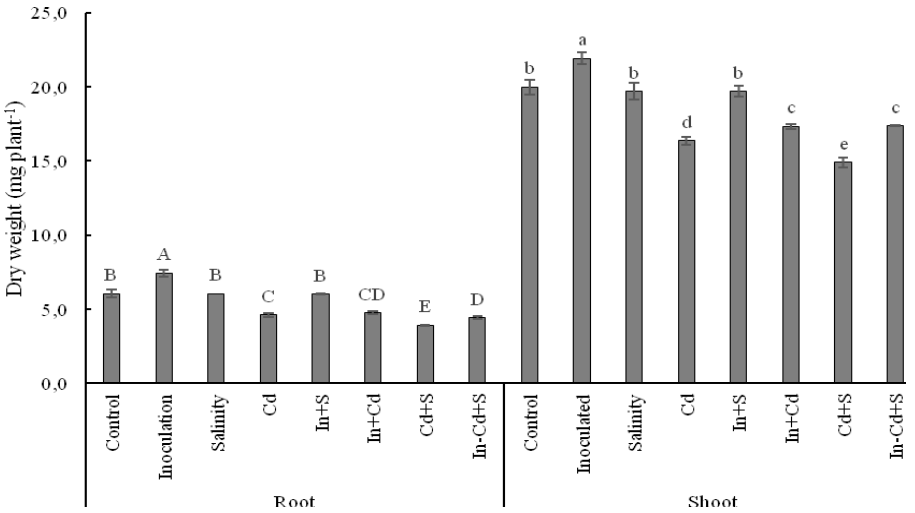

Figure 1. *Effect of inoculation, salinity and Cd on average (n = 3 ± SD) dry weight of roots and shoots of wheat seedlings. Differences in lower and upper case letters on the bar graph indicated significant difference (P < 0.05) in their mean values based on Duncan's multiple range tests.*

Root and shoot proline content

Inoculated seedlings had lower proline in the root and the shoot when compared to control plants. Salinity didn't have a significant effect on shoot proline, meanwhile, proline of root was increased from 0.7 to 1.14 μmol g^{-1} FW ($P < 0.05$, *Fig. 2)*. Cd also showed a significantly ($P < 0.05$) increase in proline of root and shoot by 60 and 44%, respectively. Dual effect of Cd and salinity causes an addition of proline in the root (21.4%) and shoot (13.0%) of non-inoculated plants compared to control seedlings.

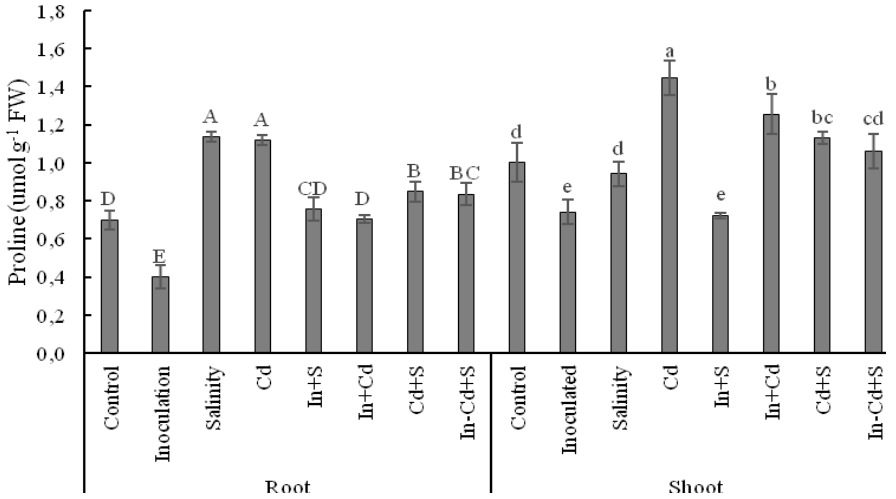

Figure 2. *Effect of inoculation, salinity and Cd on average (n = 3 ± SD) proline content of roots and shoots of wheat seedlings (n = 3 ± SD). Differences in small and cap letters on the bar graph indicated difference in their mean values based on Duncan's multiple range tests.*

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The lowest amount of proline was measured in the root and the shoot of inoculated plants not exposed to Cd and/or salinity. However, the higher values of proline in the root was measured under excess of salinity and Cd and in the shoot belonged to Cd treatment. When seedlings were inoculated with *Azospirillum brasilense* then the high proline accumulation under Cd excess and/or salinity significantly has been reduced to lower amounts which were still higher than in control plants.

