Full Length Article

Antioxidant Potential and Oxidative Stress Markers in Wheat (*Triticum aestivum*) Treated with Phytohormones under Salt-Stress Condition

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Abstract

The interactive effects of indole acetic acid (IAA) or salicylic acid (SA) (0.5 and 0.1 mM, respectively) as shoot spraying on NaCl stressed wheat (*Triticum aestivum* L.) plant organs grown in experimental pots under different salinity levels (0, 50, 100, 150 and 200 mM NaCl, respectively) were studied. The antioxidant enzymes as catalase (CAT), peroxidase (PX), and ascorbate peroxidase (APX), photosynthetic pigments, reducing sugars, proteins, amino acids (AA), and proline (Prol) contents in spike, shoot and root of salt-stressed plants were the most affected parameters, specially at high salinity levels (150 and 200 mM NaCl). Treatments with 0.5 mM of IAA or 0.1 mM of SA on stressed wheat organs mitigated the detrimental effect of NaCl. Phytohormones improved salt tolerance in wheat organs activating antioxidant enzymes, increasing photosynthetic pigments and enhancing the accumulation of non toxic metabolites. The magnitude of increase was more pronounced in SA than in IAA treated plants, and the spike was the most accumulator organ of non toxic metabolites compared to shoot and root. Finally, SA and/or IAA treatments prevent the negative effects of salt stressed wheat and these could be adopted as a potential growth regulator or antioxidant to improve wheat growth under moderate salt levels. © 2013 Friends Science Publishers

Keywords: Antioxidant enzymes; Photosynthetic pigments; Indole acetic acid; Salicylic acid; Salt; Wheat

Introduction

Most of the crop species, such as wheat, are glycophytes and generally show limited growth and development due to soil salinity that adversely influenced the growth, physiological and metabolic processes as well as crop yield (Ashraf and Harris, 2004; Saqib *et al.,* 2012). However, with increasing amounts of arable and undergoing salinization (Ghassemi *et al.*, 1995) and the increasing food demand from growing human and livestock population, there is a need to ameliorate the harmful effect of salinity using different strategies (Szabolcs, 1994).

Previous studies reported that in alfalfa the soluble sugars, proteins and total free AA including proline (Prol) were progressively accumulated as NaCl level increased (Antoline and Sauchez-Dais, 1992). Charparzadeh *et al.* (2004) stated that in Calendula high salinity depressed growth and increased lipid peroxidation and H_2O_2 levels; further, Hassanein *et al.* (2009) found a reduction in chlorophyll (Chl) *a* and *b*, carotenoids as well as total pigments in salt-stressed wheat.

Glycophytic species employ different strategies to withstand salinity stress. The increase in salt resistance may involve protection of cell membranes and accumulation of some protector components (Mansour, 2004). The main biochemical alterations in plants under environmental stress conditions is represented by the increased synthesis of reactive oxygen species (ROS; Farooq and Azam, 2006; Mallik *et al.*, 2011). So, in absence of defensive mechanisms, the plant can significantly alter its metabolism through oxidative damage by pigments, lipid and protein (Molassiotis *et al.*, 2006; Noreen and Ashraf, 2009).

Many reports indicated that antioxidants could be used as a potential growth regulator to improve salinity stress resistance in plants (Khan, 2006; Gunes *et al.*, 2007). To prevent the detrimental influences of ROS, the plant develops a useful system that include mainly antioxidants: peroxidase (POD, EC 1.11.1.7), catalase (CAT, EC 1.11.16) and ascorabate (APX, EC 1.11.1.11) (Hajiboland and Hasani, 2007; Sheteawi, 2007). Therefore, these enzymes are valuable biochemical stress markers, and their increased activity may attest to a potential for remediation (Vangronsveld and Clijsters, 1994). However, several studies indicate that ROS levels in plant cells are normally protective by antioxidant activity. Association between saline environment and endogenous level of water soluble antioxidant enzymes have been reported (Foyer *et al.*, 1993; Tsugane *et al.*, 1999; Sheteawi, 2007).

