Role of low molecular weight antioxidants in mitigating the adverse effect of salt stress in *Zea mays* seedlings

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Abstract

In order to determine fresh-dry weights, shoot-root lengths, protein content and enzymatic antioxidant activities like superoxide dismutase, ascorbate peroxidase, catalase, and glutathione reductase, as well as non-enzymatic antioxidant contents such as ascorbic acid, alpha-tocopherol, and glutathione in *Zea mays* L., an economically important cereal crop, the present work was done. To explore the defensive mechanism of the plant against stress of NaCl salt. Grains of the plant were pre-treated individually or in series before germination with ascorbic acid, glutathione, and alpha-tocopherol. After 15 days of age, seedlings that were exposed to different concentrations of 50-200 mM NaCl were examined and analysed. Results showed that the salt-affected maize seedlings responded positively to treatment with different antioxidants and had a strengthened antioxidant defense system.

Keywords: Maize, Antioxidant enzymes, Oxidative damage.

Introduction

Maize (*Zea mays L.*) is one of Egypt's most widely produced and consumed cereal crops. This productive crop, along with starch, oils, and other substances extracted for industrial uses, is primarily used as a source of staple food for humans and animal feed, all of this makes it an essential component of global food security (Campos *et al.,* 2004). A wide variety of abiotic and biotic stresses such as high salinity, extreme temperature, drought, and heavy metals can be introduced to plants (Charu *et al.,* 2011). One of the most major abiotic stresses in agriculture is the presence of salt in the soil. So, it is vital to improve the resistance of plants to salt and increase the quality and quantity of plant yields in saline soils. Almost all crops that are important to humans are vulnerable to high levels of salt in the soil (Liang *et al.,* 2018).

Globally, soil salinity is a significant environmental issue that affects many crops, including maize, in terms of growth and productivity. The main reason is considered to be inhibition of crop growth, followed by a decrease in productivity and loss of yield (Hamdia and Shaddad, 2010 and Sadak and Dawood, 2014). Great salinity stress level typically includes carbon fixation, causing the over decrease in complexes that harvest light which lead to production of ROS like superoxide ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2) and hydroxyl (OH^-) radicals (Hamed *et al*., 2014 and Hameed *et al.,* 2015). The key organelles that produce ROS during photosynthesis are chloroplasts (Asada 1999 and Orabi and Abdelhamid, 2016), which are very sensitive to stress of salt (Wang *et al*., 2014). Chlorophyll degradation caused by ROS (Yildirim *et al*., 2008). By controlling many changes such as growth-inducing hormones, polyamines, and antioxidants the adverse effects of ROS on plant output could be scavenged (El-Katony *et al.,* 2019). Antioxidants consist of two main classes; Enzymatic such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR), and non-enzymatic like ascorbate (ASA), glutathione (GSH), α-tocopherols (α-TOC), carotenoids, and phenolic compounds (Mishra *et al.,* 2009 and Hossain *et al.,* 2013). The near relationship between the activities of antioxidant mechanisms and raising resistance to environmental stresses is also underlined in numerous studies (Orabi *et al.,* 2013 and Bargaz *et al.,* 2016).

As an $O_2^{\bullet-}$ scavenger, SOD produces H_2O_2 and O_2 due to its enzymatic effect, and its three classes are known to depend on the active site metal cofactor (Mn, Fe, or Cu/Zn-SOD) (Moran *et al*., 2003). However, with different mechanisms, both the enzymes CAT and APX have the ability to scavenge toxic H_2O_2 levels (produced by scavenging the $O_2^{\bullet-}$ by SOD). APX is, in fact, more appealing to H_2O_2 and reduces it to H_2O by using ascorbate as a definite electron donor (Apel and Hirt 2004 and Sofo *et al.,* 2015). Reduced glutathione is involved in regenerating the ascorbate pool producing oxidized glutathione (GSSG). The NADPH-dependent GSSG reduction is catalyzed by GR (Edwards *et al.,* 1990). GR catalyzes the final and rate-limiting step of the enzymatic pathway of Halliwell–Asada (Bray *et al.,* 2000). Different treatments with naturally occurring compounds in plant cells are used as soaking applications in order to improve plant resistance to various abiotic stresses. Via previous work, it has been shown that the exogenous application of antioxidants of low molecular weight can reduce the adverse effects of salt stress on plant growth (Rady and Mohamed, 2015 and Rady *et al*., 2016).

Several studies have been conducted on various crops or plants and shown that the exogenously application of AsA significantly reduced the effect of salt stress and encouraged growth and yield (Hameed *et al.,* 2015 and Billah *et al.,* 2017).

In addition to regulating plant enzymes, glutathione is an important antioxidant responsible for the balance between oxidation and antioxidation, regulating many cell functions such as DNA and proteins repair and synthesis. Glutathione also protects cells from free radicals and peroxides (Pompella *et al.,* 2003) and is important for the control of stress. By quenching ROS, GSH acts as an antioxidant and is the main component of the cycle of glutathione–ascorbate, that eliminates H_2O_2 harm (Noctor and Foyer, 1998 and Kasote *et al.,* 2015).

In plants with high antioxidant potency, tocopherols, which are secondary metabolites are found to participate in protecting plants from stress, particularly ROS scavenging, through different metabolic processes, to reduce lipid peroxidation (Hasegawa *et al.,* 2000 and Ali *et al* 2020). α-TOC is commonly recognized as vitamin E, with a strong antioxidant capacity compared to other family members, but the production of α-TOC to reduce oxidative damage is cultivar-specific (Fritsche *et al.,* 2017). However, exogenous application of α-TOC was found to be beneficial in stimulating stress tolerance. There is a positive relationship between α-TOC and the antioxidant mechanism; Enzymatic and non-enzymatic antioxidants (Orabi and Abdelhamid, 2016 and Hemida *et al.,* 2017).