Shoot pigments

Inoculation caused addition (22%) of chlorophyll a when compared to control seedlings. In comparison to control plants, significantly less chlorophyll ($P < 0.05$, *Table 1*) was seen in the shoot of seedlings exposed to Cd or Cd+salinity (56.10 and 51.22%, respectively). However, inoculation was increased chlorophyll a of seedlings exposed to Cd or Cd+slinity as much as control plants. The amount of chlorophyll b was reduced more under Cd than salinity in non-inoculated plants. In contrast, chlorophyll b was increased in inoculated plants under salinity and/or Cd, but it was still lower than control plants. Total chlorophyll (a and b) showed a positive relation with inoculation while a negative relation was observed in excess of Cd and/or salinity conditions with total chlorophyll. Cd (55.36%) and Cd+salinity (53.56%) significantly decreased total chlorophyll of non-inoculated seedlings as compared to control plants.

Maximum total chlorophyll (0.65 mg $Plant^{-1}$) was observed in inoculated not exposed to any treatment. The maximum reduction of total chlorophyll was seen under excess Cd and Cd+salinity in non-inoculated seedlings when compared to control plants.

Table 1. *Effect of inoculation, salinity and Cd on the mean values (n = 3) of chlorophylls (a, b, total) and Carotenoids in the shoot of wheat seedlings. Differences in the letters on the mean values indicated the significant difference in their means based on Duncan's multiple range tests.*

Treatments	Chlorophyll a	Chlorophyll b	Total chlorophyll	Carotenoids
Control	0.41^{B}	0.15^{A}	0.56^{B}	1.35^{D}
Inoculation (In)	0.50^{A}	0.16^{A}	0.65^{A}	1.36^{CD}
Salinity (Sal)	0.40^{B}	0.12^{C}	0.52^{B}	1.56^{B}
Cadmium (Cd)	0.18^{C}	0.06^{D}	0.25^{C}	0.70^{E}
In + S	0.43^{B}	0.13^{B}	0.56^{B}	1.70^{A}
In + Cd	0.41^{B}	0.14^{B}	0.54^{B}	1.48^{BC}
Cd + Sal	0.20^{C}	0.06^{D}	0.26^{C}	0.72^{E}
In + Sal + Cd	0.40^{B}	0.14^{B}	0.54^{B}	1.40^{CD}

Antioxidant assays

In most cases, the amount of APX and CAT activities were higher in the shoot than root seedlings and also the amount of CAT activity was higher than APX. The lowest and the highest activities of APX were observed in root and shoot of control and treated seedlings expose to Cd+salinity. Their amounts in the roots were 0.41 and 1.22 μmol min^{-1} mg^{-1} protein ($P < 0.05$, *Fig. 3*) and for shoot were 0.29 and 1.42 μmol min^{-1} mg^{-1} protein, respectively. This is almost 197.5% addition in roots and 389.6% for shoots. Inoculation did not have any effect on APX activity of seedlings compared to control.

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However, inoculation caused reduction of APX activities of the seedlings exposed to Cd and/or salinity but their amounts still were more than control seedlings.

Change in CAT activities under different conditions was approximately similar to APX activities of the roots and shoots. The lowest and the highest activities of CAT were observed in root and shoot of control and treated seedlings expose to Cd+salinity. Their amounts in the roots were 0.5 and 1.35 μmol min^{-1} mg^{-1} protein ($P < 0.05$, *Fig. 3*) and for shoot were 0.25 and 1.17 μmol min^{-1} mg^{-1} protein, respectively. This is almost 170.0% addition in roots and 368% for shoots. Inoculation did not have any effect on CAT activity of seedlings compared to control. However, inoculation also showed no changes in CAT activities of the seedlings exposed to Cd or salinity but their amounts still were more than control seedlings.

