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Protective Effects of Salicylic Acid on Physiological Parameters and Antioxidants Response in Maize Seedlings under Salinity Stress

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ABSTRACT

Salinity is an important abiotic stress which could restrict plants growth and productivity through several physiological and biological processes. Salicylic acid (SA) is known as an effective alleviating factor for plants under stress conditions. The present investigation was conducted In order to evaluate the effects of different levels of SA on physiological response of maize under different salinity levels. Total dry matter and shoot dry matter were significantly affected by different salinity levels, but SA alleviated these harmful effects. SA also increased RWC which was highly declined due to salinity. Proline accumulation was decreased on 1 mM SA level, but it rose again on 200 mM salinity and 2 mM SA application. Chlorophyll a and b content of stressful plants were destructively affected, but it was reorganized and increased along with increasing SA concentration. Antioxidants system activities were strongly responded to the stress conditions and SA application. An elevated activity of catalaze (CAT) and Ascorbate peroxidase (APX) were observed caused by simultaneous effect of salinity and SA application and it was amplified as the SA concentration increased. Salinity motivated Superoxide dismutase (SOD), Peroxidase (POX), and Glutathione reductase (GR) activity, while they had a declining trend as a consequence of increasing SA level. It may be a result of ameliorative role of SA and a sign of alleviated oxidative damage. Overall, it can be concluded that SA could improve physiological properties of maize seedlings under saline conditions. Further investigation to evaluate long term salinity effects is recommended.

KEYWORDS: Antioxidants, Salicylic acid, Salinity, Proline

1- INTRODUCTION

Plants encounter various kinds of environmental stresses during their life. Salinity is one of the most important abiotic stresses which affect many aspects of plants metabolism and reduce growth and crop production (Zhu, 2001; Kilik and others 2008). About one-third of the world's irrigated land was estimated to be unsuitable for crop production (frommer and others 1999). Salinity causes injurious effects on plant growth and productivity mainly through changes at physiological, biochemical and molecular level (Khan and others 2009; Syeed and others 2010). The physiological processes affected by salt stress include ion toxicity, osmotic stress, nutrient deficiency and especially oxidative stress (Flowers, 2004). Chlorophyll content and photosynthesis rate was also reported to decrease due to salinity (Lee and others 2004; Kao and others 2006).

Salicylic acid (SA) is an important plant hormone which affects various physiological and biochemical processes in plants. As an important signaling molecule, it has various effects on biotic and abiotic stress tolerance (Arfan and others 2007).

Exposure of plants to unfavorable environmental conditions such as temperature extremes, heavy metals, drought or salt stress can increase the production of ROS. To protect themselves against these toxic oxygen intermediates, plant cells and its organelles like chloroplast, mitochondria and peroxisomes employ antioxidant defense systems. A great deal of research has established that the induction of the cellular antioxidant machinery is important for protection against various stresses (Tuteja, 2007; Khan nad Singh, 2008; Gill and others 2011; Singh and others 2008). Enzymatic antioxidants such as SOD, CAT, APX and GR and non-enzymatic antioxidants include GSH, carotenoids and tocopherols are the components of antioxidants defence system (Mittler and others 2004). Salicylic acid has an affinity to bind with the enzymes like CAT, APX and carbonic anhydrase (Durner and Klessig, 1995; Ruffer and others 1999; Slaymaker and others 2002) and some of these enzymes are involved in ROS metabolism and in redox homeostasis. Alteration in this homeostasis leads to induction of a defense response in plants (Mittler, 2002; Torres and others 2002; Durrant and Dong, 2004).

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Exogenous application of salicylic acid enhanced the photosynthetic rate and also maintained the stability of membranes, thereby improved the growth of salinity stressed barley plants (El Tayeb, 2005). SA application was also alleviated the destructive effects of salinity in Arabidopsis seedlings (Borsani and others 2001). osmotic potential, shoot and root dry mass, K^+/Na^+ ratio and contents of photosynthetic pigments in wheat seedlings under salinity stress was completely affected by exogenous SA (Kaydan and others 2007). Another report implied that B. juncea seedlings under saline conditions showed an increase on antioxidants enzymes such as CAT and SOD and also on proline content when they treated with SA foliage spraying (Yusuf and others 2008). Accumulation of large amounts of osmolytes (proline) is an adaptive response in plants exposed to stressful environments (Rai, 2002). Wheat seedlings accumulated large amounts of proline under salinity stress which was further increased when salicylic acid was applied exogenously, thereby alleviating the deleterious effects of salinity (Shakirova and others 2003).