In addition to their endogenous defense mechanisms, to strengthen their defense system against stress, plants need some exogenous help, such as antioxidants. Therefore, the purpose of the current study was to investigate the effect of salinity on fresh-dry weights, shoot-root lengths, the activity of antioxidant enzymes, non-enzymatic antioxidants, and protein expressed in seedlings of maize. Maize seeds were either not soaked or soaked in AsA, GSH, and α-Toc at a rate of 0.50 mM each to achieve this goal. Such soaking treatments were individually or sequentially applied.

Materials and Methods

Maize (*Zea mays* L.) grains used in this research were secured from Institute of Horticultural Science, Agricultural Research Center, Agriculture Ministry, Giza, Egypt. Antioxidants (α-tocopherol; α-TOC, glutathione; GSH, and ascorbic acid; AsA) were purchased from Sigma Aldrich, Egypt.

Seed germination experiments

A homogeneous lot of maize grains were selected for uniform size, shape, and viability. Prior to germination, the surface of grains was sterilized by soaking for 3 min in a 2.5% sodium hypochlorite solution, after which they were washed several times with distilled water to get rid of the hypochlorite and then left to dry for 1 h. Next, grains were then split into five sets for five soaking treatments. The first grain set was soaked in distilled water to serve as a control. Three sets of grains were soaked in different antioxidants; α-TOC, AsA, and GSH at a concentration of 0.5 mM each as individual treatments. The fifth grain set was soaked in the three antioxidants in a sequential manner starting with AsA for 2.5 h, then with GSH for 2.0 h, and finally with α-TOC for 1.5 h using the same concentration of individual treatments. Antioxidant concentration and soaking periods were chosen for either individual treatments or for sequential treatment on the basis of a preliminary study (data not shown). Treatments were designed in randomized complete blocks. After the soaking periods were completed, the grains were left to air-dry overnight in preparation for sowing.

The five maize grain sets were transferred to sterile 15-cm Petri dishes, containing two sheets of Whitman No.1 filter paper each. The filter paper was moistened in each Petri dish with 10 ml of distilled water or different concentrations of NaCl salt (50, 100, 150, and 200 mM). Ten grains were assigned to each Petri dish, 5 of which were allocated to each treatment. The grains were allowed to germinate at 25 ± 2 °C in the dark and 2 ml of distilled water or NaCl solutions was added to each petri dish on the third day of the germination. After normal germination of all Petri dishes, they were exposed to a 14 h photoperiod. The experiment was continued until the seedlings were 15-day-old and then collected for different measurements.

Seedling growth measurements

The 15-d-old seedlings were selected at random from each of the various treatments and weighed (fresh weight; FW), then placed in an oven at 70 °C to reach a constant dry weight (DW) which was recorded. Also, shoot and root lengths were recorded.

Enzyme activity assay

Antioxidant enzymes (e.g., SOD, APX, CAT, and GR) were extracted from seedling tissue samples (0.5 g) by homogenization in ice-cold 0.1 M phosphate buffer (pH 7.5) and 0.5 mM EDTA (Ethylene diamine tetra acetic acid). On 4 °C, each homogenate was then centrifuged at 15,000 $\times g$ for 15 min. The supernatant was decanted and used to measure enzymatic activities

(Esfandiari *et al.,* 2007). SOD, CAT, APX, and GR activities were assayed according to procedures detailed in SenGupta *et al.,* 1993, Aebi, 1984, Yoshimura *et al.*, 2000, and Sairam *et al.*, 2002, respectively.

Determination of ascorbic acid

Total ascorbic acid content was estimated using Folin-Denis reagent according to Jagota and Dani, 1982.

Determination of glutathione

Glutathione content was estimated according to Anderson, 1985.

Determination of α-tocopherol

α-tocopherol content was assayed as described by Philip *et al.*, 1954.

Determination of protein

Protein content was estimated according to the Lowry *et al.,* 1951 method.

Statistical analysis

All data were subjected to analysis of variance (ANOVA) for a split-plot system in a randomized complete blocks design, after testing for the homogeneity of error variances according to the procedure outlined by Gomez and Gomez, 1984 using InfoStat estadistico (2016) software. The significant differences between the treatments at $P \le 0.05$ were compared by Duncan's Multiple Range Test.

Results

Effects of seed soaking with antioxidants on growth of maize seedlings grown under salt stress

Changes of growth parameters (fresh-dry weights and shoot-root lengths) of *Zea mays* seedlings as a result of pre-soaking their grains in antioxidants individually or in sequence for 6 hours, followed by treatment with different NaCl salt concentrations throughout 15 days of growth were represented graphically in Figures (1-4). Data showed that the fresh-dry weights and shoot-root lengths of *Zea mays* seedlings markedly decreased significantly with increasing levels of NaCl. The higher NaCl concentration (200 mM), showed sharply reduction than lower concentration. Pretreatment of the maize grains with antioxidants ASA, GSH, and α-TOC singly or in sequence as grains presoaked considerably increased seedlings fresh-dry weights and root-shoot lengths under each saline level. Fresh-dry weights and root-shoot lengths of maize seedlings still showed

a decrease with increasing levels of NaCl, although their grains treated with antioxidants. Generally, treatment of grains with antioxidants alleviated the adverse effects of NaCl on *Zea mays* seedlings growth when compared with either control or salinized seedlings. The antioxidants, applied in sequence, ASA-GSH-α-TOC, was more effective than singly antioxidants application.