In recent years, phytohormones are the subject of

interest because of their capacity to produce stress-resistance in plants (Levent Tuna *et al.*, 2007). Phytohormones, such as IAA and salicylic SA, may serve as modulator by decreasing or improving the stress conditions in plant (Popova, 1995). However, little literature was found on the relation between salt-stress and auxin concentrations in plants, and the function of auxin in improving salinity stress. It was previously reported that the exogenous application of IAA resulted in improved roots and shoots growth in salt-stressed wheat (Egamberdieva, 2009). Therefore, IAA application offers an valuable solution to stress (Javid, 2011). Salicylic acid (SA) represents an endogenous growth phenolic regulator involved in many physiological patterns (Hayat *et al.*, 2010) and also it defends against salt-stress in plants (Hussein *et al.*, 2007). The positive effects of SA have been well reported, including salinity tolerance in different plants (Tari *et al.*, 2002; Gunes *et al.*, 2007; Azooz, 2009).

Therefore, the aim of the present work was to investigate the ameliorative effect of foliar application of IAA and SA on antioxidant enzymes, photosynthetic apparatus and some metabolic constituents in salt-stressed wheat.

Materials and Methods

Plant Material, Treatments and Experimental Design

Wheat (*Triticum aestivum* L. cv Giza 186) was obtained from breeding program of Seeds Center of Beni Suef, Egypt. Wheat grains were surface sterilized by immersion in a mixture of ethanol 96% and H_2O_2 (1:1) for three times, followed by several washings with sterile distilled water. Ten seeds were sown per pot. Each pot contained 2 kg of clay soil. All forty five pots (3 treatments $\times$ 5 levels $\times$ 3 replicates) were weekly irrigated with normal tap water to achieve soil water field capacity for three weeks. Then, pots were randomly classified into three treatment groups with three replicate each.

Treatment groups were as follows:
I. *Salt treatments* (reference group).
a) 0 mM NaCl (control)
b) 50 mM NaCl
c) 100 mM NaCl
d) 150 mM NaCl
e) 200 mM NaCl
II. *Indole acetic acid (IAA) + salt treatments* (treated plants were sprayed once a time with 0.5 mM IAA).
a) 0.5 mM IAA+0 mM NaCl.
b) 0.5 mM IAA+50 mM NaCl .
c) 0.5 mM IAA+100 mM NaCl.
d) 0.5 mM IAA+150 mM NaCl.
e) 0.5 mM IAA+200 mM NaCl.
III. *Salicylic acid (SA) + salt treatments* (treated plants were sprayed once a time with 0.1 mM SA).

a) 0.1 mM SA+0.0 mM NaCl.
b) 0.1 mM IAA+50 mM NaCl.
c) 0.1 mM IAA+100 mM NaCl.
d) 0.1 mM IAA+150 mM NaCl.
e) 0.1 mM IAA+200 mM NaCl.

Plants of the three groups (I, II and III) were harvested after 70 days and dried in an oven at 70°C to constant mass to obtain plant extract for chemical analyses. The chlorophyll (Chl) *a, b* and carotenoids of fresh leaves were determined using a spectrophotometer (Metzner *et al.*, 1965) and reducing sugars were analyzed according to Fales (1951). Soluble proteins, amino acids (AA) and Prol were also determined according to Moore and Stein (1948), Lowery *et al.* (1951) and Bates *et al.* (1973), respectively.