There were various attempts to improve the salinity tolerance of a variety of crops by traditional breeding programs, but commercial success has been limited so far. There are many reports implied that exogenous application of salicylic acid could alleviate injurious effects of salinity on crops under saline conditions. So it can be considered as a successful approach to increase salinity tolerance and improve crops productivity. Thus, the main object of this study was to evaluate ameliorative effects of SA on physiological responses of maize seedlings under saline and none saline conditions.

2- MATERIALS AND METHODS

2-1- plant materials

Seeds of maize (*Zea mays* L., Single cross 704) were surface sterilized with %1 Sodium hypochlorite (NaClO) for 5 minutes and rinsed twice completely. Salicylic acid treatments were applied as 0, 0.5, 1 and 2 mM SA solutions. The soil which includes peat, perlite and sand with the same proportions was air-dried, sieved to 2mm and each pot contained 1000 g of soil. Seeds were soaked on each solution for 10 minutes, air dried and sown on plastic pots. Four seeds was sown an each pot and thinned to two seedling after 8 days. Seedlings were irrigated with distilled water for 10 days in order to improve plant development. Salinity treatments were applied on three levels of 0, 100 and 200 mM of NaCl concentration, which added to Hoagland nutrient solution (Hewitt, 1966). Each pot was supplied with 200 ml of nutrient solution every four day and 150 ml deionized water every alternate day. Plants were harvested after 30 days and Fresh weights of whole plant and shoot tissue were measured. For determination of dry weight, plant tissues were dried two days at 70℃.

2-2- Relative water content determination

Relative water content (RWC) was determined using fresh leaf discs with 2 cm^2 diameter. After weighting, they floated on deionized water for saturation until 24 hours. Saturated leaf weight was recorded and the Dry mass was noted after dehydration at 70 ◦C for 48 h. the following formula was used to calculate RWC (Hayat and others 2007):

$$RWC = \frac{\text{Fresh weight-dry weight}}{\text{Turgor weight-dry weight}}$$

2-3- Chlorophyll measurement

Samples (100 mg leaves) were homogenized in chilled 80% (v/v) acetone and centrifuged at 10 000 g for 10 min at 4 °C. Absorbance of the acetone extracts was measured at 663 and 645. The contents of chlorophyll a and chlorophyll b were calculated as described by Lichtenthaler (1987).

2-4- Proline measurement

Proline content was determined based on the method of Bates et al. (1973). 100 mg of Leaf tissue was homogenized with 10 ml of 3% aqueous sulfosalicylic acid and centrifuged at 10,000×g for 10 min, 2ml of supernatant were mixed with 2ml of glacial acetic acid and 2ml of acid ninhydrin for 1 h at 100 ◦C. The developed colour was extracted in 4ml toluene and measured colourimetrically at 520nm. A standard curve with l-proline was used for the final calculations. Content of proline was expressed as mol g−1 FW (fresh weight).

2-5- Enzyme assays

Leaves tissue (100mg FW) were placed into liquid nitrogen and then homogenized with a prechilled mortar and pestle under ice cold-conditions in 4 ml 50 mM potassium phosphate buffer, pH 7.0, with the addition of 1 mM EDTA. The homogenate was centrifuged at 15 000 rpm, at 4 ◦C for 20 min. The supernatant was stored at -20 ◦C and used for the assay of enzyme activity.

Superoxide dismutase (SOD, EC 1.15.1.1) activity was determined by measuring the inhibition of photochemical reduction of NBT (Giannopolitis and Ries, 1977). The color was developed by adding the following

reagents: 2.4mL of 50mM potassium phosphate buffer solution (pH 7.8), 0.2mL of 195mMmethionine, 0.1mL of 0.3mMEDTA, 50µL enzyme extract, 0.2mL of 1.125mM NBT and 0.2mL of 60µM riboflavin. Reaction mixtures were illuminated for 15min at light intensity of 5000 lux. The absorbance of solution was measured at 560 nm. One unit of SOD was defined as the amount of enzyme causing half-maximal inhibition of the NBT reduction under the assay condition.

