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Impact of biological and chemical treatments on the improvement of salt tolerance in wheat

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Abstract
Salinity stress has been known as an important constraint limiting agricultural production especially in arid and semi-arid regions. Among several strategies to improve crop growth under salt stress, using of salinity tolerant *Trichoderma* isolates and silicon application could be an effective and easily adaptive strategy. In order to evaluate silicon and *Trichoderma virens* inoculation effects on some physiological and morphological properties of wheat grown under saline condition, a greenhouse experiment arranged as factorial based on completely randomized design with three replications was carried out. The factors included three levels of salinity (E1:3, E2:7 and E3: 10 dS m^{-1}) from NaCl, CaCl2 and MgCl2 sources (3:2:1 ratio, respectively), two levels of Si, 0 (S1) and 1.5 mM (S2), from the source of Na2SiO3 and two levels of *Trichoderma virens* (with and without inoculation). It was shown that salt stress caused very significant reduction in plant height, chlorophyll content, grain yield and other measured properties. Salinity stress increased proline and soluble sugar concentration, Na/K and Na/Ca ratios in leaves. Application of Si to the growth medium significantly increased chlorophyll content, grain yield of wheat grown under normal as well as under saline environments, but those influences were lower than the fungus effect. These results seem to show that silicon may alleviate salt stress in wheat due to decreased Na/K and Na/Ca ratios and proline concentration in leaves. *Tirchoderma* inoculation significantly increased chlorophyll content and grain yield of wheat under salt stress. *Trichoderma virens* deteriorate salt stress by significantly decreasing Na/K and Na/Ca ratios and proline concentration and increasing soluble sugar in the leaves.

Keywords: Chlorophyll; Grain yield; Plant height; Proline; Soluble sugars

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Introduction

Salinity stress limits crop production in arid and semi-arid regions (Etesami and Beattie 2018). It is beneficial for agricultural development in arid and semi-arid areas to use proper plant species and soil microorganisms to maintain soil fertility and alleviate stresses. Crop yield decrease in saline soils occurs because of a number of physiological and biochemical dysfunctions. The negative effects of salinity stress on plants include osmotic stress, ion (Na^+, Cl) toxicity and nutritional disorders (Jamil *et al.* 2011). Plants accumulate several osmolytes in response to abiotic stresses (Chen and Murata 2002). The increase in proline content has been considered as one of the salt tolerance mechanisms in wheat (*Triticum aestivum* L.) plants (Tuna *et al.* 2008). High proline and K^+ accumulation as well as decrease in Na^+ concentration have been shown to mediate salt tolerance (Gharsallah *et al.* 2016). Results of Khan *et al.* (2009) have shown that higher grain yield in wheat is associated with higher K/Na ratio, proline and chlorophyll contents.

Wheat is adversely affected by a number of abiotic stresses including salinity, drought and heat. Various chemical, physical and biological measures can be considered for economic crop production in such soils. Si as non-nutritional element for plant growth has improved the growth of higher plants under stressed environments (Liang *et al.* 2006; Broadley *et al.* 2012). It is beneficial to plants, especially under stress conditions, and reduces the toxic effects of abiotic stresses, such as salt stress, and protects plants from pests and diseases (Broadley *et al.* 2012; Luyckx *et al.* 2017). Physical and mechanical protection by SiO_2 deposits and biochemical response of plants that trigger metabolic changes are commonly considered as major mechanisms contributing to stress resistance (Luyckx *et al.* 2017). The protective roles of Si was first attributed to the physical barrier protecting the cell wall, e.g., against fungal pathogens, however, further studies have indicated the more complex role of this element on plants (Luyckx *et al.* 2017). Silicon impacts on endogenous phytohormones are commonly connected with resistance of plants to pathogens (Fauteux *et al.* 2006) or heavy metals (Kim *et al.* 2014).

The positive effect of Si to reduce salt stress has been revealed in various plant species including sorghum (Yin *et al.* 2013), maize (Moussa 2006) and wheat (Tuna *et al.* 2008). Some mechanisms by which Si may ameliorate salinity stress in plants are the improvement of water status (Romero-Aranda *et al.* 2006), increase of photosynthetic activity (Ma and Takahashi 2002), stimulation of antioxidant system (Zhu *et al.* 2004) and decrease in Na uptake (Tahir *et al.* 2006; Tuna *et al.* 2008) or enhance the activity of H-ATPase and increase the uptake and transport of K from roots to shoot under salt stress (Liang 1999). Si decreases salt uptake by forming complexes with sodium in the root zone (Ahmad *et al.* 1992). Saqib *et al.* (2008) demonstrated that salt stress alleviation in plants by Si depends on increasing cell wall Na^+ binding and reducing cell sap Na^+ concentration.