Assays for Antioxidant Enzyme Activity

Enzymes extraction was carried out as described by Mukherjee and Choudhuri (1983). Catalase activity (CAT; EC 1.11.1.6) was evaluated in a reaction solution (3 mL) composed of 50 mM at pH 7 phosphate buffer, 30% (w/v) H_2O_2 and 0.5 mL enzyme extract (Aebi, 1984). It was estimated by the absorbance reduction at 240 nm, as result of H_2O_2 utilization and it was reported as units per mg of protein (Havir and Mellare, 1987). Total PX (EC 1.11.1.6) was determined using guaiacol reaction solution (3 mL) containing 10 mM KH_2PO_4 at pH 7, 10 mM H_2O_2, 20 mM guaiacol and 0.5 mL crude extract (Maehly and Chance, 1954). The ascorbate peroxidase activity (APX, EC: 1.11.1.11) was determined from decrease in absorbance of ascorbic at 290 nm (Asada and Chen, 1992). The enzymes activity was assayed in 3 mL containing 50 mM phosphate buffer at pH 7, 0.5 mM ascorbic acid and 0.5 mM H_2O_2. The reaction was started by addition of H_2O_2.

Statistical Analysis

One-way analysis of variance (ANOVA) test was used to analyze all data and means were compared by the Tukey–Kramer's test, at $P < 0.05$ level of significance, using MINITAB Software Version no. 14.

Results

Antioxidant Enzymes Activity

Table 1 showed that phytohormones, salinity and their interactions significantly affected the studied antioxidant enzymes. Catalase activity (P=0.0001). Salinity induced a significant increase in CAT activity in wheat plants, particularly at high salinity level (150 and 200 mM NaCl). Treatments with IAA, in most salinization levels, resulted in a marked stimulation of CAT activity as compared with control salt group. The same trend was reported for plants sprayed with SA at moderate salinity level (100 mM NaCl) when compared to untreated plants (Fig. 1).

Phytohormones, salinity and their interaction significantly affected PX activity (Table 1). Salinity stress resulted in significant increase of PX activity in wheat compared to control plants. Spraying wheat plants with both phytohormones resulted in increase of PX activity, which was more prominent SA than IAA treated plants (Fig. 1).

The effects of phytohormones, salinity and their interaction APX activity in wheat are reported in Table 1. The APX activity of salt stressed wheat plant increased as compared with untreated plants ($P = 0.0005$; Fig. 1). The highest value of APX activity (0.84 units per mg of proteins) was recorded at the lower salinity levels, and with 0.1 mM SA it was about four times higher than control plants (0.22 units per mg of proteins). Adding any of the two growth regulators resulted in a considerable increase in APX activity in wheat plants.

The correlation coefficients of data revealed that among the antioxidant defense system, PX was positively correlated with salinity, but negatively with salinity + IAA as well as salinity + SA; whereas, APX results not significantly correlated to salinity, salinity + IAA nor salinity + SA. The CAT was positively correlated with salinity, but negatively with salinity + SA (Table 2). It was observed that, among the antioxidant defense system, PX was the higher, followed by CAT and APX, respectively. On the other hand, in spite of both selected phytohormones (SA and IAA) significantly reduced the effect of salinity, the SA was the more effective compared to IAA.

Photosynthetic Pigments

Table 1 showed that treatments significantly affected both Chl *a* and *b* as well as carotenoids. These photosynthetic pigments tend to increase with salinity level. Treating salt-stressed wheat plant with SA or IAA significantly increased the production of photosynthetic pigments (Chl *a* > Chl *b*>carotenoids, respectively) compared with control plants (Fig. 2).

The correlation coefficients of data revealed that among the photosynthetic pigments, Chl *b* was not significantly correlated with both treatments. On the other hand, Chl *a* positively correlated with salinity and Salinity + SA treatments, whereas with salinity + IAA treated plants Chl *a* was negatively correlated. Carotenoids were positively correlated with salinity, but negatively with SA sprayed plants (Table 2). It was interesting to note that in salt-stressed wheat treated with SA (at 50, 100 and 150 mM NaCl, respectively) the concentration of Chl *a* was higher than Chl *b*, conversely carotenoids resulted in lower concentration.