For Ascorbate peroxidase (APX, EC 1.11.1.11) activity measurement the reactive solution contained 50mM sodium phosphate buffer (pH 7.0), 0.5mM ascorbate, 0.1mM H_2O_2 and 10µL of enzyme extracts. The decrease in absorbance at 290nm was read. Activity was calculated using the extinction coefficient (2.8mM−1 cm−1). One unit of APX was defined as the amount of degrading 1µmol of ascorbate min−1 mg protein−1 under the assay conditions (Nakano and Asada, 1981).

Catalase (CAT, EC 1.11.1.6) activity was determined by following the consumption of H_2O_2 at 240nm for 1 min (Aebi, 1984). The reaction mixture contained 100 mM potassium phosphate buffer (pH 7.0), 15 mM H_2O_2 and 50µl of enzyme extract in a 3 ml volume. The enzyme activity was calculated using the extinction coefficient (39.4mM−1 cm−1) and expressed as units (1µmol of H_2O_2 decomposed per minute) per mg protein.

Glutathione reductase (GR) activity was determined according to Jablonski and Anderson (1978). The reaction mixture consisted of 10 mM GSSG, 1 mM EDTA, and 200 mM phosphate buffer. The supernatant was preincubated at 25 °C for 5 min. The reaction was initiated by an addition of 1 mM NADPH, and the rate of oxidation of NADPH was monitored at 340 nm. The enzyme activity is expressed as µmol NADPH min−1 mg−1 protein.

Peroxidase (POD) activity was assayed using the method of Polle et al (1990). Absorbance Changes of guaiacol in the presence of H_2O_2 at 460 nm were noted and POD activity were calculated.

2-6- Statistical analysis

The experiment was a 3*4 factorial based on a randomized complete block design with four replications and two seedlings on each pot. All data were analyzed using Analysis of Variance (ANOVA) and the LSD was calculated at P = 0.05.