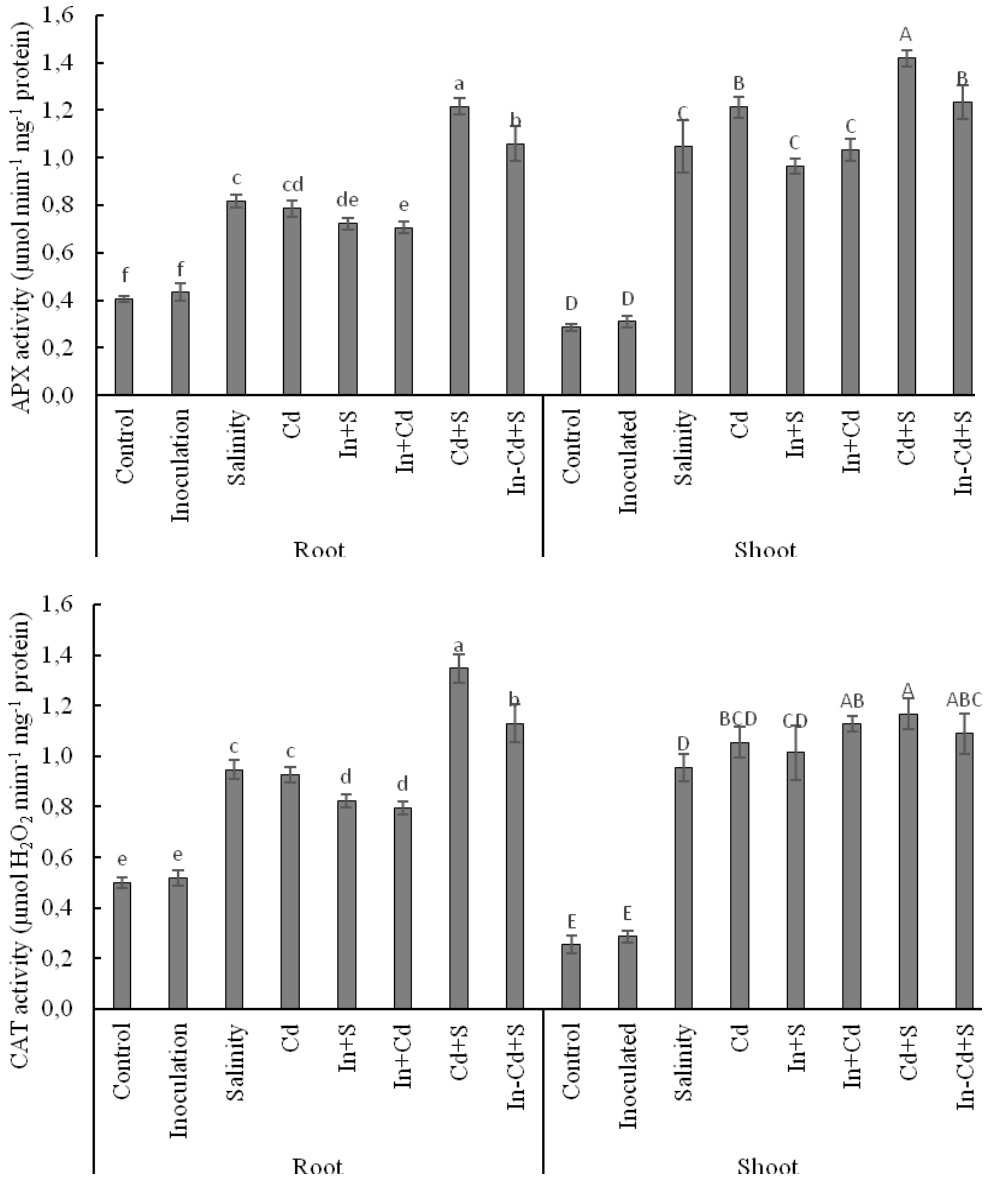

Figure 3. *Effect of Salinity (Sal) and Cd, inoculation (In) and their interactions on the activities of APX and CAT in the shoot of wheat seedlings in inoculated and non-inoculated condition. Mean values (n = 3) with the same letter are not significantly different at P < 0.05.*

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Cadmium, sodium, potassium, calcium and iron of root and shoot

There was not any Cd in the root of seedlings of non-inoculated or inoculated plants under control and salinity conditions. While its amount in the root of treated seedlings was increased to 32.18 mg g^{-1} (Cd-treated), 28.27 (Cd and inoculated), 38.49 (Cd and salinity) and 33.29 mg g^{-1} DW for inoculated+Cd+salinity treated seedlings. It was found that the amount of Cd in Cd+salinity treated of non-inoculated plants was the highest and significantly ($P < 0.05$) higher than the other treatments. Also, inoculation reduced the Cd concentration of plant exposed to Cd. It is interesting that Cd in the shoot of inoculated seedlings (7.52 mg g^{-1} DW) did not show significant reduction compared to non-inoculated plants exposed to Cd (7.01 mg g^{-1} DW). At non-inoculation condition, the Cd of roots and shoots raised due to the combined effect of Cd+salinity.

In non-inoculated seedlings, the amount of Fe in the roots and shoots (*Table 2*) was the highest (0.38 and 0.16 mg g^{-1} DW, respectively) and a significant reduction was observed in roots treated with Cd (21.05) and salinity (23.68%) stress, but inoculation couldn't help the Fe uptake by the roots under Cd and/or salinity conditions. However, *Azospirillum* helped to increase the amount of Fe in unstresses seedlings to more than control plants and reached to 0.51 mg g^{-1} DW (the highest amount). Fe in the shoots and roots of inoculated and non-inoculated seedlings followed a similar trend.