Effects of seed soaking with antioxidants on enzymatic antioxidant activities and protein content of maize seedlings grown under salt stress

Changes in activities of superoxide dismutase (SOD), ascorbate peroxidase (APX), catalase (CAT), and glutathione reductase (GR) of *Zea mays* seedlings which their grains either non-soaked or soaked with the antioxidants, were assayed after 15 days of treatment with different NaCl salt levels and represented in Figures (5-8). Figures showed that activities of enzymes SOD, APX, CAT, and GR were found to be significantly increased with increasing NaCl levels up to 100 mM, then decreased at higher levels (150, and 200 mM) in seedlings of maize that their grains are non-treated with antioxidants. Using of antioxidants as grains pre-soaked, individually or in the sequence enhanced activities of the above enzymes in seedlings of maize exposed to each level of NaCl and results showed highly activities of both SOD and APX than CAT and GR with respect to the control. The increase in the activity of preceding enzymes continued with increasing levels of NaCl up to 100 mM, then decreased at high salt levels (150, and 200 mM). The sequenced application of ASA-GSH-α-TOC was more effective than singly applications.

The effect of presoaking in antioxidants (ASA, GSH, and α-TOC) individually or in sequence on protein in salinized seedlings of *Zea mays* was graphically represented in figure (9). As NaCl salt concentrations increased, the total protein content seems to be decreased in the non-treated maize grains seedlings. Furthermore, using of antioxidants as grains pre-soaking individually or in sequence, increase the total protein content of maize seedlings, at each NaCl level. In spite of using antioxidants treatments with grains, the increase in the total protein content of seedlings continued up to 100 mM, and then decreased at higher salt levels. The sequenced application of AsA-GSH-α-TOC was more effective than singly antioxidants application.

Effects of seed soaking with antioxidants on non-enzymatic antioxidant contents of maize seedlings grown under salt stress

The role played by antioxidants in modifying salt stress induced variations in the biosynthesis of ASA, GSH, and α-TOC graphed in figures (10-12). The present study indicated that increasing in

NaCl salt levels significantly reduced ASA, GSH, and α-TOC contents in maize seedlings, growing from non-antioxidants treated grains, compared to respective controls. Grains soaking application with antioxidants individually or in sequence sharply increased the endogenous antioxidant contents in seedlings of maize concomitant with each corresponding NaCl level. The increase in antioxidants contents continued with rise NaCl levels up to 100 mM, then decreased in higher salt concentrations (150, and 200 mM) but still higher than corresponding NaCl. The higher endogenous antioxidant contents, found in the seedlings that their grains treated with antioxidant sequenced series.

Figure 1. Effect of treatment with AsA, GSH and α-TOC singly or in sequence and different concentrations of NaCl on fresh weight of *Zea mays* seedlings after 15 days of salt treatment.
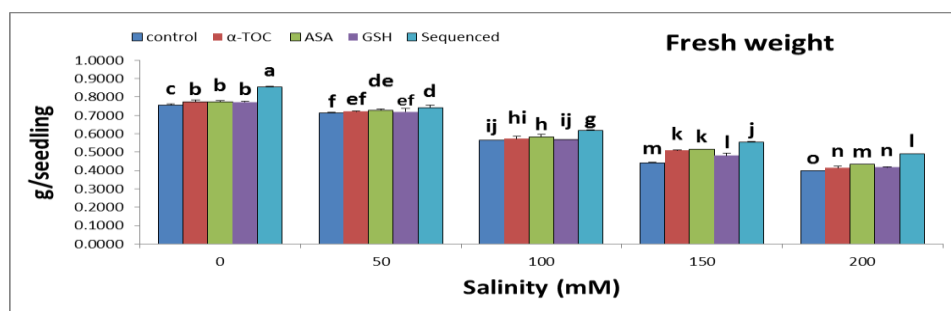

Figure 2. Effect of treatment with AsA, GSH, and α-TOC singly or in sequence and different concentrations of NaCl on dry weight of *Zea mays* seedlings after 15 days of salt treatment.
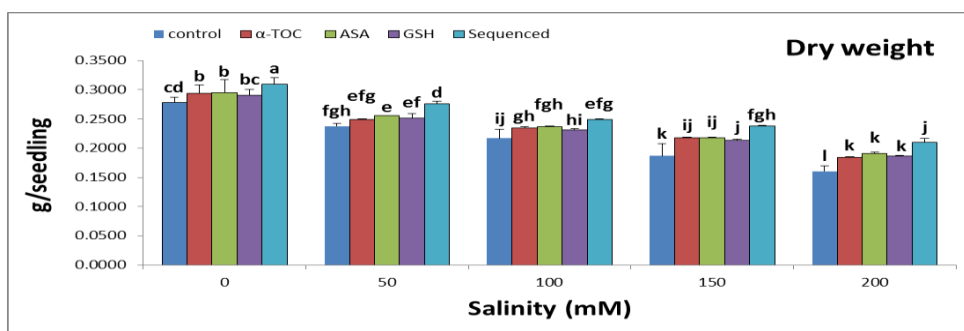