Integrated stress management can be a favorable technique to sustain crop yields through the balanced use of chemical and biological agents. The effect of fungi was indicated to increase plant tolerance to abiotic stresses through improved root growth and water-holding capacity of plants (Bae *et al.* 2009), or the increase in nutrient uptake, such as potassium (Yildirim *et al.* 2006).

Trichoderma spp. are free-living fungi in ecosystems of soil, rhizosphere and root surfaces (Harman *et al.* 2004). Increased plant growth due to association of *Trichoderma* strains with plants is higher under biotic, abiotic, or physiological stresses (Harman *et al.* 2004; Bae *et al.* 2009).

Reduction of the negative impacts of salinity stress on wheat (*Triticum aestivum* L.) has been reported by seed biopriming with *Trichoderma harzianum. Trichoderma* spp. allowed proline accumulation in wheat faced with salt stress condition (Rawat *et al.* 2011).

The present research was carried out to assess *Trichoderma virens* inoculation and silicon use on K and Na uptake and some physiological and biochemical attributes of wheat exposed to normal and saline conditions.

Materials and Methods

Experimental design and treatments

Effect of silicon and *Trichoderma virens* inoculation on some physiological and morphological properties of wheat (*Triticum aestivum* L.) grown under saline condition was studied by conducting a factorial experiment in greenhouse using randomized complete design with three replications. The factors included three levels of salinity (E1: 3, E2: 7 and E3: 10 dS m^{-1}) from NaCl, CaCl2, MgCl2 sources (3:2:1 ratio, respectively), two levels of Si, 0 (S0) and 1.5 mM (S1), as Na_2SiO_3, and two levels of *Trichoderma virens* (F0, with fungus inoculation and F1, without fungus inoculation).

Preparation of *T. virens*

Trichoderma virens Ham 65 (Iranian type culture collection) was used for this study. *Trichoderma* isolate was cultured on potato dextrose agar medium plates (PDA) and incubated at 30 °C. After 7 days, fungal plugs were mixed with sterilized cooked wheat grain and incubated for 15 days at 30 °C. Sand (2-4 mm in diameter) was mixed to prevent the sticking of wheat grains. The prepared inoculum contained approximately 2×10^9 CFU g^{-1} *T. virens* and was given to the pots (F1 treatment) at the concentration of 10 g kg^{-1} soil before sowing.

Plant culture and treatment application

A surface soil (0-30 cm) was collected from the research field of Faculty of Agriculture, Shahid Chamran University of Ahvaz. Collected soil was air dried and passed from 2 mm sieve to measure some soil properties. Electrical conductivity, pH, organic matter, calcium carbonate, Olsen P, available K and total nitrogen were determined (Gupta 2004). Some soil properties of the experimental field are shown in Table 1. Four kg of the soil was filled in each of the 36 plastic pots lined with polyethylene bags. Silicon was added to the soil according to the treatments used in the experiment.

Table 1. Some chemical and physical characteristics of the soil.

Soil texture	pH	EC (dS m^{-1})	OC (%)	Total N (%)	Available P (mg kg^{-1})	Available K (mg kg^{-1})	$CaCo_3$ (%)
Clay Loam	7.8	3.2	0.52	0.04	10	290	41