Table 2. *Effect of various tested treatments on roots and shoots nutrients of seedlings wheat plants. Values with the same letter are not significantly different at $P < 0.05$.*

	Root (mg g^{-1} DW)					Shoot (mg g^{-1} DW)				
Treatments	**Cd**	**Fe**	**Ca**	**Na**	**K**	**Cd**	**Fe**	**Ca**	**Na**	**K**
Non-inoculated	0.00^{D}	0.38^{B}	1.88^{B}	1.32^{E}	6.30^{B}	0.00^{C}	0.16^{B}	2.22^{B}	0.49^{B}	18.28^{B}
Inoculated (In)	0.00^{D}	0.51^{A}	2.14^{A}	0.80^{F}	9.87^{A}	0.00^{C}	0.22^{A}	2.46^{A}	0.20^{E}	22.50^{A}
Salinity (Sal)	0.00^{D}	0.29^{D}	1.15^{D}	1.83^{A}	4.81^{D}	0.00^{C}	0.11^{C}	1.53^{D}	0.60^{A}	15.70^{DE}
Cadmium (Cd)	32.18^{B}	0.30^{CD}	1.07^{D}	1.76^{AB}	4.83^{D}	7.01^{B}	0.12^{C}	1.57^{D}	0.58^{A}	15.50^{DE}
In + Sal	0.00^{D}	0.32^{C}	1.52^{C}	1.59^{D}	5.35^{C}	0.00^{C}	0.13^{C}	2.04^{BC}	0.30^{D}	17.08^{BC}
In + Cd	28.27^{C}	0.31^{CD}	1.55^{C}	1.62^{CD}	5.34^{C}	7.52^{AB}	0.13^{C}	1.95^{C}	0.33^{CD}	16.67^{CD}
Cd + Sal	38.49^{A}	0.30^{CD}	1.13^{D}	1.71^{BC}	4.72^{D}	7.66^{A}	0.11^{C}	1.60^{D}	0.56^{A}	15.30^{E}
In + Sal+ Cd	33.22^{B}	0.31^{CD}	1.56^{C}	1.55^{D}	5.26^{C}	7.71^{A}	0.12^{C}	1.92^{C}	0.37^{C}	16.18^{CDE}

Calcium concentration in the roots and shoots of inoculated plants not exposed to salinity and/or Cd increased by 13.83 and 10.81%, respectively when compared to control plants. Cd, or salinity and also salinity+Cd in both roots and shoots of seedlings caused a significant reduction in Ca content. However, inoculation was improved its amount in the root and shoot but their amount was still less than the control ones.

In non-inoculated condition, the amount of Na in the root and shoot was 1.32 and 0.49 mg g^{-1} DW. While application of salinity increased Na of the roots (38.63%) and shoots (22.45%) in non-inoculated plants. Dual effect of Cd and salinity show the highest amount of Na in the roots and shoots; however, its amount was less in inoculated plants exposed to Cd and/or salinity. When plants were inoculated then the amount of Na in the roots and shoots decreased by 39.4% and 59.2%, respectively. Simultaneously K of the roots and shoots increased by 56.6 and 23%, respectively. Inoculation decreased harmful effects of Cd and/or salinity via less Na and increase in K uptake and its accumulation in the roots and shoots of inoculated plants. In whole plant (root and shoot), the best and the

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worst conditions for Na content were measured in inoculated (0.28 mg g^{-1} DW) and salinity (2.43 mg g^{-1} DW) seedlings, respectively. However, the worst and the best conditions for K were seen in seedlings treated with Cd+salinity (20.1 mg g^{-1} DW) and inoculated but not exposed to any treatment (32.3 mg g^{-1} DW), respectively.

TaSOS1 and TaTM20 genes expression

In non-inoculated condition, there was not any significant expression and difference for *TaSOS1* of roots in control and inoculated seedlings (*Fig. 4*) while Cd (14.13 fold change) and salinity (32.43 fold change) significantly caused addition of expression of *TaSOS1*. In inoculated seedlings, the *TaSOS1* experession significantly was decreased in Cd-treated seedlings as equal as control seedlings, but there was higher expression of *TaSOS1* under saline condition (61.03). In dual effects of Cd and salinity, the *TaSOS1* expression was the highest (72.89 fold changes) and inoculation cause addition of its upreggulation under salinity but no effect under other treatments.