Figure 3. Effect of treatment with AsA, GSH, and α-TOC singly or in sequence and different concentrations of NaCl on shoot length of *Zea mays* seedlings after 15 days of salt treatment.
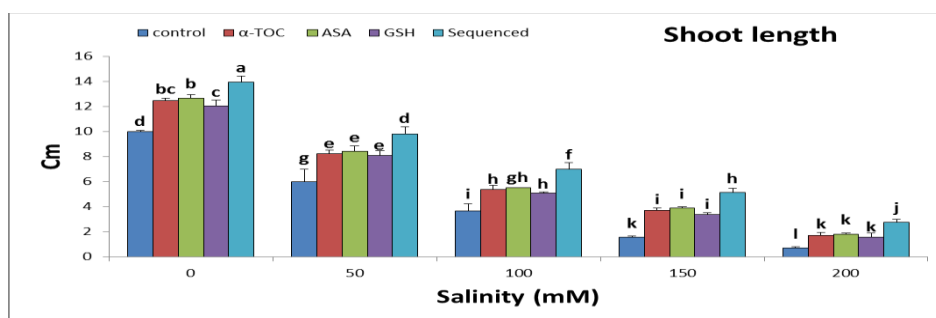
Figure 4. Effect of treatment with AsA, GSH, and α-TOC singly or in sequence and different concentrations of NaCl on root length of *Zea mays* seedlings after 15 days of salt treatment.
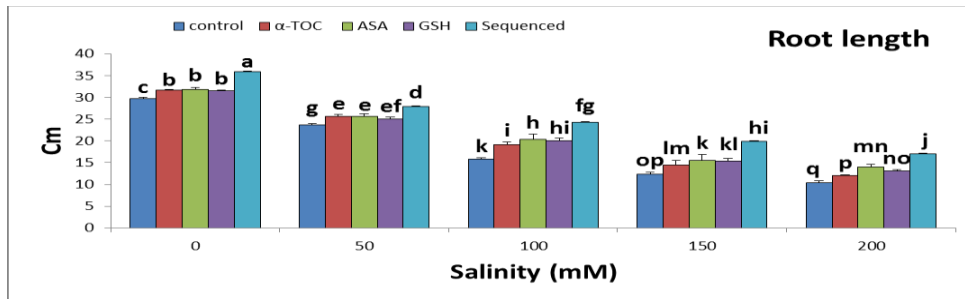

Figure 5. Effect of treatment with AsA, GSH, and α-TOC singly or in sequence and different concentrations of NaCl on superoxide dismutase; SOD activity of *Zea mays* seedlings after 15 days of salt treatment.
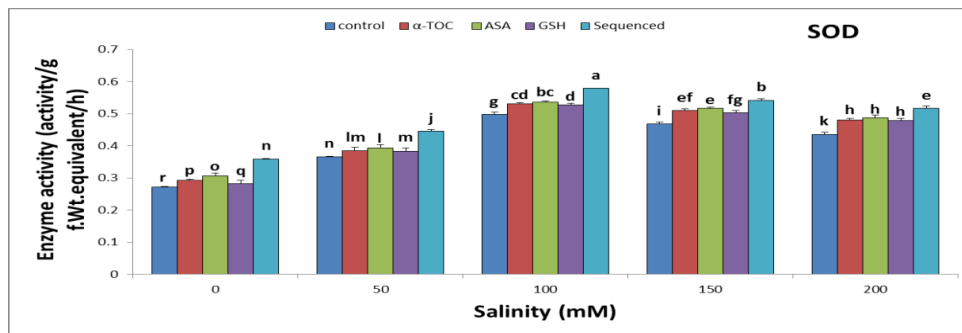

Figure 6. Effect of treatment with AsA, GSH, and α-TOC singly or in sequence and different concentrations of NaCl on ascorbate peroxidase; APX activity of *Zea mays* seedlings after 15 days of salt treatment.
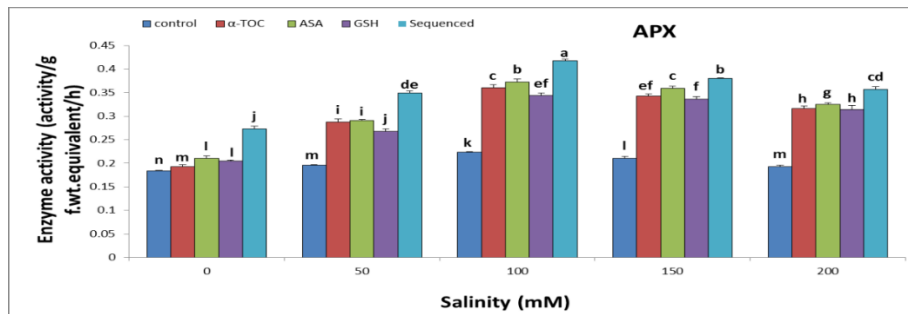

Figure7. Effect of treatment with AsA, GSH, and α-TOC singly or in sequence and different concentrations of NaCl on glutathione reductase; GR activity of *Zea mays* seedlings after 15 days of salt treatment.
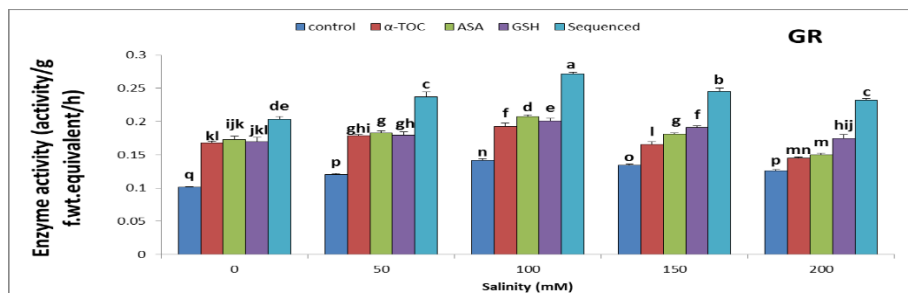
Figure 8. Effect of treatment with AsA, GSH, and α-TOC singly or in sequence and different concentrations of NaCl on catalase; CAT activity of *Zea mays* seedlings after 15 days of salt treatment.
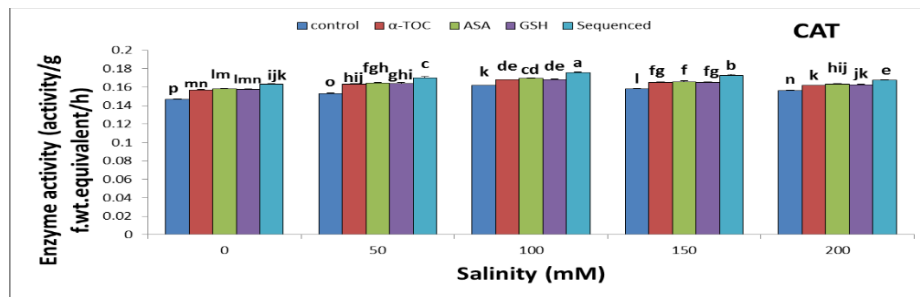