Metabolic Constituents

ANOVA showed that phytohormones, salinity and their interaction had significant effects on reducing sugars (Table 1). Among the three organs of salt-stressed wheat, spike showed the higher reducing sugars accumulation than shoot

and root, respectively. The reducing sugars of spike decreased as salinity increased only in NaCl stressed plants (control group). Foliar application with phytohormones showed a marked and progressive increase in reducing sugar in the spike, specially in SA treated plants at high salinity level (0.1 mM SA + 150 and 200 mM NaCl, respectively) than IAA treated plants at no or low salinity dose (0.5 mM IAA + 0 or 50 mM NaCl, respectively). Reducing sugar in shoot and root, it was observed that SA treated shoot was accumulator than IAA treated shoot, while in root vice versa was observed (Fig. 3). As salinity increased the content of reducing sugars in the root become close to the value of control plants. The correlation coefficient of selected organs (Table 2) showed the reducing sugar in spike and shoot were correlated with all treatments, while roots were correlated only with IAA treated plants.

The ANOVA showed that all treatments significantly affected the accumulation of soluble protein (Prot) in wheat organs (P=0.000; Table 1). Salt stress induced a pronounced increase in Prot concentration in the spike, and gradually it decreased in shoot and root of wheat plants (Fig. 4). The Prot content in general was higher in spike than shoot and root organs, especially at moderate and high salinity levels. However, Prot was higher in SA than IAA treated plants. The correlation matrixes for Prot showed that it was correlated in both shoot and root organs with all treatments, whereas in root it was not significantly correlated with SA treated plant (Table 2).

Data showed that phytohormones, salinity and their interaction significantly influenced AA accumulation in spike, shoot and root organs (Table 1). Generally, the increase in AA contents was registered in spike than other studied organs (spike > shoot > root). Exogenous application with any of the studied phytohormones led to a significant increase in AA content in spike, shoot and slightly in root (Fig. 5).

Correlation matrix of IAA treated plants showed a significant correlation with all studied organs, conversely SA treated plants showed none correlation. Salt treated plants (reference group) showed correlation only with root organ. The ANOVA showed that all treatments and their interaction had significant affect on Prol accumulation in studied organs (P= 0.000; Table 1). The Prol concentration was higher in spike than shoot and root in all treatments.

Exogenous application of hormones significantly retarded the accumulation of Prol in shoot and root organs of salt-stressed wheat plants (Fig. 6). Correlation coefficients of data showed that Prol accumulation in spike was significantly correlated all treatments, while in shoot it was no correlated. On the other hand, roots were correlated with salinity + IAA treated plants (Table 2). Generally, it was observed that spikes were the most accumulator organs among selected organs and that SA was more significant phytohormone affecting the studied antioxidants, photosynthetic pigments and metabolites compared to IAA hormone.

Table 1: Analysis of variance for antioxidant enzymes, photosynthetic pigments and some metabolic constituents in spike, shoot and root of wheat plants

Item	Treatments		
	Phytohormones (PH)	Salinity (S)	PH × S
CAT	0.0001***	0.0001***	0.0001***
PX	0.0001***	0.0061***	0.0001***
APX	0.0001***	0.0001***	0.0005***
Chl *a*	0.0001***	0.0001***	0.0001***
Chl *b*	0.0001***	0.0001***	0.0001***
Carotenoids	0.0001***	0.0004***	0.0001***
RS, spike	0.000***	0.000***	0.000***
RS, shoot	0.000***	0.000***	0.000***
RS, root	0.000***	0.000***	0.000***
Protein, spike	0.000***	0.000***	0.000***
Protein, shoot	0.000***	0.000***	0.000***
Protein, root	0.000***	0.000***	0.000***
AA, spike	0.000***	0.000***	0.000***
AA, shoot	0.000***	0.000***	0.000***
AA, root	0.000***	0.000***	0.000***
Prol, spike	0.000***	0.000***	0.000***
Prol, shoot	0.000***	0.000***	0.000***
Prol, root	0.000***	0.000***	0.000***