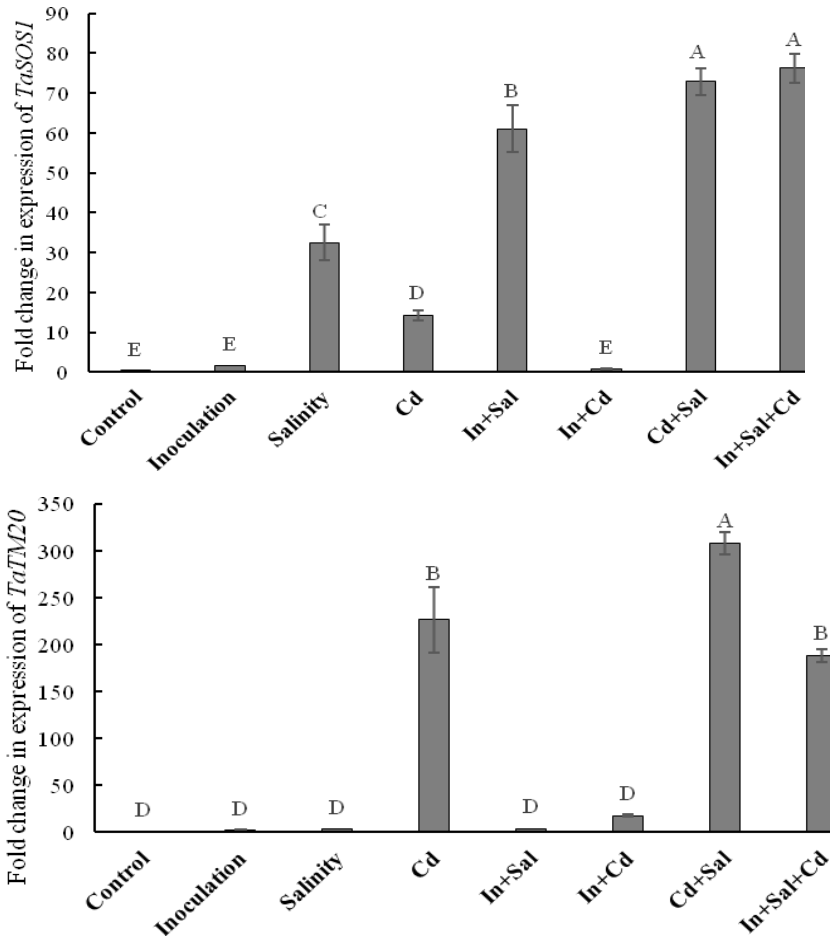

Figure 4. *Fold change expression of TaSOS1 and TaTM20 genes in the root of inoculated (A. brasilense, 10^7 CFU ml^{-1}) and non-inoculated wheat seedlings grown under saline and non-saline (200 mmol NaCl) condition. The plant samples were obtained five days after salt application. The growth conditions were light density of 650 µmol $m^{-2}S^{-1}$ and temperature of 25 ºC. Each value represents mean of three individual measurements ± SE. Different letters represent significant differences at the 95% confidence interval.*

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There were no significant differences in *TaTM20* gene expression in non-inoculated and inoculated seedlings when seedlins were not exposed to Cd or salinity. Simultaneously, salinity had no significant effect on *TaTM20* expression either in inoculated or non-inoculated seedlings not exposed to Cd. However, Cd by itself could increase *TaTM20* expression significantly (226.3 fold change) in non-inoculaed seedlings but when inoculated with *A. brasilense* there was significantly less *TaTM20* expression when compared to control seedlings. The highest effect in *TaTM20* expression was seen in combined treatments of Cd and salinity (308.3 fold change). While this effect was lower (188.3 fold change) in dual effects under inoculated with *A. brasilense.*

Discussion

A large portion of agricultural land suffers from salinity, which causes a reduction in agricultural production (Siadat, 1998). Simultaneously, the deficiency of plant nutrients causes fertilizer application to increase crop yields (Adams, 1991; Dobermann, 2000). Most chemical fertilizers especially phosphorous and also manure mainly contains unwanted elements such as Cd and nickel (He and Singh, 1994). This is because of the waste application and industrial activities. Many researchers have pointed out that crops are affected by the excess of salinity and heavy metals (Corwin and Ahmad, 2015; Leblebici et al., 2011; Shah et al., 2011). Cd and NaCl have been identified as important abiotic stresses in crop production (Corwin and Ahmad, 2015; Mahajan and Tuteja, 2005).

Our results indicated that root and shoot dry weight decreased under excess of Cd and salinity+Cd. This result is similar to that of Leblebici et al., (2011) who showed the relative growth rate of *Lemnaceae* plants (*Spirodela polyrrhiza*) was decreased under salinity+Cd condition. Excess Cd in the soil can reduce the growth of plants by direct or indirect effect on photosynthesis pigments, gas exchange parameters as well as plant nutrient imbalance (Gohar et al., 2003; Parida et al., 2003; Shah et al., 2011). Moreover, it is possible that shoot and root dry weight affected by the toxic effects of Cd due to mainly the chlorocomplexes formation (Weggler-Beaton et al., 2000). In contrast, plant growth promotion rhizobacteria (PGPRs) can reduce the harmful effects of salinity and Cd stress (El-Dengawy et al., 2011; Stout et al., 2010). Our results show *Azosirilium brasilense* improved salinity and/or Cd adverse effects on root and shoot dry weight. These results are accordance with the result obtained by Giller et al., (1998) who showed that bacteria can reduce the phytotoxicity of the contaminated soil by heavy metals. In addition, Stout et al., (2010) indicated that bacteria could serve as a phytoprotective factor in their relationship with *Lemna minor*, preventing uptake of toxic Cd. Also, El-Dengawy et al., (2011) reported that inoculation of Carob seedlings (*Ceratonia siliqua* L.) with *A. lipoferum* under saline condition could improve the reduction of seedling growth rate, K^+/Na^+ content and root characters. PGPRs can help the plant to grow better by different mechanisms such as efficient mineral uptake (Askary et al., 2009), more phytohormones production (Kang et al., 2014) and better tolerant to abiotic stresses (Vargas-Garcia et al., 2012).