Figure 9. Effect of treatment with AsA, GSH and α-TOC singly or in sequence and different concentrations of NaCl on protein content of *Zea mays* seedlings after 15 days of salt treatment.

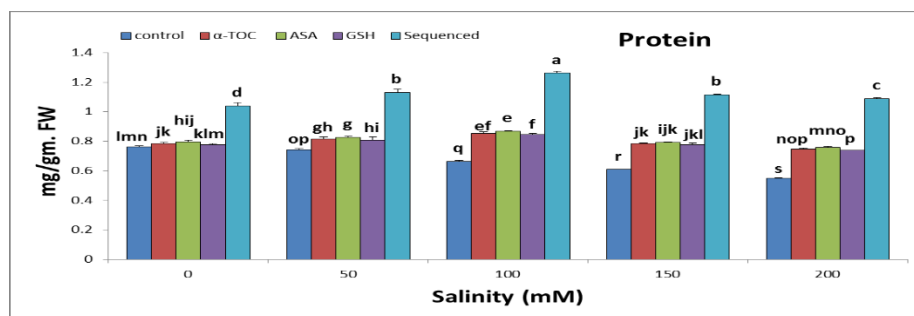**Figure10.** Effect of treatment with AsA, GSH, and α-TOC singly or in sequence and different concentrations of NaCl on ascorbic acid content of *Zea mays* seedlings after 15 days of salt treatment.
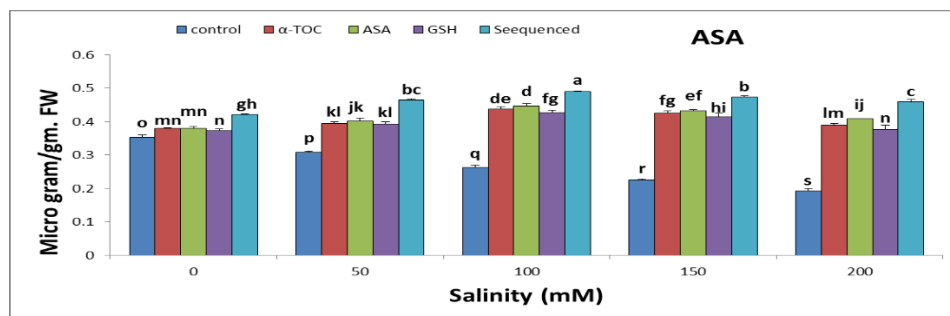

Figure11. Effect of treatment with AsA, GSH, and α-TOC singly or in sequence and different concentrations of NaCl on glutathione content of *Zea mays* seedlings after 15 days of salt treatment.
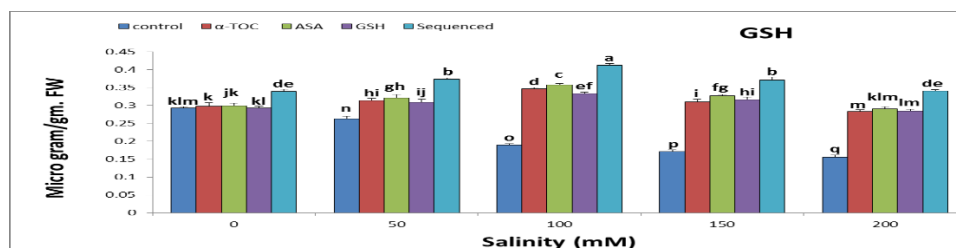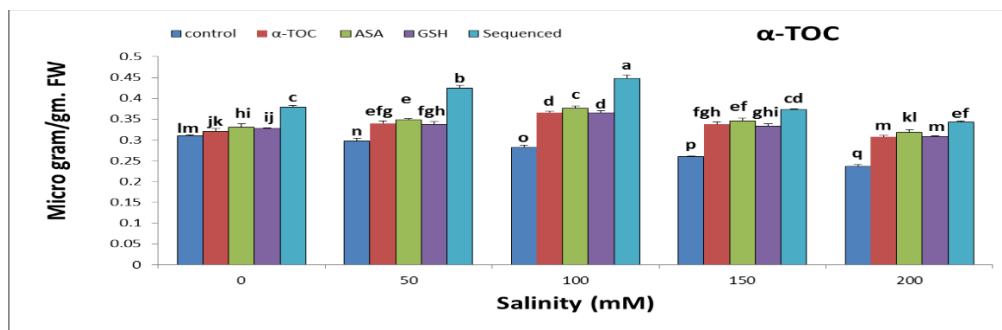
Figure12. Effect of treatment with AsA, GSH and α-TOC singly or in sequence and different concentrations of NaCl on α-tocopherol content of *Zea mays* seedlings after 15 days of salt treatment.