CAT, catalase; PX, peroxidase; APX, ascorbate peroxidase; Chl, chlorophyll; RS, reducing sugars; AA, amino acids; Prol, proline; PH × S, Phytohormones × Salinity interaction; *** Significance level at $P < 0.001$

Table 2: Correlation between Salinity, IAA + Salinity and SA + Salinity with antioxidants, photosynthetic pigments and some metabolites of wheat plant organs

Item	Treatments		
	Salinity	0.5 mM IAA+Salinity	0.1 mM SA+Salinity
CAT	0.765(**)	-0.339	-0.638(*)
PX	0.865(**)	0.673(**)	-0.893(**)
APX	0.468	-0.09	-0.068
Chl *a*	0.641(*)	-0.518(*)	-0.762(**)
Chl *b*	0.479	-0.393	-0.165
Carotenoids	0.520(*)	-0.193	-0.728(**)
RS, spike	-0.674(**)	-0.796(**)	0.805(**)
RS, shoot	-0.937(**)	0.675(**)	-0.919(**)
RS, root	0.221	0.873(**)	0.209
Protein, spike	0.880(**)	0.885(**)	-0.776(**)
Protein, shoot	0.878(**)	0.820(**)	0.036
Protein, root	0.852(**)	0.854(**)	0.577(*)
AA, spike	-0.428	0.856(**)	-0.362
AA, shoot	0.214	0.863(**)	-0.154
AA, root	0.889(**)	-0.719(**)	-0.285
Prol, spike	0.518(*)	-0.554(*)	0.640(*)
Prol, shoot	-0.486	0.022	0.416
Prol, root	-0.898(**)	0.755(**)	-0.34

CAT, catalase; PX, peroxidase; APX, ascorbate peroxidase; Chl, chlorophyll; RS, reducing sugars; AA, amino acids; Prol, proline; (*) $P < 0.05$; (**) $P < 0.01$

Discussion

Tolerance to salt-stress in higher plants correlates to the levels of antioxidant systems and substrates (Jahnke and White, 2003). Modifications in antioxidants contents represent a signal of plant's tolerance to stress environment. Thus, changes in enzymes activity are strictly related to
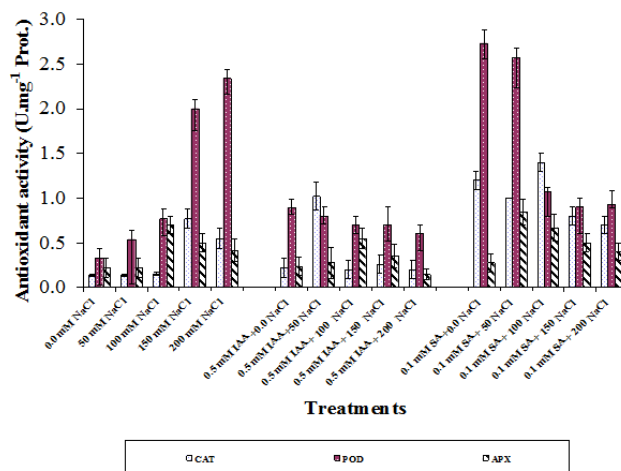
Fig. 1: Effect of different salinity levels, combined IAA and salinity effect and combined SA and salinity effect on antioxidant activity of wheat. Vertical bars represent the standard deviation
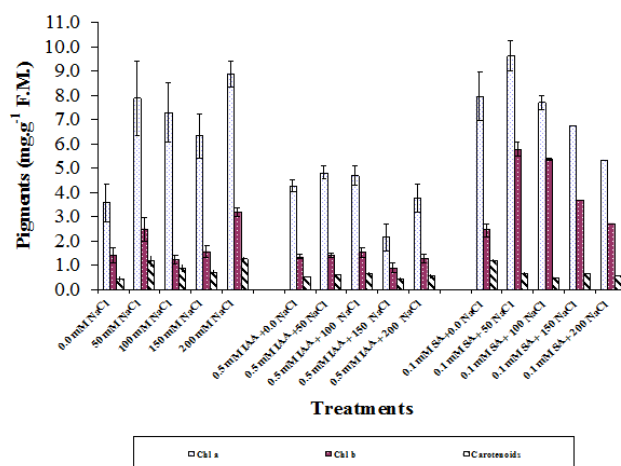
Fig. 2: Effect of different salinity levels, combined IAA and salinity effect and combined SA and salinity effect on photosynthetic pigments of wheat. Vertical bars represent the standard deviation