3- RESULTS

3-1- Analysis of total dry matter and shoot dry matter

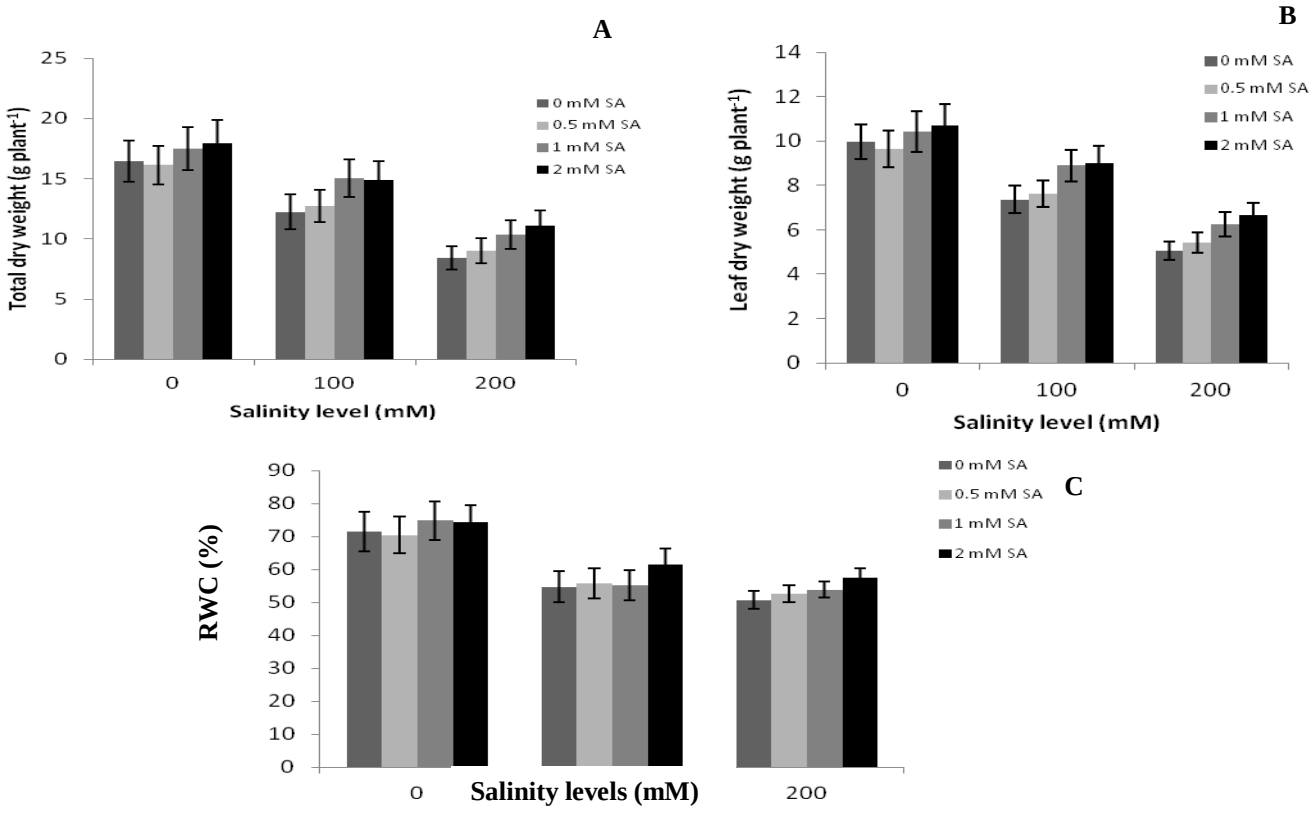

Figure 1. Total dry matter (A), Shoot dry matter (B) and relative water content (C) of maize seedlings under different levels of salinity and salicylic acid (SA). Data are the mean value of three replications±SE which represented by the vertical bar in each graph.

Total dry matter (TDM) and shoot dry matter (SDM) were significantly affected by different salinity levels (Fig. 1A). TDM was highly decreased on 200 mM salinity treatment compared with control. Salicylic acid alleviated salinity harmful effects on both 100 mM and 200 mM salinity levels. Along with increasing SA concentration, TDM was improved on both salinity levels. There was a little decrease at 100 mM salinity and 2 mM SA on TDM, but not significant. There was a little decrease at 100 mM salinity and 2 mM SA on TDM, but not significant. There was also a decline on shoot dry matter of the seedlings on salinity treatments (Fig. 1B). The lowest SDM (5.07 gr) was related to the 200 mM salinity and no SA application. SDM was improved with SA application on both saline and non saline conditions. Higher SA levels had more ameliorative effects and the higher amounts of SDM. These results show that although TDM and SDM were significantly affected by salinity levels, but SA could improve these parameters and this effect enhanced with increasing SA concentration.

It is obviously cleared that the plants being exposed to stressful environments such as saline conditions, result in a reduced metabolic activity, thereby leading to retarded overall growth (Ramagopal, 1987). However, salinity induced retardation of growth in wheat was extremely alleviated by salicylic acid application (Shakirova, 2007). It was also reported that SA could enhanced the leaf area and dry mass production in corn and soybean (Khan and others 2003). Another study by Fariduddin et al. (2003) showed that the dry matter accumulation was significantly increased in Brassica juncea with SA spray application. So, it can be concluded that salicylic acid could acts as an endogenous regulator and affects plants growth and productivity. Alleviative effect of SA could also be contributed to the role of SA on preventing IAA and cytokinin level reduction on plants under salinity stress which leads to improve cell division and so plant growth (Shakirova and others 2003).

3-2- Relative water content

Seedlings under stress conditions showed a drastic decreased on relative water content on all treatments. The highest (71.5%) and lowest (50.7%) relative water content were obtained on control treatment and 200 mM salinity with no SA application, respectively (Fig. 1C). SA treatment could alleviate reduced RWC to a great extent. In fact, RWC had a rising trend on both SA treatments. Leaf RWC could be considered as a valuable parameter to determine plants water status (Flower and Ludlow, 1986). Reduced RWC could be a result of lower water availability under saline conditions (Shalhevet, 1993). It may also a consequence of inefficient root system which could not retrieve the water losses because of decreasing its absorbing surface (Gadallah, 2000). Reduced RWC due to the salinity stress were also reported by many other researchers (Thind and Malik, 1988; Srivastava and others 1998).

3-3- Proline

Proline accumulation was significantly affected by salinity stress. Salinity increased proline amount on plants under stress. The highest proline accumulation was observed on plants under 200 mM salinity and 2 mM SA application (Fig. 2A). SA had also increased proline accumulation on plants under non saline conditions which treated with SA. But, it was decreased on 1 mM SA application on both 100 and 200 mM salinity. It could be contributed to the alleviative effect of SA. In fact, it may be a sign of stress alleviation by SA. Osmolytes accumulation such as proline is an adaptive mechanism on plants under stress conditions (Rai, 2002). Salinity increased proline accumulation in wheat seedlings and there was further increase with exogenous SA application (Shakirova and others 2003). There is also other studies reported increased accumulation of proline on plants exposed to salinity stress which was amplified by SA treatment (Szepesi and others 2005).