Discussion

The objective of the current investigation was to determine the effect of antioxidants (ASA, GSH, and α-TOC singly or in sequence) on maize grains treated with various levels of NaCl. Data in Figures (1-4), indicated that growth characters, fresh-dry weights, shoot and root lengths of maize seedlings that their grains non-antioxidant soaked were significantly decreased by increasing NaCl salt levels throughout the experiment. The decrease in the growth parameters in maize seedlings exposed to NaCl salt stress is often associated with a reduction in photosynthetic pigments, and a decrease in chlorophyll content, and/or with the accumulation of Na^+ and Cl^- ions that prevents the metabolic activities in seedlings, particularly at high NaCl levels, it may work to increase ROS under salt-stress, causing a nutritional imbalance and desiccation of the tissues via lower water potential (Agami, 2014; Gul *et al.*, 2015 and Billah, *et al.,* 2017). Also, this decrease could be attributed to the osmotic effect caused by salt stress, rising in the growth inhibitors (i.e., abscisic acid), decreasing in the growth promoters [i.e, indole-3-acetic acid and gibberellins] (Rady *et al*., 2013 and Semida and Rady, 2014).

The decrease in both fresh and dry weights of the maize seedlings reveals the influence of water in motivating and regulating the photosynthetic enzymes and growth promoting hormones, which therefore influence dry matter production (Monti *et al.*, 2006). The reduction of photosynthesis and metabolic changes caused by NaCl salt may limit seedlings growth, and this was reflected in decreasing the dry weight.

Moreover, the reduction in shoot and root lengths in seedlings in response to NaCl salt stress may be due to either decrease in cell elongation resulting from the inhibiting effect of water shortage of metabolic processes, resulting from the existence of toxic Na^+ and Cl^- ions which, in turn, led to a reduction in each of cell turgor, cell volume and eventually cell growth (Banon *et al.*, 2006). Our results are in harmony with Metwali *et al.*, 2015; Rahman *et al*., 2017 and Yassin *et al.*, 2019 who observed that the growth and biomass of various plants declined by salt stress.

Application of antioxidants ASA, GSH, and α-TOC, as grains antioxidants soaked, either singly or in sequence, increased growth parameters, fresh and dry weights, shoot and root lengths of maize seedlings and the same parameters also increased in seedlings exposed to NaCl salt levels.

The increase in fresh weight of seedlings may be due to the capability of the seedlings to increase their water content, which in turn dissolves salt ions that have accumulated and causes the consequent rise in fresh weight (Munns, 2002). The promote effect of antioxidants on increasing shoot length and root length may be the result of increasing cell division and cell enlargement, or may induce osmotic adjustment activity, or may due to accumulation of non-toxic compatible solutes such as proline, soluble sugars in seedlings, leading to increase in water content and provide the turgor needed for cell expansion (Munns and Termaat, 1986). Antioxidants in sequence seemed to be more effective than application of the antioxidants alone, and this may be due to the synergistic effects of antioxidants on growth parameters. The antioxidants may able to catch the free radicals, or the active oxygen species produced in maize seedlings as an effect of NaCl salt stress. Our results are in harmony with Ejaz *et al.*, 2012 and Alhasnawi *et al.,* 2015 who found that treatment with AsA significantly enhanced the all studied growth parameters. Also with, Sakr and El-Metwally, 2009 and Orabi and Abdelhamid, 2016 who stated that treatment with α-TOC recorded significant increments in growth parameters. Moreover, treatment with GSH has been recommended to promote growth of plant and enhance stress tolerance by detoxifying stress induced-ROS (Cao *et al*., 2015 and Ding *et al*., 2016).

One of the routes of plant salt stress tolerance may depend on the amelioration of its antioxidant enzyme activities. So, we try to assess their impacts to cope with the oxidative stress, the responses of SOD, APX, GR, and CAT enzyme activities of maize seedlings exposed to NaCl salt stress with different concentrations refer that the

oxidative stress is an important component of salt stress and it must be studied how the maize seedling overcome it.

Data of the current study (Figures 5-8) showed that increased NaCl salt concentrations up to 100 mM led to a significant increasing in activity of the tested antioxidant enzymes in non-treated maize seedlings, compared to control seedlings (unstressed). The high concentrations (150, and 200 mM), showed a diminution in the seedlings enzyme activities. Also, results clarified that pre-soaking maize grains in $AsA_{0.50}$, $GSH_{0.50, and}$ α-Toc $_{0.50}$ individually or in sequence increased the activities of SOD, APX, CAT and GR compared to the salt stressed-control seedlings. On the other hand, the pre-soaking maize grains in a sequence of antioxidants showed a highly significant increase in activities of tested enzymes than those pre-soaked individually. These increased activities of antioxidant enzymes may contribute to give maize seedlings the ability to cope with the harmful influence of salinity stress, together with increasing of endogenous antioxidant compounds (Figures 10-12). The complex network of antioxidant enzymes and non-antioxidant metabolites works in a concerted and organized way to prevent overproduction of ROS (Asada, 2006).

NaCl salt stress may increase toxic compounds such as ROS that induce metabolic variations in the maize seedlings. SOD, APX, CAT and GR are responsible for ROS-scavenging (Sadak and Abdelhamid, 2015). The increasing in the preceding antioxidant enzymes activities, remove the hazard ROS from the maize seedlings. SOD is the first enzyme scavenges high ROS, particularly superoxide radical caused by NaCl stress thus the oxidative stress and damage of cells decreased, this leading to increase of maize seedlings tolerance to NaCl oxidative stress, hence, SOD is considered, as one of maize seedlings, defense mechanism against NaCl salt stress. APX is another one of maize seedlings defense; it is the primary enzyme of ASA-GSH cycle. The increased activity of APX of maize seedlings, grown either from antioxidants treated or non-treated grains, may utilize ASA, as a specific electron donor to reduce H_2O_2 produced by SOD to water and oxidized ascorbate (ASA), to monodehydroascorbate radical, as explained by Asada, 2006 and Caverzan *et al.,* 2012.