oxidative stress adaptation of plant (Sudhakar *et al.*, 2001). Mittler (2002) reported that changes in the content of antioxidants levels may act as a signal for ROS scavenging processes and ROS transduction. In our results, the activity levels of antioxidant enzymes, as CAT, PX and APX, showed progressive increases with increasing concentration of NaCl specially at high salinity level (up to 200 mM NaCl) compared to control plants. Our data agree the findings of Lee *et al.* (2001) which observed that salt-stress spread mitochondrial activities and chloroplastic antioxidant enzymes in leaf, offering a protection for cells against superoxide. Moreover, our results showed a linear decrease in CAT activity in SA treated plants especially at high salinity levels (150 and 200 mM NaCl), but still higher
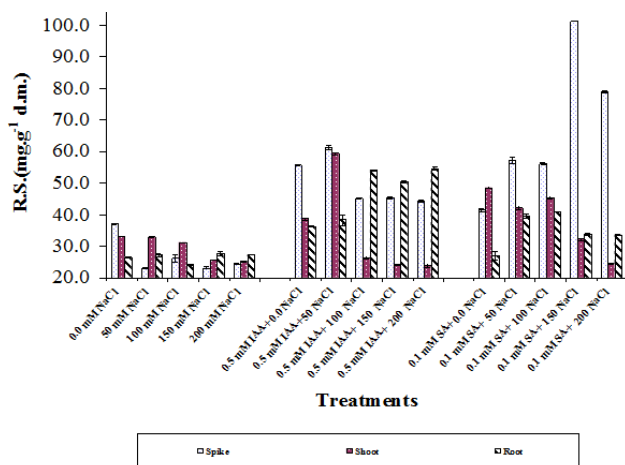
Fig. 3: Effect of different salinity levels, combined IAA and salinity effect and combined SA and salinity effect on reducing sugars (RS) accumulation of different wheat organs. Vertical bars represent the standard deviation
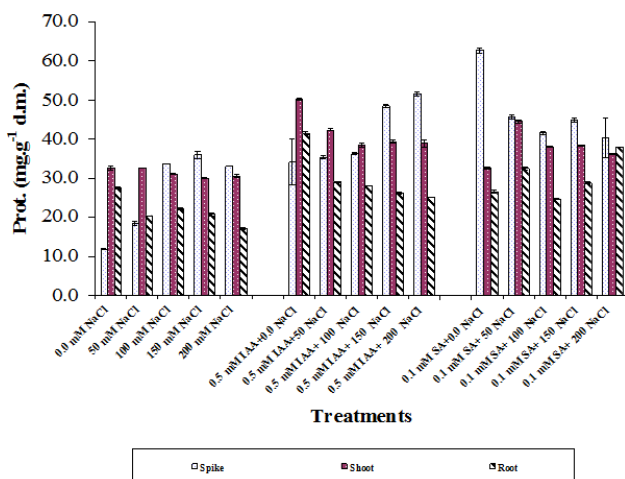

Fig. 5: Effect of different salinity levels, combined IAA and salinity effect and combined SA and salinity effect on soluble amino acids (AA) accumulation of wheat. Vertical bars represent the standard deviation