Salinity could lead to osmotic stress (Carillo et al., 2008), disruption of homeostasis and ion distribution (Zhang, 2008) as well as formation of reactive oxygen species (Matysik, 2002) in plant cell that has harmful effects on plant growth and its productivity. Proline production is one of the mechanisms that enable the plant to tolerate adverse effect of environmental stresses. Proline is thought to contribute to

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osmotic adjustment, detoxification of ROS, and protection of membrane integrity (Heuer, 2010). Addition of proline in our experiment is similar to Tavakoli et al., (2016) who showed saline condition could accumulate the proline content of three Iranian wheat cultivars. In addition, Sharmila et al., (2017) also demonstrated that Cd-induced iron deficiency and promotes proline accumulation in *Brassica juncea* plants. Although there are numerous reports related to accumulation of proline in plant cells after inoculation with some *Azospirillum* Spp.. Our result showed a negative correlation between proline content of non-inoculated wheat seedlings and inoculation ones with *A. brasilense* Sp7. This result may be due to the fact that *Azospirillum* Spp. may facilitate its beneficial effects in different ways on the host plant.

Many studies have shown a reduction in photosynthetic pigments in saline soil (Leblebici et al., 2011; Shafi et al., 2009; Tiwari, 2010 ; Jamil et al., 2007). Our result also showed a reduction in photosynthesis pigments of shoots under saline condition which is similar to the result of El-Dengawy et al., (2011) who demonstrated chlorophyll content decreased in response to salinity in leaves of carob seedlings. It seems that reduction in chlorophyll could be due to less number of chloroplast and disorganization of thylakoid's membrane structure in leaves under salt stress. In addition, our results are consistent with Cheng et al., (2013) who showed an increase in carotenoids of shoot as a way to cope with the saline condition, especially in glycophyte plants. Photosynthetic pigments may be negatively affected by Cd stress in different ways. For example, Cd prevented the chlorophyll production by affecting the synthesis of 5-aminolaevulinic acid, disabling the protochlorophyllide reductase (Stobart et al., 1985) and Fe deficiency (Fodor et al., 2005; Wallace et al., 1992). In addition, the increase in Cd concentration caused loss of chlorophyll and damage to membrane in two maize cultivars (Ekmekçi, et al., 2008). The result of this experiment showed a reduction of shoot chlorophyll due to excess of salinity and/or Cd, meanwhile, *A. brasilense* was able to prevent further reduction of shoot chlorophyll under dual treatments (*Table 1*). This result in some way is similar to Zhang et al., (2008b) who indicated *B. subtilis* GB03 increases photosynthetic efficiency by increasing chlorophyll content in *Arabidopsis*. In addition, Wani and Khan (2010) showed that photosynthetic pigments of chickpea (*Cicer arietinum* L.) improved by *Bacillus* species under different Cd concentration. Reduction of chlorophyll in our experiment under Cd or salinity conditions should be one of the reasons for dry weight reduction and the higher reduction of dry weight in Cd+salinity could be due to more solubility of Cd under higher NaCl of the media.

Antioxidant enzymes activity have been studied specially under stress conditions (Asada, 2006; Shafi et al., 2009). It has been reported that antioxidant enzymes activity could be increase due to stressful condition. Our result clearly demonstrated that salt and/or Cd significantly have increased APX and CAT activity, but their amounts were decreased when seedlings were inoculated with *A. brasilense* under stress condition. Similar results are reported by different researches. Shafi et al., (2009) showed salinity and Cd stress caused an increase in enzymatic antioxidants such as CAT in three wheat cultivars. In addition, Asada (2006) indicated that APX plays an important role in plant defense against oxidative stress by scavenging H_2O_2. Salinity and Cd generate oxidative stress in plants, leading to a limited growth and even plant death. Oxidative stress is one of the main causes of cellular damage in all organisms exposed to a wide variety of stress conditions (Lin and Kao, 2001; Liu, 2006). It is well known that some of PGPRs such as *Azospirillum* Spp. could help and improve the adverse effect of oxidative stress.