The nitrogen and phosphorus fertilizers were added to soil as urea (49.4 mg/kg) and triple superphosphate (24.7 mg/kg) before seed sowing. Initial analysis indicated that potassium was enough in the soil for wheat growth, therefore potassium fertilizer was not applied. The soil was inoculated by *Trichoderma* before planting. Seeds of Chamran cultivar were sterilized with 10% sodium hypochlorite solution for 10 min and then rinsed with sterilized distilled water (Tale Ahmad and Haddad 2011) and planted at about 2 cm depth. To prevent osmotic stress due to salinity, the amount of each salt at each salinity level was gradually added to the irrigation water during the fourth week of plant growth. Salt solutions were provided by dissolving NaCl, CaCl2 and $MgCl_2$ (3:2:1 ratio, respectively) in distilled water based on each salinity level (0, 15 and 30 Meq kg^{-1}). In order to maintain the moisture content at 80% of field capacity, distilled water was used by regular weighting (without drainage). Pots with no plants were used as negative controls for soil salinity levels during the experiment. The range of soil saturated electrical conductivity in E1, E2 and E3 treatments during the experiment were (3-3.3), (6.8-7.5) and (9.5-10.6) dS m^{-1}, respectively. During the experiment leaf area and chlorophyll index (SPAD) and at the end of the experiment, plant height and grain yield were measured. Proline was determined based on Bates *et al.* (1973). About 0.5 g of fresh plant material was homogenized in 10 mL of 3% aqueous sulfosalicylic acid and filtered by Whitman's No. 2 filter paper. Two milliliters of filtrate was mixed with 2 mL acid-ninhydrin and 2 mL of glacial acetic acid in a test tube. The mixture was put in a water bath for 1 h at 100 °C. The mixture of the reaction was extracted with 4 mL toluene. The chromophore having toluene was aspirated and cooled to room temperature, and the absorbance was measured with a Shimadzu UV 1601 spectrometer at 520 nm. Appropriate proline standards were used to calculate proline in the samples. Soluble carbohydrates were measured by phenol-sulphuric acid method (Dubois *et al.* 1956). A standard curve was presented to measure hexose and pentose. The extract from leaves was centrifuged at 3000 rpm for 5 min. A pure sulphuric acid and phenol (5%) were added to the extract and then the absorbance was measured at 490 nm.

To measure Na, K and Ca of the leaves, about 0.5 g of ground samples were digested at 550 °C for 2 h. The ash was dissolved in 10 ml of HCl (2 M), then filtered into a 50 mL flask and made up to 50 mL with distilled water. Na and K were determined by a flame photometer and Ca by titration (Gupta 2004).

Statistical analyses

After analysis of variance, the means were compared using Duncan's multiple range tests at 0.05 probability level. SAS statistical software (SAS Institute, Inc. 2000) was utilized to analyze the data.

Results and Discussion

This experiment showed that salt stress in wheat makes very considerable decrease in plant height. Plants' height was significantly lower when grown in saline soil (EC 10 dS m^{-1}) than those grown in the normal condition (EC 3 dS m^{-1}) and reduced about 17.5% (Figure 1). *T. virens* inoculation increased plant height significantly (Figure 1). Interactions of *Trichoderma* × salinity and salinity × *Trichoderma* × silicon were not significant on plant height. The positive effect of *Trichoderma harzianum* on shoot length was shown in wheat under saline condition (Rawat *et al.* 2012). The use of *Trichoderma* strains increases deep roots of plants, helping more water uptake and enables the plants to tolerate abiotic stress (Shukla *et al.* 2012).

Increase of salinity level from 3 to 10 dSm^{-1} significantly decreased wheat leaf area. *Trichoderma* and silicon effect at each salinity level enhanced leaf area, significantly. The application of *Trichoderma* and Si increased leaf area about 38% at 10 dSm^{-1} salinity compared to the treatment without presence of *Trichoderma* and Si (Figure 3).

Chlorophyll was lower in plants grown under saline condition. The instability of pigment complexes and destruction of chloroplast structure have been reported under saline condition (Singh and Dubey 1995). Si and *Trichoderma* significantly increased SPAD number at each salinity level (Figure 4). Similar result was reported about the positive effect of silicon on chlorophyll content under salt stress condition in wheat (Tuna *et al*. 2008) and barley (Liang 1999).

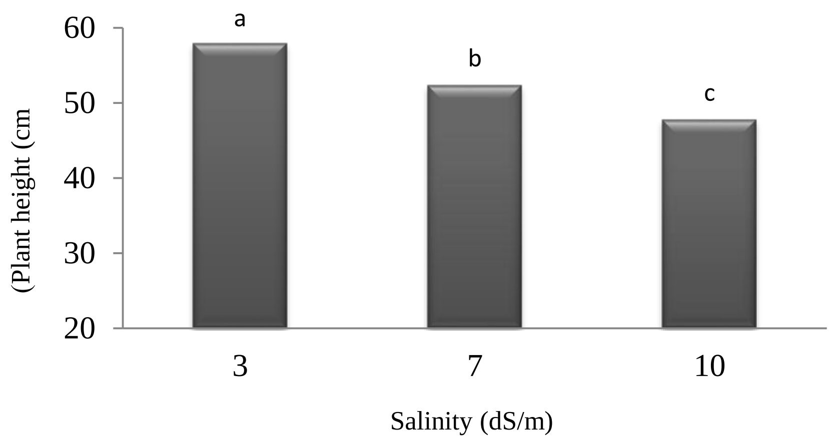

Figure 1. The effect of salinity treatment on plant height; Means followed by the same letter are not significantly different at 5% probability level using Duncan's multiple range test.