Fig. 4: Effect of different salinity levels, combined IAA and salinity effect and combined SA and salinity effect on soluble proteins (Prot) accumulation of wheat. Vertical bars represent the standard deviation

Fig. 6: Effect of different salinity levels, combined IAA and salinity effect and combined SA and salinity effect on proline (Prol) accumulation of wheat. Vertical bars represent the standard deviation

(about three times) than reference group, which led to the accumulation of toxic level of H_2O_2 (Lee *et al.*, 2001). On the other hand, CAT activation by salt-stress and IAA treated plants (set II) may be due to synthesis of new enzymes (Feierabend and Dehne, 1996) or CAT photo activation (Polle, 1997).

The shoot spraying of phytohormones under different salt levels produced a significant stimulation in the CAT, PX and slightly increase in ascorbate activity as antioxidant defense compound in wheat plant. Therefore, treatments with any of our tested phytohormones alleviated the negative effects of salt on photosynthetic pigments, growth traits and metabolic processes by diminishing the build-up of ROS at high salinity levels, and thus enhancing the
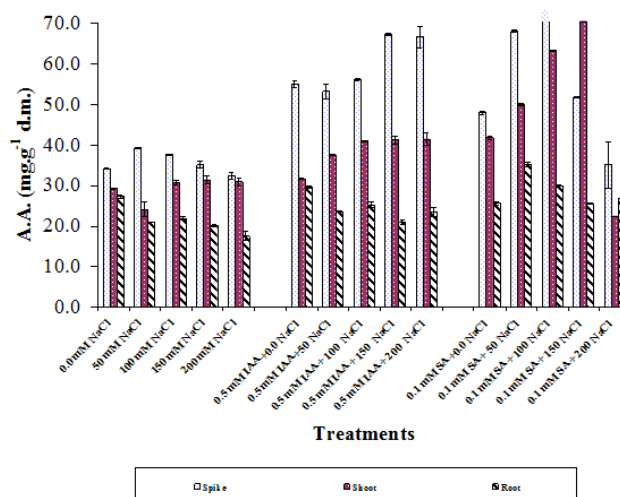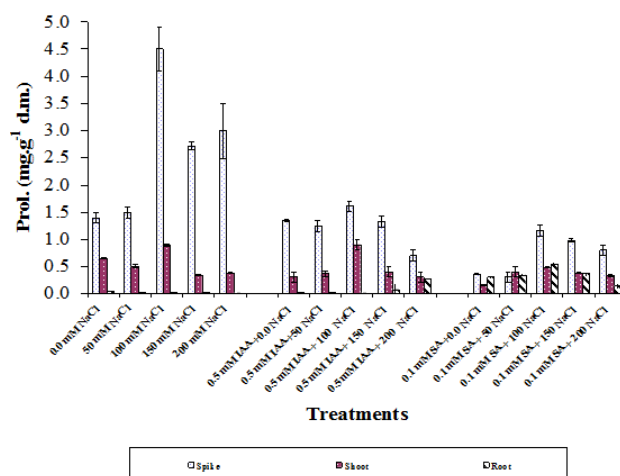
tolerance to salinity (Hassanein *et al.*, 2009). These observations were confirmed by the correlation coefficient, which reported that CAT and PX were positively correlated with salinity. Our correlations were also confirmed by previous literature data (Jebara *et al.*, 2005). Furthermore, a rise in the PX activity with salinity has also been reported in *Morus alba* (Sudhakar *et al.*, 2001), *Glycine max* (Ghorbanli *et al.*, 2004) and *Lycopersicon esculentum* (Rahnama and Ebrahimzadeh, 2005).

In our wheat plants, the significant decrease of Chl *a*, *b* and carotenoids in control treatments may be attributed to the toxic role of salt on pigments synthesis. These results are in agreement with those observed by Rao and Rao (1981)

and Quartacci and Navari-Izzo (1992). The application of any of the two phytohormones in most cases did not only alleviate the inhibitory effect of salt-stress on the biosynthesis of photosynthetic pigments, but also induced a significant stimulatory effect greater than observed in the corresponding controls, a response which may directly contribute to the effectiveness on photosynthetic apparatus and in some way can alter plant productivity.