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For instance, Bashan et al., (2005) showed inoculation of wheat plants with *Azospirillum* could increase auxiliary photoprotective pigments in shoot of plant which may protect chlorophyll from oxidation during exposure to salt stress. Furthermore, Othman (2015) showed the ability of *A. brasilense* Sp7 in production of ACC deaminase which may be responsible for better growth of rice seedlings. Under stress condition, excess ROS production leads to an increase in plant hormone such as ethylene to prevent the ROS induced damage to reduce the enzymatic antioxidants production (Cho and Seo, 2005). Afterward, degradation of the ethylene precursor ACC by bacterial ACC deaminase releases plant from ethylene induced stress (Figueiredo et al., 2008). In this experiment, inoculation could have alleviated the negative effects of salinity and/or Cd stress (*Fig. 3*). It appears that reduction in the activity of antioxidant enzymes may be due to the reduction of severity of stress experienced by inoculated seedlings. Omar et al., (2009) and Othman (2015) indicated that antioxidant enzyme activity significantly decreased in barely plants inoculated with *A. brasilense*.

Uptake of mineral by plant's root and its distribution was affected by a variety of factors such as heavy metals and salinity (Daneshbakhsh et al., 2013; Dražić et al., 2004; Khoshgoftar et al., 2004; Khoshgoftarmanesh, 2009). Concerning the concentration of Cd of the roots (32.18) and the shoots (7.01 mg g^{-1}DW) of non-inoculated seedlings, a higher portion of the Cd taken up by wheat seedlings would remain in the roots rather than moving into shoots. This may be because of the cell wall polyanions and also accumulation of Cd in the vacuole of the root cells (Stolt, 2003). Liu et al., (2009) reported similar conclusion. They show that the accumulation of toxic heavy metals in winter wheat roots was significantly higher than in the aerial parts. Addition of Cd was most seen in the roots (19.61%) and then in shoots (9.27%) in non-inoculated seedlings under salinity+Cd condition when compared to Cd-treated seedlings. Sodium chloride can increase the adverse effect of Cd via more mobilization of Cd in the soil solution and consequently more uptake of Cd by plant roots can happen (Khoshgoftar et al., 2004). In addition, Fe, Ca and K of root in non-inoculated seedlings significantly decreased due to the excess of salinity and/or Cd in the root media. Therefore excessive Cd and/or salinity affects the rate of uptake and distribution of certain essential nutrients in plants, and consequently, this phenomena may be responsible for mineral deficiency/imbalance which causes limited plant growth. Uptake of Cd occurs via the same transmembrane carriers used to uptake Ca^{2+} and Fe^{2+}, so addition of Cd can decrease the concentration of calcium and iron in plant (Dražić et al., 2004; Roth et al., 2006). Same conclusion reported by different researchers. Chang et al., (2012) found Fe^{2+} and K^{+} content of root and shoot significantly decreased in Cd-treated rice seedlings. Also, Astolfi et al., (2012) showed that Fe deficiency enhanced in barley plants under excess of Cd availability.

Under saline condition, the competition between uptake of sodium and potassium by non-inoculated plants favored sodium ions, but potassium uptake was increased in the wheat seedlings inoculated with *Azospirillum* (*Table 2*). Inoculation improved harmful effects of Cd and/or salinity via less Na and hight K uptake and accumulation in the roots and shoots of inoculated plants. At the same time, less sodium entry into the cell and less potassium leakage out of the cell maybe happen. Reduction of sodium in the roots of inoculated seedlings may be the result of higher expression of *TaSOS1* under excess of sodium.

PGPRs can affect the uptake of nutrient by changing root-uptake characteristics (Martinez-Toledo, 1991), and consequently change in relative growth rate (Rodelas et

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al., 1999; Tinker, 1984). We observed that *A. brasilense* could help to prevent sodium uptake by plant in inoculated wheat seedlings. It has been reported that production and secretion of bacterial exopolysaccharides to the root environment and in turn reducing the availability of Na^+ in the media is the cause of less Na uptake. This conclusion can be made on the basis of the result obtained by Upadhyay et al., (2011). They indicated that some of the native strains of bacteria which separated from the wheat rhizosphere of the soils were able to establish salt tolerance by bacterial secretion such as exopolysaccharides.

Lin et al., (1983) reported that inoculation of Zea and Sorghum bicolor roots with *A. brasilense* increased the uptake of several mineral ions such as NO^{3-}, K^+, and $H_2PO_4^-$. Also, Chang et al., (2012) showed that Cd could decrease Fe and K^+ concentration, increase APX and CAT activity antioxidant enzymes and also improve Fe status which is able to reduce the toxicity of rice seedlings. Furthermore, the low iron content of plants that are grown under the high level of heavy metals generally shows chlorosis, since iron deficiency inhibits both chloroplast development and chlorophyll biosynthesis (Imsande, 1998).