The increase in the CAT enzyme activities may decrease the H_2O_2 formed in maize seedlings as a result of NaCl level exposure and acquired their salt-resistance (Lopez-Huertas *et al.,* 2000). In

harmony with our results, rises in the activity of CAT, have been described in Chinta *et al*., 2001 and Sayed *et al.*, 2016 under salinity stress.

In case of GR, which is one of antioxidant enzymes that participates in ASA-GSH cycle, it is responsible for converting GSSG to GSH. Hence, in our experiment, the increasing of GR enzyme activity may increase GSH that acts as antioxidant involved in reducing ROS when the seedlings exposed to NaCl salt stress. Also, Noctor *et al*., 2012 and Ghosh and Biswas, 2017 both elucidate that GR plays a vital role in maintaining GSSG/GSH levels under stressed conditions.

The results are in harmony with Mekki *et al.,* 2015 who explained that SOD is the first defense agent against ROS as it is the chief scavenger of $O_2^{\bullet-}$, also with Aghaleh *et al*., 2014and Nahar *et al.,* 2015. Data explained that the application of $AsA_{0.50}$ - $GSH_{0.50}$- α-Toc $_{0.50}$ in a sequence is more effective than their single application and this may be referred to the integrative mode of actions of these antioxidants and to their synergistic function (Shao *et al.*, 2008). Data of our experiment were concomitant with results obtained by Li *et al.*, 2008 and Akladious and Abbas, 2013.

The decrease of antioxidant enzyme activities, resulting from the influence of high NaCl salt levels (150, and 200 Mm) may probably due to the inhibition of enzyme synthesis under salt-stress conditions. Application of α-TOC as pre-soaking of maize grains increased the antioxidant enzymes that giving maize seedlings their resistance to overcome the NaCl salt stress. The gained result may suggest that the rise in the activities of the antioxidant enzymes to be stimulated by α-TOC treatment, increasing their antioxidative role to quench and scavenge off the undesirable ROS caused by salinity levels. Reports exist that α-TOC is also an excellent quencher and scavenger of singlet oxygen by controlling the lifetime of ROS (Fahrenholtz, *et al.,* 1974), and it was confirmed by Ali *et al.,* 2020. In harmony with our findings, Sakr and El-Metwally, 2009 and Orabi and Abdehamid, 2016 recorded rises in the antioxidant enzymes in response to α-TOC application on wheat and faba bean plants against oxidative stress.

Similarly to our findings, Athar *et al*., 2009; and Billah *et al*., 2017 reported a rise in antioxidant enzyme activities in plants after an ASA application. Hasanuzzaman *et al*., 2011 and Zhou *et al.*, 2018 observed that enzymes activity increased after the application of GSH under the salinity.

Also, findings are in harmony with Özdemir *et al.* 2004 who reported that ASA, α-TOC, and GSH react directly or via enzyme catalysis with OH, H_2O_2, or O_2.

The data (Figure 9) indicated that NaCl salt with different levels, significantly suppressed the total proteins in seedlings of maize that their grains non-antioxidants soaked. These findings are compatible with El-Mashad and Mohamed, 2012 in that the total proteins of cowpea leaves were decreased with rising concentrations of NaCl in comparison with control plants, and confirmed with Ertani *et al.*, 2013 and Gul *et al.*, 2017 in maize.

The decrease in total protein content may be either due to effects of sodium chloride salt on lowering the synthesis of protein and denaturation of the enzymes participate in protein biosynthesis Surendar *et al.,* 2013 and Hemida *et al.,* 2014 or as a result of the increasing activity of protease enzymes to keep osmotic stress during exposure to NaCl levels (Parida *et al.,* 2002). The accumulation of Na^+ due to NaCl salt stress, may compete with K^+ binding in protein compounds, leading to inhibition of synthesis of protein and the metabolic enzymes (Pardo and Quintero, 2002 and Pandey *et al.*, 2016).

Furthermore, the higher concentrations of NaCl could affect synthesis of protein and stimulate its sharp decline (Caplan *et al.*, 1990). El-Mashad and Mohamed, 2012 showed that protein degradation in a saline environment might be due to the quicker proteolysis, reduction in the availability of amino acid and denaturation of enzymes involved in synthesis of protein. El-Zeiny *et al.*, 2007 and Orabi and Abdelhamid, 2016, stated that the decrease in protein content under salt stress of seawater might be due to the disturbance in nitrogen metabolism or inhibition of nitrate absorption.

Also, results in (Figure 9) showed that low NaCl salt treatments, up to 100 mM, significantly improved the total protein content in seedlings that their grains soaked in antioxidants either singly or in sequence, whereas, the higher salt levels (150, and 200) resulted in decreasing the protein content, despite of using antioxidants. The increment in total protein may involve in osmotic regulation in seedlings, this gives it an essential role in plant resistance to NaCl salts (Bartels and Sunkar, 2005), also proteins may use as a defensive strategy to scavenge Na^+ toxicity (Chen *et al.*, 2002 and Metwali *et al.*, 2015).

The sequential application of antioxidants, showed significantly effective in improving proteins of seedlings, better than the other single antioxidants application, under NaCl salt stress conditions. This is because the cooperation of antioxidants with each other against the effect of NaCl salt on maize seedlings. The use of antioxidants may enhance protein synthesis as a route to defend the maize seedlings against harmful effect of NaCl concentrations, some protein compounds that are specially responded to stress are induced in several plants normally or with application of antioxidants (Ekmekçi and Karaman, 2012 and Hemida *et al.,* 2014). Increasing protein content due to using α-TOC may be due to its roles in improving membrane permeability and increasing protein concentrations which protected membranes and membrane-bound enzymes. So α-TOC protected maize seedlings against salt toxicity and controlling the uptake of Na^+ and other toxic ions (Buschmann and Lichtenthaler, 1979).