3-4- Chlorophyll content

Salinity had a destructive effect on chlorophyll a and b content of stressful plants (Fig. 2B and 2C). Although SA treatment reorganized seedlings chlorophyll structure and chlorophyll content was increased along with increasing SA concentration, but decreasing trend on two saline conditions compared with control treatment were remarkable. Chlorophyll a/b ratio was also significantly affected by salinity (Fig. 2D). There was a noticeable decrease on 200 mM salinity and 2 mM SA treatment and had the lowest chlorophyll a/b ratio. According to the Sabater and Rodriguez (1978), chlorophyll degradation may be due to the formation of proteolytic enzymes like chlorophyllase. It may also be a result of chlorophyll components degradation (Yasseen, 1983). There is a challenge in terms of SA effects on photosynthesis pigments for stressed plants. There are some studies such as Hayat et al (2005) which reported the enhancement effect of SA on pigment contents for wheat seedlings under salinity stress. Chlorophyll content was also significantly increased on Brassica juncea plants under salinity and foliar application of SA treatment (Fariduddin and others 2003). Similar results were obtained by Ghai and others (2002) for Brassica napus stressed plants. Despite these evidences, reduced chlorophyll content due to the SA pretreatment was also observed by some researchers (Anandhi and Ramanujam, 1997; Pancheva and others 1996). Additionally, Moharekar et al. (2003) reported the simultaneous decrease in chlorophyll pigments and chlorophyll a/b ratio in wheat and moong.