The cell membrane plays an important role in metal homeostasis, via preventing or stimulation of nutrient passage into cell. Cd could alter the plasma membrane permeability by lipid peroxidation, reduction of ATPase activity (Fodor et al., 1995) and altering the activities of genes related to Na homeostasis. Our data showed Cd enhanced sodium of plant tissue (almost 24%) and eventually, the *TaSOS1* gene expression significantly increased to 14.3 fold change in Cd-treated seedlings compared to control plants. Our result show under saline condition, the competition between uptake of sodium and potassium by non-inoculated plants favored sodium ions (Wakeel, 2013; Tester and Davenport, 2003). Thus, antiporters of plant cell plasma membrane (*SOS1*) should be involved to maintain the plant cell ion homeostasis. Our results are consistent with Taherinia (2015), Ramezani et al., (2013) and Xu et al., (2008) who demonstrated salt stress could increase the relative *TaSOS1* gene expression. In addition, we observed inoculation of seedlings with *A. brasilense* Sp7 can contribute to salt tolerate via overexpression of *TaSOS1* gene. Although there is not any report related to the effects of *A. brasilense* on the upregulation of *TaSOS1* in wheat seedlings, Vargas-Garcia et al. (2012) showed ethylene receptors genes expression in rice plants upregulated due to the inoculation with *A. brasilense* Sp245. There are some other reports relate to an increase in transcripts of some other genes involved in nutrient uptake in response to *A. brasilense* (Cavalcante, 2007; Kim, 2015; Nogueira, 2001).

Plant cell reduces the adverse effects of Cd through different mechanisms like secretion of organic acids (Nigam, 2001), using Casparian strip of the root plant cell (Lux, 2004), ABC transporters (Bovet et al., 2005; Verbruggen et al., 2009), antioxidant enzymes (Mishra, 2006) and expression of some genes which *TaTM20* gene is one of them (Kim et al., 2008). So it is predictable that the *TaTM20* gene expression for inhibition and detrimental effects to plant would be increased. *Figure 4b* shows not only Cd increased the relative *TaTM20* gene expression to 226.32 fold change but also an additive effect was observed in its expression under combined Cd+salinity (308.14 fold change). When we use manure containing Cd in agricultural land, especially under saline condition, potassium content of the root reduces because of chloride complexation of Cd (Ciećko, 2004; Smolders et al., 1998). Also, Kim et al., (2008) demonstrated Cd stress could increase the relative *TaTM20* gene expression as well as Cd tolerance through the stimulation of Cd efflux from the cell.

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Under Cd+salinity stress, the relative *TaTM20* gene expression in inoculated seedlings (188.36) was less than non-inoculated ones (308.14 fold change). Simultaneously we obtained better growth and less Na and higher potassium in the shoots and roots which indicated that inoculation provides a better condition for plant to grow. This result is in some ways similar to the result of Belimove and Dietz (2000) who showed that associative bacteria were capable of decreasing partially the toxicity of Cd for the barley plants through the improvement of nutrient uptake. Since the *TaTM20* gene is known recently, so there is not enough information to present a good conclusion. However, it is possible that *Azospirillum* contributed to plants to tolerate the stress condition through the extension of the root system, secretion of exopolysaccharides and alteration of *TaTM20* expression, which in turn decreasing the Cd uptake and its accumulation in the cytosol of a cell.

Conclusion

In non-inoculated condition, a reduction of Fe, Ca, K content and addition of Na, Cd, proline, antioxidant enzyme (CAT and APX), chlorophyll (a and b) and carotenoid in the root and shoot were observed under excess of Cd. However, the changes in the measured indexes were higher for the excess of Cd under salinity. Losing of dry weight of roots and shoots was significantly correlated to measured indices. In excess of salinity or Cd, *TaSOS1* expression was upregulated in the root of seedlings, and reached its maximum under dual effect of Cd and salinity (72.89 fold change). However, *TaTM20* upregulation was more Cd dependent than salinity or inoculation but Cd had higher effect on *TaTM20* upregulation under saline condition which might be because of more Cd solubility in the media.

Inoculation with *A. brasilense* improved root and shoot dry weight, caused more accumulation of Fe, Ca and potassium but less uptake of Cd and Na in seedlings not exposed to Cd and salinity. Simultaneously, in inoculated seedlings there were less adverse effects on shoot's pigments such as chlorophyll a and carotenoid under excess of salinity and Cd. These events coincided with upregulation of *TaSOS1* under saline and/or Cd stress conditions.

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