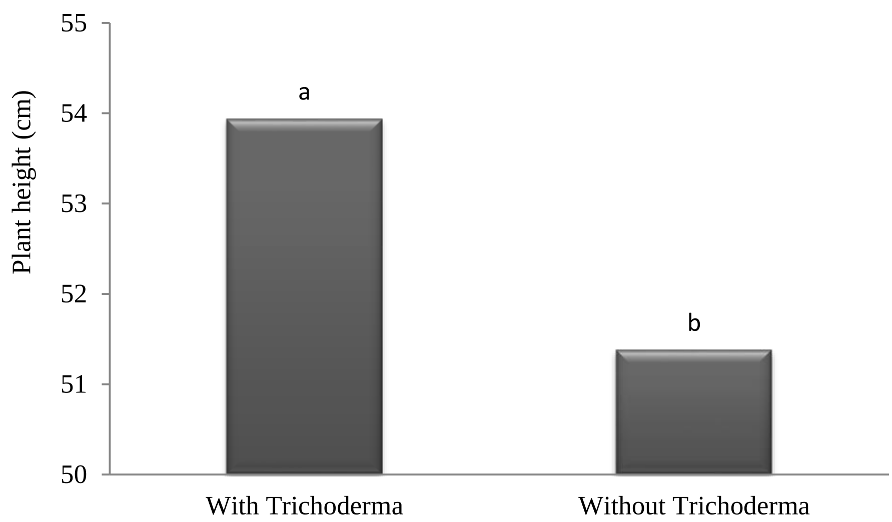

Figure 2. The effect of *Trichoderma virens* inoculation on plant height; Means followed by the same letter are not significantly different at 5% probability level using Duncan's multiple range test.

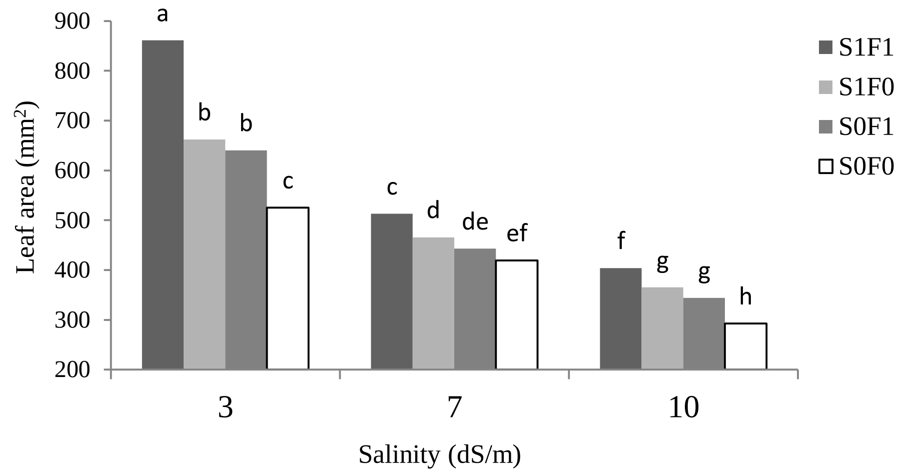

Figure 3. The effect of treatment combinations of salinity, fungus and silicon on leaf area; Means followed by the same letter are not significantly different at 5% probability level using Duncan's multiple range test; S0: without Si, S1: 1.5 mM Si; F0: without inoculation, F1: with *Trichoderma virens* inoculation.