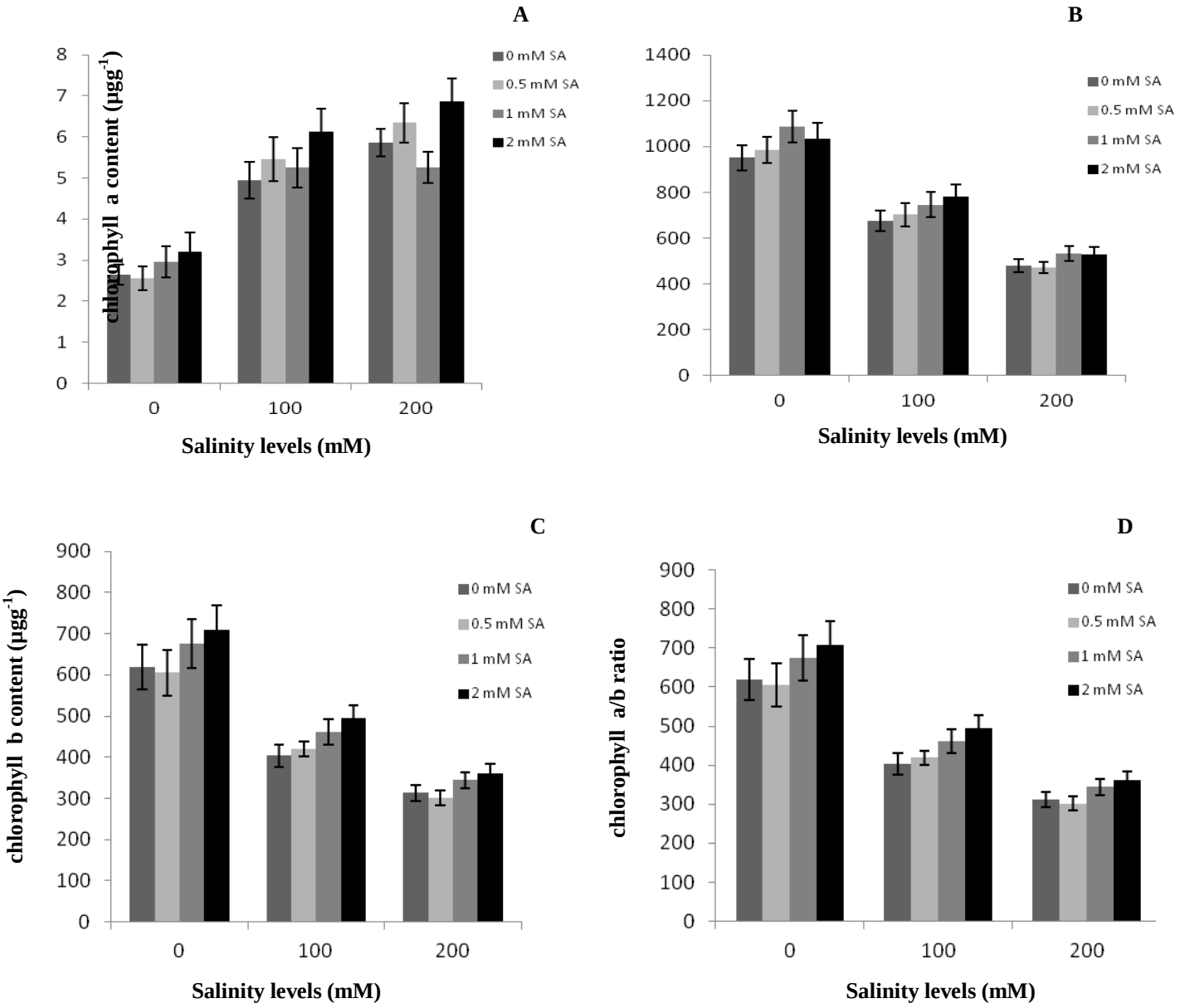

Figure 2. Proline content (A), chlorophyll a (B), chlorophyll b (C) and chlorophyll a/b ratio (D) of maize seedlings under different levels of salinity and salicylic acid (SA). Data are the mean value of three replications±SE which represented by the vertical bar in each graph.

3-5- Analysis of antioxidants activity

Antioxidants system activities were strongly affected by salinity stress. There were significant differences between the treatments for all evaluated antioxidants (figure 3 and 4). In fact, this result indicated that oxidative stress is one of the main salinity stress consequences on maize and SA has an ameliorative effect on this process. SA application may cause a temporary and low level of oxidative stress in plants, which acts as a hardening process, improving the antioxidative capacity of plants and helping to induce the synthesis of protective compounds and, therefore, the acclimation to stress (Janda and others 2007).