Many authors found that salicylic acid caused significant increased in chlorophyll content in wheat (Iqbal *et al.*, 2006) and maize (El-Mergawi and Abdel-Wahed, 2007) plants. The pigments' concentration, as a result of salicylic acid utilization, can be due to the improvement of photosynthetic effectiveness, as further demonstrated by augmenting in both Chl *a*, *b* and carotenoids levels in salt-stressed plant leaves. Furthermore, Tari *et al.* (2002) also observed that SA provides a pool of compatible osmolyts in the presence of salinity. The increase in production of photosynthetic pigments in SA treated plants was associated with the accumulation of saccharides and growth yield of wheat plant under the different salinity levels.

Regarding metabolic constituents, carbohydrates play a key-role in the acclimatization of roots by the production of precursors of most chemical synthases, production of metabolic energy and consequently maintained osmoregulation in roots. Data on reducing sugars in the present study in wheat plant organs grown under salt-stress showed a significant decrease of reducing sugars. This trend in wheat organs led to conclude that the photosynthetic efficiency was decreased in response to salinity and thus led to retard the biosynthesis of carbohydrates, which are utilized in growth of wheat plants (Patricia *et al.*, 1992; Singh and Dubey, 1995). Foliar application of phytohormones activated the concentration of carbohydrates in stressed wheat organs serving as bioactivators of carbohydrates (Kodandaramaiah, 1983). Moreover, the accumulation of carbohydrates plays a key-role in reducing salt-stress via osmotic regulation (Ackerson, 1985; Srivastava *et al.*, 1995).

We observed a variable response in the distribution of saccharides, proteins and AA in the three wheat organs with increasing salinity stress. Protein content decreased in root and shoot, but increased in spike in all treatments. Further, we observed an increase of AA content in shoot and spike and a decrease in root. Conversely, the saccharides content decreased in shoot and spike. This situation can be due to the conversion of saccharides into AA and proteins increased in shoot and spike, which may also increase the osmotic potential of wheat organs and the osmotic tolerant of plants through the shoot and spike (Hamdia, 2004). The recorded promotion in saccharides, proteins and AA after SA or IAA treatments was associated with a great promotion in wheat yield. The physiological importance of Prol concentration results questionable. In a study, Rai *et al.* (2003) found that the increase of Prol represented a signal of stress; conversely, Silveira *et al.* (2003) reported that high Prol levels act as solute of cellular osmotic modification.

Based on our findings, Prol showed a variable strategy in wheat organs of the same plant, thus it can be considered a stress sign in spike, while in shoot behaves as a sign of sensitivity. Therefore, the treatment with SA determines the concentration of Prol in presence of salt. In agreement with the findings of Sakhabutdinova *et al.* (2003), also our data suggest that Prol plays a fundamental role in salicylic acid mediated defensive processes in wheat against salt, reducing the detrimental influence of stress. The Prol was found to increase in shoot, but to decrease in root. This opposite behavior of Prol in our study decrease its physiological significance; we take into consideration that the absolute amount of Prol was very rare to play a role in osmoregulation or other roles, which have been also previously reported by Hamdia *et al.* (2004).

In conclusion, the use of phytohormones in wheat, under salinity-stress conditions, stimulated the plants salt tolerance through an improvement of antioxidant enzymes, enhancement of photosynthetic pigments biosynthesis and thereby increasing the plant carbohydrate contents and rate as well as enhancing the accumulation of nontoxic metabolites, which reflect an increase the production wheat plant organs. Thus, our tested phytohormones prevent the deleterious effects of salinity in wheat and they could be adopted as growth regulators or antioxidants in order to improve plant growth under different salinity levels.

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