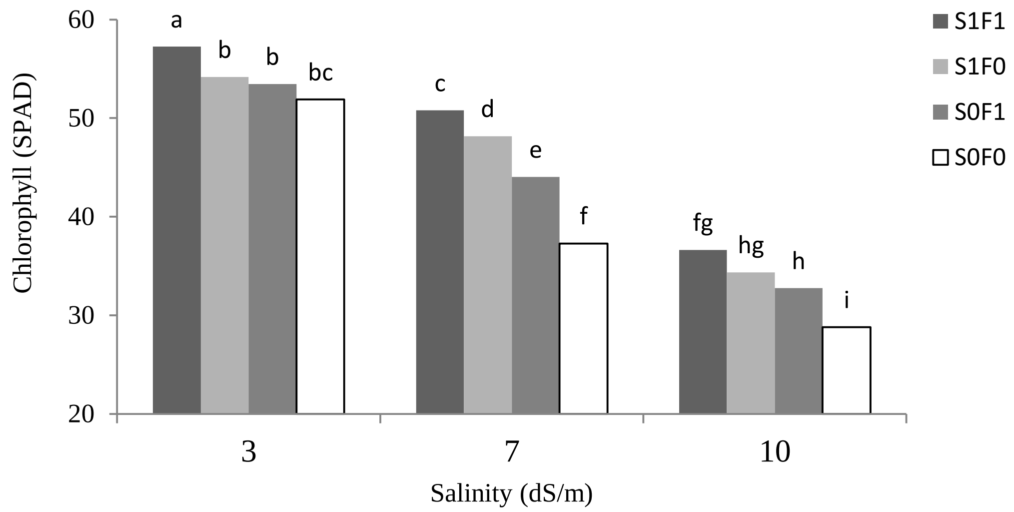

Figure 4. The effect of treatment combinations of salinity, fungus and silicon on chlorophyll; Means followed by the same letter are not significantly different at 5% probability level using Duncan's multiple range test; S0: without Si, S1: 1.5 mM Si; F0: without inoculation, F1: with *Trichoderma virens* inoculation.

Salt stress affected grain yield significantly. Grain yield decreased about 60% by increasing salinity from 3 to 10 dS m^{-1}. Si and *Trichoderma* improved grain yield about 21% at 10 dS m^{-1} salinity level compared to the treatment without *Trichoderma* and Si application. The enhancement of grain yield by fungus at different salinity levels was more than the silicon effect (Figure 5). *Trichoderma* releases different compounds inducing resistance to biotic stresses (Harman *et al.* 2004) which consequently increases plant yield.

Tahir *et al.* (2006) showed the negative effect of salt stress (EC 10 dS m^{-1}) on grain yield of two wheat genotypes (Auqab-2000, SARC-5), particularly in SARC-5, while the use of silicon in the growth medium enhanced grain yield of both genotypes grown in non-saline and/or in saline conditions.

Figure 6 shows that salt treatments greatly increased wheat proline content. The low concentration of proline in the control and high concentration in the stressed plants have been reported by many studies about salt tolerance (Ashraf and Foolad 2007; Tuna *et al.* 2008; Datta *et al.* 2009; Szabados and Savoure 2010). Using Si and *Trichoderma* decreased proline accumulation at each salinity level. Some researchers indicated that supplementary silicon reduced the effects of stress accompanied by the decrease in proline level in soybean (Lee *et al.* 2010) and wheat (Pei *et al*. 2010). However, Yin *et al.* (2013) reported no considerable effect of silicon on proline content of sorghum under salt stress. Proline accumulation has been demonstrated in plants inoculated with *Trichoderma harzianum* (Rawat *et al.* 2011), *Azospirillum* and *Piriformospora indica* (Zarea *et al.* 2012). Proline accumulation under stress has been reported in stress-tolerant plants than in stress sensitive plants (Madan *et al.* 1995), while other studies suggested that proline accumulation is a stress injury symptom rather than indicating stress tolerance (Lutts *et al*. 1999; de Lacerda *et al*. 2003). Our findings support the view that accumulation of proline exposed to stress is a reaction to injury rather than an osmotic adjustment.

Total concentration of soluble sugars increased significantly in wheat leaves under salinity (Figure 7) which are in line with Yin *et al.* (2013) and Nemati *et al.* (2011) showing that salinity stress increased the soluble carbohydrates in sorghum and rice, respectively. There are several reports showing soluble sugars accumulation in plants under salinity stress to reduce the osmotic potential of plant as important mechanism to protect plants against stress (Ezz and Nawar 1994; Al-Garni 2006). The results indicated that Si application increased total concentration of soluble sugars. Similar observations showed that under drought stress in wheat (Pei *et al*. 2010) and sorghum (Sonobe *et al*. 2010) and under salt stress in sorghum (Yin *et al*. 2013), silicon increased total amount of soluble sugars

Application of *Trichoderma* in our study enhanced total soluble sugars. It has been reported that arbuscular mycorrhiza improve salinity resistance of host plants by increasing soluble sugars (Sheng *et al.* 2011; Zhu *et al.* 2016). A positive correlation was reported between total carbohydrates and mycorrhization of the host plants (Thomson *et al.* 1990; Porcel and Ruiz-Lozano 2004; Al-Garni 2006). However, some researchers showed no significant differences in sugar concentrations exposed to saline conditions or differences between *Piriformospora indica* inoculated and non-inoculated plants (Zarea *et al.* 2012).

Salinity increased Na/K and Na/Ca ratio but silicon and fungus application decreased the ratios (Figure 8 and 9). Tahir *et al.* (2006) also reported the increase of Na:K ratio in wheat exposed to salinity stress. However, this ratio decreased when Si was added to the medium. Salinity stress has enhanced Na concentration of leaves significantly in sorghum (Yin *et al*. 2003) and wheat (Tahir *et al.* 2006).