Our results are in agreement with previous findings reported by Orabi and Abdelhamid, 2016, who found that foliar application of α-TOC at 50 or 100 mg L^{-1} exhibited opposite trends to salinity influences producing significant increments in protein content not only relative to corresponding stressed plant kinds but also to the non-treated plant kinds irrigated with tap water, (Rady *et al.*, 2011) stated that pretreatment of both sunflower seed cultivars with α-TOC (50 mg/l) induced significant rises in total protein content of sunflower plant under all salinity levels as compared with the corresponding salinity level. Cvetkovska *et al.*, 2005, and El-Bassiouny and Sadak, 2015, stated that ascorbic acid or α-TOC reacting directly or indirectly with ROS, so contribute to preserve the integrity of cell structure such as protein compounds, lipids and nucleic acids from impairment which promoted by salinity stress. Azooz *et al.*, 2013 report that presoaking in ASA increased content of protein of faba bean, These findings were supported with Abdel-Hafeez *et al.,* 2019 who stated that content of protein of sunflower significantly increased with ASA application.

A primary biological function of non-enzymatic antioxidant compounds is to overcome the oxidative stress. Under the no-stress conditions, plants induce antioxidants production to cope with any excess of ROS, which may generate due to the different physiological activities. Under the conditions, which promote oxidative stress, particularly salinity, endogenous antioxidants are produced in higher concentrations in order to avoid the bad influence of oxidative stress (Ozgur *et al.,* 2013).

The data of our experiment showed that endogenous ASA, GSH, and α-TOC contents were decreased by increasing salinity levels in maize seedlings which their grains, non-treated with antioxidants through the experimental period. This diminution of the antioxidants may because of their consumption to resist the bad effects of NaCl concentrations.

Salinity stress considerably declined content of ASA as aresult of extra production of ROS or by impaired biosynthesis or regeneration of ASA under the severe conditions of stress (Song *et al*., 2005).These results are compatible with Nazara *et al*., 2015 and Abdel-Hafeez *et al*., 2019 who revealed that the content of AsA decreased in wheat, mustard plant and leaves of sunflower compared with the non-stressed control. Also, Jaleel *et al.*, 2007 and Billah *et al*., 2017 reported that reduced glutathione content diminished significantly under stress of salt when compared with control.

It was evident from Figures (10-12) that grains soaking application with antioxidants $ASA_{0.50}$, $GSH_{0.50}$, and $α\text{-Toc}_{0.50}$ singly or in sequence, stimulated the accumulation of endogenous ascorbic acid (ASA), glutathione (GSH), and α-tocopherol (α-TOC), contents in salinized maize seedlings as compared with that of the un-salinized control. The increment in the preceding endogenous antioxidants in seedlings continue with increasing the NaCl salt level up to 100 mM, then they decreased at higher NaCl level (150, and 200 mM). Also, the sequential application of antioxidants, showed significantly effective in improving non enzymatic antioxidants contents better than the other single antioxidants application

The gained results may suggest that the increase in the endogenous α-TOC content in salinized maize seedlings, may be due to the increasing in GSH synthesis, or decreasing in its degradation, or may act to enhance the activities of antioxidant enzymes that have a role to quench the undesirable ROS caused by salinity levels. While, the increased contents of both ASA and GSH, may be because of its improved biosynthesis and their important pivotal role of the ascorbate-glutathione cycle, for scavenging ROS, that formed by the influence of NaCl salt stress. ASA, α-TOC, and GSH may react directly or via enzyme catalysis with superoxide, hydroxyl radicals and singlet oxygen and reduce H_2O_2 to water.

The explained results are matched with Khafagy *et al.*, 2009 who showed that many compounds are being used to cope with the toxic effects of salinity including ASA, GSH, and α-TOC. Shao *et al.*, 2008 stated that ASA protects metabolic processes against H_2O_2 and other toxic derivatives of oxygen affecting many enzyme activities, minimizes the damage caused by oxidative processes through synergistic function with other antioxidants, and stabilizes membranes. Noctor and Foyer, 1998 and Özdemir *et al.*, 2004 showed the role of enzymes APX, GR and SOD which involved in the regeneration of glutathione and ascorbate that are important in detoxification of ROS. The most recent studies on maize have shown that the elevated antioxidant levels can protect the photosynthetic apparatus from oxidative damage (Diao *et al.,* 2014).

Also, It was confirmed by Taha *et al,* 2018 and Ali *et al.,* 2020 that the positive impacts of α-TOC arose through its role in increasing osmotic tolerance and/or through regulating processes such as the up taking of nutrients from solution of the soil. Our results are in harmony with Semida *et al.*, 2016 who found that application of α-TOC under stress of salinity rised AsA and GSH contents. Also, exogenous treatment of ASA increased content of ASA as reported in Hameed *et al.,* 2015 and Abdel-Hafeez *et al.*, 2019, and improved content of GSH in maize as found in Billah *et al*., 2017. Moreover, exogenous GSH application increased the ASA content as found in Khattab, 2007 and in Nahar *et al.*, 2015 in mung bean seedlings also, it increased the content of endogenous GSH as documented in Khattab, 2007 ; Nahar *et al*., 2015 and Zhou *et al.*, 2018). At higher NaCl levels (150, and 200 mM), that decrease in the endogenous antioxidants concentrations may due to the severe effect of the salt on the metabolic processes of seedlings.

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