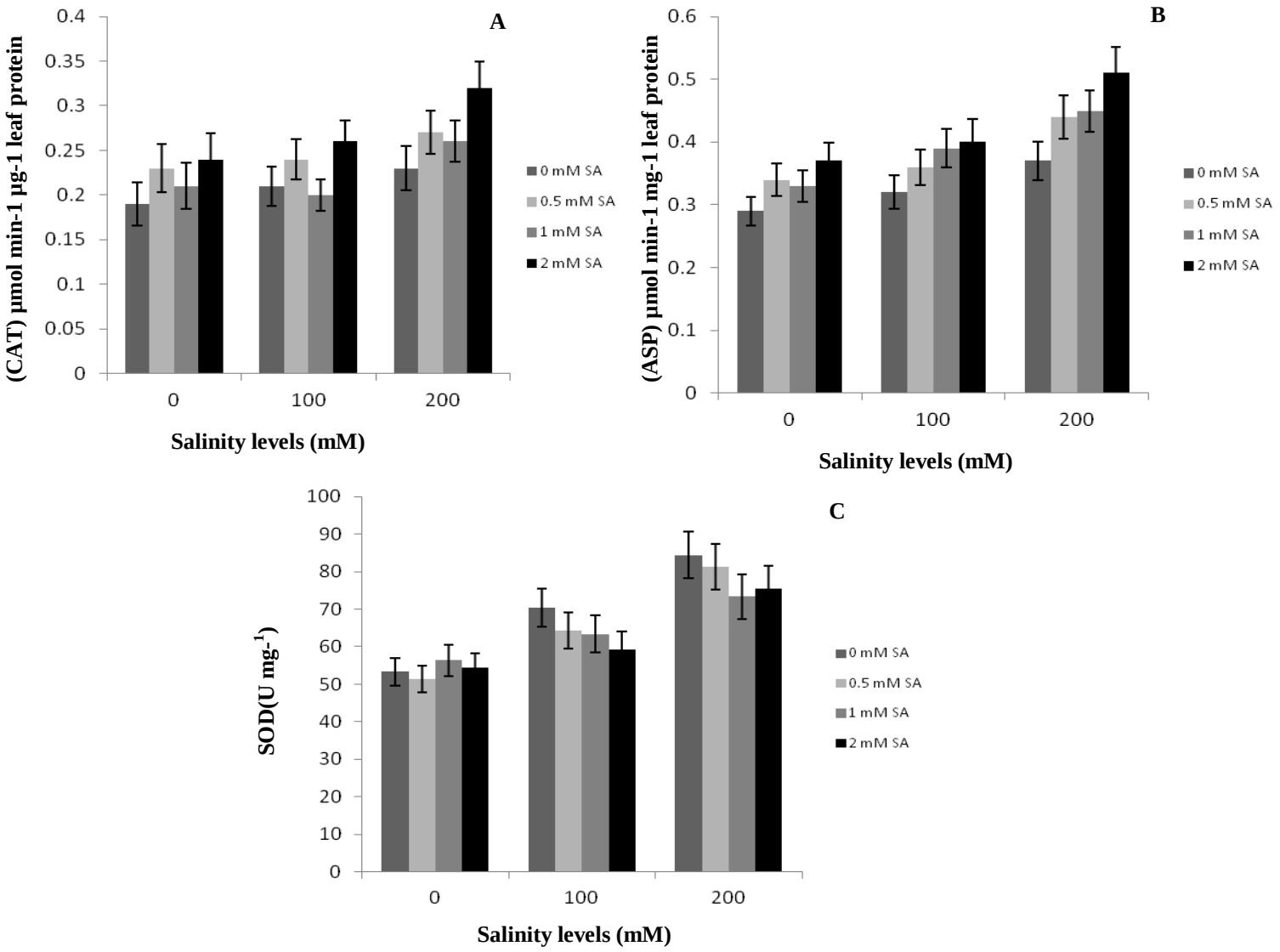

Figure 3. Activity of antioxidative enzmes including Catalaze (CAT) (A), Ascorbate Peroxidase (ASP) (B) and Superoxide dismutase (SOD) (C) of maize seedlings under different levels of salinity and salicylic acid (SA). Data are the mean value of three replications±SE which represented by the vertical bar in each graph.

3-5-1- Catalaz

Results of enzyme assessment showed a drastic increase on CAT activity (Fig. 3A). SA treatment was also an amplification factor on CAT activity and Along with increasing SA concentration, CAT activity was also increased. There were an unexpected decline on CAT activity when 1 mM SA was applied on both saline and none saline conditions. It seems that this respond is because of the reduction of oxidative stress severity. The highest CAT activity was related to the 200 mM salinity and 2 mM SA treatment. Stressful environments induce the generation of reactive oxygen species (ROS) and so causes oxidative damage in plants (Prasad and others 1999; Panda and others 2003a,b) which could harm biochemical compounds and thus changing redox homeostasis (Smirnoff, 1993; Gille and Singler, 1995). It was confirmed that SA exogenous application could improve antioxidants activity in plants (Knorzer and others 1999). There was a transitory reduction on CAT activity as a result of SA exogenous treatment (Janda and others 2003). While Yusuf and others 2008 found that SA application could increase antioxidants activity such as CAT, peroxidase (POX) and superoxide dismutase (SOD) in B juncea stressed plants.

3-5-2- Ascorbate peroxidase activity

APX activity was also affected by the applied treatments. Salinity increased APX activity at both applied level and there were significant differences between the two salinity level treatments (Fig. 3B). SA application had an

additive effect on APX activity and along with increasing SA level, APX activity was also increased. These results indicated that increased APX activity could be an adaptive mechanism to an increased oxidative stress which caused by salinity. Furthermore, SA intensified APX activity in order to facilitate oxidative damage protection. Salicylic acid has an affinity to bind with the enzymes like APX and CAT (Ruffer and others 1999; Slaymaker and others 2002) which are involved in ROS metabolism and redox homeostasis. Alteration in this homeostasis leads to induction of a defense response in plants (Mittler, 2002; Durrant and Dong, 2004). Increasing APX activity as a consequence of exogenous SA application was also reported by Krantev et al. (2008).