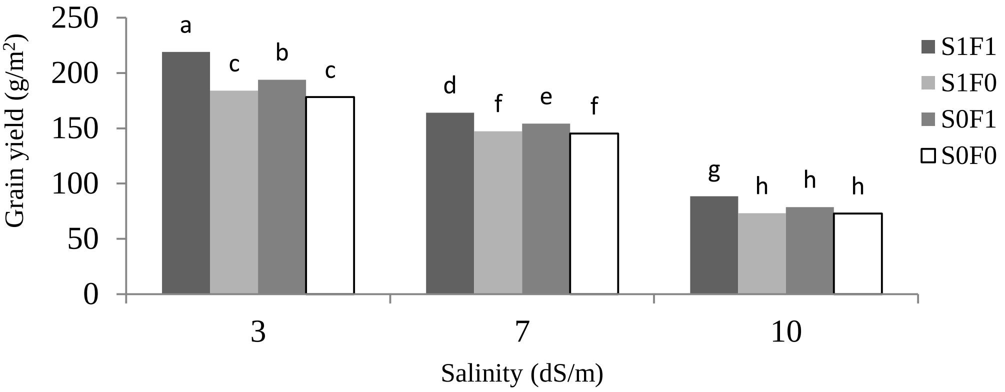

Figure 5. The effect of treatment combination of salinity, fungus and silicon on grain yield; Means followed by the same letter are not significantly different at 5% probability level using Duncan's multiple range test; S0: without Si, S1: 1.5 mM Si; F0: without inoculation and F1: with *Trichoderma virens* inoculation.

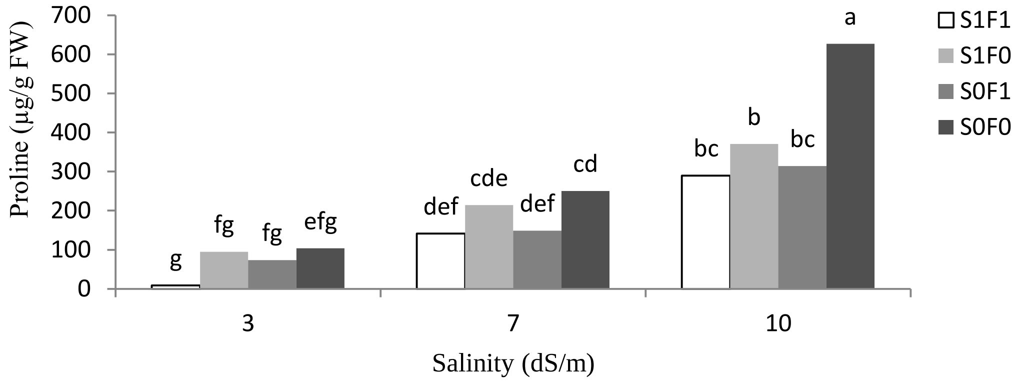

Figure 6. The effect of treatment combination of salinity, fungus and silicon treatment on proline; Means followed by the same letter are not significantly different at 5% probability level using Duncan's multiple range test; S0: without Si, S1: 1.5 mM Si; F0: without inoculation and F1: with *Trichoderma virens* inoculation.

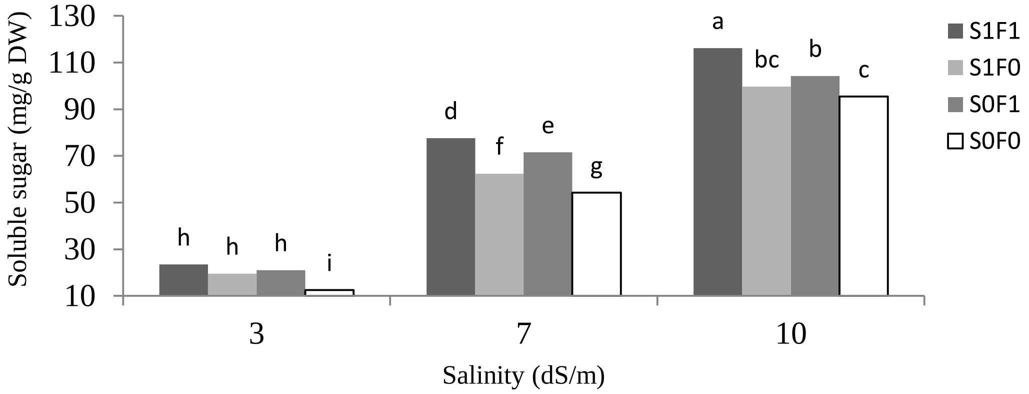

Figure 7. The effect of treatment combination of salinity, fungus and silicon treatment on soluble sugars; Means followed by the same letter are not significantly different at 5% probability level using Duncan's multiple range test; S0: without Si, S1: 1.5 mM Si; F0: without inoculation and F1: with *Trichoderma virens* inoculation.

Positive influences of silicon on reduction of Na content in wheat (Tahir *et al.* 2006; Tuna *et al.* 2008) and alfalfa (Wang and Han 2007) were reported. The mechanism by which Si inhibited Na uptake is poorly understood. Silicon deposition in exodermises and endodermis reduces apoplastic sodium uptake by roots (Gong *et al.* 2006).

Potassium plays a considerable role in improving plant water situation and reducing the toxic effects of sodium. K^+ uptake suppression happens by excess sodium in the environment (Assaha *et al.* 2017). In our study, Si and fungus increased K^+ content in plants exposed to stress condition. Enhanced K^+ content due to silicon application has been demonstrated in the shoots and leaves of salt tolerant alfalfa under salt stress (Wang and Han 2007) and in wheat (Tuna *et al.* 2008).

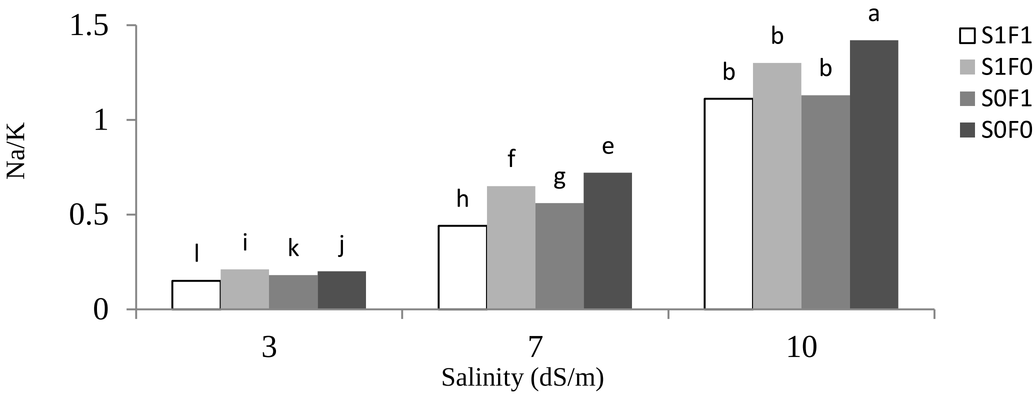

Figure 8. The effect of treatment combination of salinity, fungus and silicon treatment on Na/K; Means followed by the same letter are not significantly different at 5% probability level using Duncan's multiple range test; S0: without Si, S1: 1.5 mM Si; F0: without inoculation and F1: with *Trichoderma virens* inoculation.

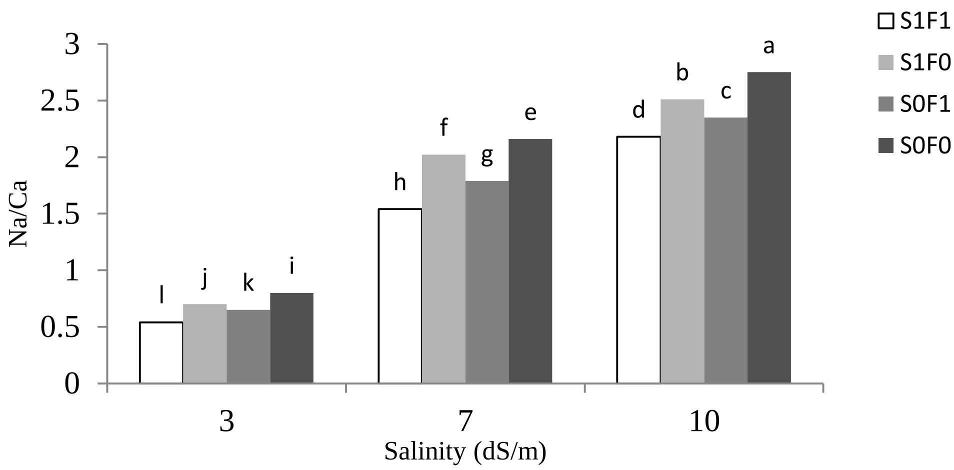

Figure 9. The effect of treatment combination of salinity, fungus and silicon treatment on Na/Ca; Means followed by the same letter are not significantly different at 5% probability level using Duncan's multiple range test; S0: without Si, S1: 1.5 mM Si; F0: without inoculation and F1: with *Trichoderma virens* inoculation.