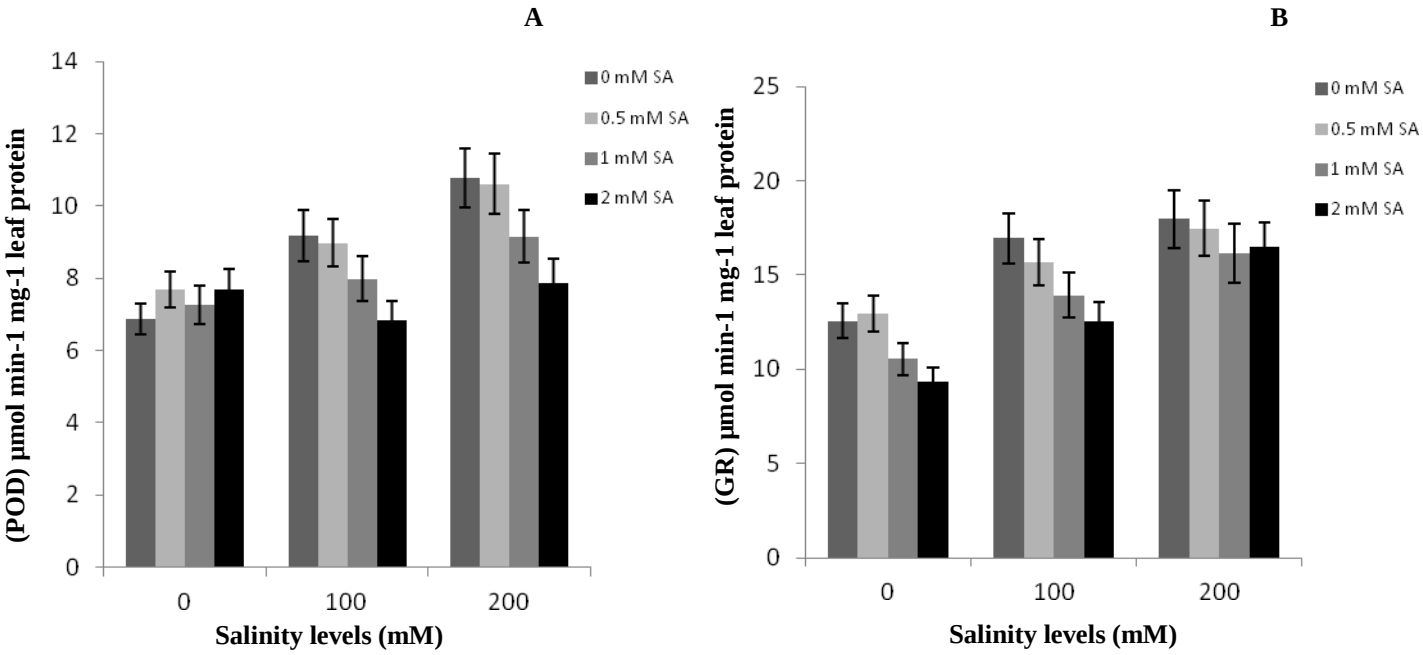

Figure 4. Activity of Peroxidase (POD) (A) and Glutathione reductase (GR) (B) of maize seedlings under different levels of salinity and salicylic acid (SA). Data are the mean value of three replications±SE which represented by the vertical bar in each graph.

3-5-3- Superoxide dismutase activity

As a result of salinity, Superoxide dismutase (SOD) activity was increases on salinity stressed seedlings compared with control plants (Fig. 3C). But, despite the previous antioxidants, SA application not only did not increase SOD activity, but also the activity of SOD was decreased along with increasing applied SA concentration under saline conditions. Reduced SOD activity could be a symptom of decreased oxidative stress severity which could be a result of SA application. Thus, the necessity of SOD activity is reduced. Meloni and others (2003) found that SOD activity increased in cotton cultivars under salinity stress. Decreasing SOD activity due to SA application was also reported by Choudhury and Panda (2004), when rice seeds were primed with SA treatment and exposed to oxidative damage.

***3-5-4-* Peroxidase activity**

Based on the Peroxidase (POX) assessment results, there were significant differences between the treatments about POX activity (Fig. 4A). Salinity increased POX activity comparing with non saline treatments. But similarly with SOD, a decrease on POX activity in salinity and exogenous SA application was observed. Higher SA concentration had the lower POX activity on both saline conditions. It can be concluded that SA treatment could lowered reactive oxygen species on seedlings under salinity stress and this leads to a lower POX activity and protecting against oxidative damage. These findings had conformity with the results of Choudhury and Panda (2004).

***3*-5-5- Glutathione reductase**

Salinity induced Glutathione reductase (GR) activity on both two salinity treatments (Fig. 4B). Higher salinity level had the higher GR activity, but like the previous, SA application leads to a lower GR activity on salinity stressed seedlings. It could also be a result of reduced oxidative damage due to SA application and so this caused a decreased GR activity.

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