Ca^{2+} reduction due to the increase of sodium content has been reported in maize (Turan *et al*. 2010), sorghum (Netondo *et al.* 2004), rice (Nemati *et al*. 2011) and wheat (Tuna *et al.* 2008).

Ca^{+2} content of the leaves increased in Si-receiving plants compared with the medium without Si. Our findings confirmed the reports of Tuna *et al*. (2008) who found that the addition of Si increased both leaf and root calcium in wheat.

Fungus increased Ca^{+2} content in plant leaves by the increase of salt stress. Ca^{2+} functions in plants include salinity stress alleviation, maintaining membrane stability, improved plant growth (Tuna *et al.* 2007), better sensing and responding (Kader and Lindberg 2010) and enzyme activation (Hu *et al.* 2016).

Conclusions

Our findings revealed the significant effect of *Trichoderma* and silicon on salinity tolerance. *Trichoderma* was more efficient than Si to increase grain yield, which could be related to better water condition and higher photosynthetic pigments content. Furthermore, *Trichoderma* inoculation and silicon addition to the soil increased the total soluble sugars accumulation. For the survival of plants under salt stress conditions, intracellular adjusting of leaf osmotic potential is crucial.

Since proline biosynthesis demands a great energy, the reduction of proline could be may be useful to plant by storing more energy to tolerate stresses. The reduction of proline accumulation happened in plants inoculated with *Trichoderma* under saline condition. Our results recommend more investigations to assess different mechanisms in plants at the cell level in which *Trichoderma* and Si reduce salinity stress.

Acknowledgements

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اثر تیمارهای بیولوژیکی و شیمیایی بر بهبود تحمل به شوری در گندم

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چکیده

تنش شوری به عنوان یک محدودیت مهم در تولید محصولات کشاورزی به ویژه در مناطق خشک و نیمه خشک شناخته شده است. در بین راهکارهای بهبود رشد گیاه در شرایط تنش شوری، استفاده از جدایه‌های *تریکودرمای* قادر به تحمل شوری و کاربرد سیلیسیوم می‌تواند راهکاری موثر و سازگار باشد. به منظور ارزیابی اثرات مایه‌زنی *تریکودرما ویرنس* و کاربرد سیلیسیوم بر برخی از ویژگی‌های فیزیولوژیکی و مورفولوژیکی گندم در شرایط شور، آزمایش گلخانه‌ای به صورت فاکتوریل در قالب طرح کاملاً تصادفی با سه تکرار انجام شد. فاکتورها شامل سه سطح شوری (E1: ۳، E2: ۷ و E3: ۱۰ $dS\ m^{-1}$) از منابع NaCl، $CaCl_2$ و $MgCl_2$ (به نسبت ۳: ۲: ۱) ، دو سطح سیلیسیوم از منبع Na_2SiO_3 شامل صفر (S1) و ۱/۵ (S2) mM و دو سطح مایه‌زنی قارچ (با و بدون مایه‌زنی) بود. نتایج نشان داد که تنش شوری موجب کاهش معنی‌دار ارتفاع گیاه، میزان کلروفیل، عملکرد دانه و سایر ویژگی‌های مورد اندازه گیری شد. تنش شوری سبب افزایش غلظت پرولین و قند، نسبت Na/K و Na/Ca در برگ شد. کاربرد سیلیسیوم مقدار کلروفیل و عملکرد دانه گندم را در شرایط شور و غیرشور به‌طور معنی‌داری افزایش دادف ولی تاثیر آن بر ویژگی‌های ذکر شده کمتر از قارچ بود. نتایج نشان داد که سیلیسیوم با کاهش نسبت‌های Na/K و Na/Ca و غلظت پرولین در برگ باعث کاهش تنش شوری در گندم شد. مایه‌زنی *تریکودرما* به طور معنی‌داری میزان کلروفیل و عملکرد دانه گندم را در محیط شور افزایش داد. *تریکودرما ویرنس* با کاهش معنی‌دار نسبت‌های Na/K و Na/Ca و غلظت پرولین و افزایش قند محلول در برگ باعث کاهش تنش شوری شد.

واژه‌های کلیدی: ارتفاع بوته؛ پرولین؛ عملکرد دانه؛ قندهای محلول؛ کلروفیل