ROLE OF CHELATED ZINC (ZN-HEDTA) FOLIAR SPRAY IN REGULATION SALT STRESS IN MAIZE PLANTS

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Abstract. Salinity is an important constraining factor in the way of crop production around the world. Plant nutrition management could alleviate the harmful effects of salinity. In order to evaluate the interaction effects of Zinc availability and salinity, an experiment was conducted using Zinc HEDTA (20 µmol) and 100mmol of NaCl salinity with two cultivars (single cross -10 and single cross-162) of maize plants. Results implied that chlorophyll contents and carbonic anhydrase (CAA) which destructively affected by salinity were improved by Zn foliar spray. According to the results, antioxidant activity of peroxidase (POD) increased by the combination of salinity and Zn to a great extent compared to sole salinity. Our results indicated that the accumulation of 22 KD M.W of proteins was in response to salinity stress. The results showed that Zn (II)HEDTA induced the synthesis of new protein bands. According to the results obtained, it seems that Zn (II)HEDTA can be used as novel chelates to supply Zn and to increase tolerance to salt-stress of maize in salinized hydroponic nutrient solution.

Keywords: Salinity; Zn-HEDTA; Photosynthesis; carbonic anhydrase; peroxidase; maize; cultivars.

INTRODUCTION

Salinity causes numerous physiological and biochemical changes in plants like reduced leaf size, stem extension, root proliferation, reduced water use efficiency [12]. Alteration in metabolic activities [13], inhibition of enzymatic activities [10], ionic imbalance and disturbances in solute accumulation [11, 17] or a combination of all these factors. Zinc (Zn) is an essential trace element for plants, being involved in many enzymatic reactions and is necessary for their good growth and development. It is well known that zinc is an important component of many important enzymes, and is astructural stabilizer for proteins, and membrane and DNA-binding proteins (Zn-fingers). Zinc is known to have a stabilizing and protective effect on bio membranes against oxidative and peroxidative damage, and loss of plasma membrane integrity, as well as on membrane permeability alteration [32].

In salt affected areas, zinc application could alleviate Na^+ and Cl^- injury in plants and depressed Na^+ transport in plants grown in salinized solutions, with improvement in plant growth [2].

Zinc ions bind to ligands containing sulfur, nitrogen, and to a lesser extent oxygen, and preferentially bind to the membrane proteins [2]. Therefore, Zn may have a role in modulating free radicals and their related damaging effects by enhancing plants' antioxidant systems.

The present study focused on the influence of spraying Zn-HEDTA treatment on photosynthesis processes and "hydrogen peroxide-scavenging enzyme activity", in maize seedlings under salt stress. Also, to determine whether Zn is involved in regulation salt stress in maize plants.

MATERIALS AND METHODS

Maize cultivars

The seeds of maize cultivars named single cross -10 and single cross -162 were obtained from Agriculture Research Centre, Ministry of Agriculture, and Giza, Egypt. The seeds were surface sterilzed in 0.1% $HgCl_2$ solution for 3 min, washed thoroughly with distiled water before cultivation.

Plant culture and treatments

The experiment was carried out at Fertilization Technology Department, National Research Centre, Cairo, Egypt. Seeds of two cultivars of maize (single cross-10) and (single cross-162) were washed and soaked for several hours in aerated tap water. The germination was carried out in plastic dishes at 28 ºC in dark. Three days-old seedlings were put to grow in plastic pots filled with one-tenth concentration of [15] solution (pH 6.0). Treatments were (1) control(C): nutrient solution alone, (2) salt stress (S): 100 mM NaCl, (3) C + Zn-HEDTA 20µmol (4) NaCl + Zn-HEDTA. Plants were sprayed with N-{2-[Bis (carboxymethyl)amino]ethyl}-N-(2-hydroxyethyl)glycine (Zn –HEDTA) solution (50 ml/pot) once a week from day 10 after germination up to day 21. It was used to correct the nutrient imbalance caused by salt stress conditions. All chemicals used were of analytical reagent grade, and composition of nutrient solution was): 5 mM$Ca(NO_3)_2.4H_2O$, 5 mM KNO_3, 1 mM KH_2PO_4, 2 mM $MgSO_4.7H_2O$ and micronutrients in µM : H_3BO_3 -10, $MnCl_2$ -0.5, $ZnSO_4$ - 0.5, $CuSO_4$- 0.2, Na_2MoO_4 - 0.1, Fe (III)-HEDTA - 20. The seedlings were grown in an environmental chamber under 16h light at PPFD of 120 µmol m^{-2} sec^{-1} provided by fluorescent tubes, 16h light and 8h dark, 60% RH, at 25 ºC day /20 ºC night temperature. At 21 days old leaves were collected for different analysis.

Extraction and Estimation of Chlorophyll

One plant per replicate was used for chlorophyll determination. Prior to extraction, fresh leaf samples were cleaned with deionized water to remove any surface contamination. Chlorophyll extraction was carried out on fresh fully expanded leaf material; 1 g leaf sample was ground in 80% acetone using a pestle

and mortar. The absorbance was measured with a UV/Visible spectrophotometer (Pye Unicam SP6-550, UK) and chlorophyll concentrations were calculated using the equation proposed by [19].

Chl. *a* (mg/ gfw^{-1}) = 11.64 × (A663) − 2.16 × (A645)

Chl. *b* (mg /gfw^{-1}) = 20.97 × (A645) − 3.94 × (A663)

Total chlorophyll (mg/gfw^{-1}) = Chl.a+ Chl. b

where (A663) and (A645) represent absorbance values read at 663 and645 nm wavelengths, respectively.

Extraction of Cytosolic Fraction

A plant material (5g) was excised and homogenized in 10 ml of ice-cold grinding buffer containing 0.4 M sucrose and 25 mM Tris (pH 7.2). The homogenate was passed through 4 layers of cheat cloth and centrifuged at 12,000 × g for 15 min at 4°C. The resulting supernatant was used for determination of enzyme activities and protein contents.

Assay of peroxidase activity (EC 1.11.1.7)

Peroxidase activity (POD) was assayed by monitoring the increase in absorbance at 430 nm due to the oxidation of pyrogallol (ε= 2.6 mM^{-1} cm^{-1}) [3]. The reaction mixture consisted of 50 mM potassium phosphate buffer (pH 7.0), 20 mM pyrogallol, and 5mM H_2O_2 and 20 µl of enzyme extract. POD activity was expressed as EU/g f.w. One unit of enzyme was the amount necessary to decompose 1 µmol of substrate per minute at 25°C.

Assay of Carbonic anhydrase activity (EC 4.2.1.1)

Leaf tissue (100 mg FW) was placed into liquid nitrogen and homogenized with a buffered solution (pH 8.3) that contained 50 mM veronal-H_2SO_4, and 0.2% (w/v) PVP under ice cold-conditions. The homogenate was centrifuged at 12,000*g* for 2 min, and the supernatant was used for the determination of CA activity [25].

Protein extraction

SDS – PAGE analysis was done to identify the qualitative and quantitative differences of leaf protein from different treatments [27]. A known quantity of leaf sample was homogenized in phosphate buffer (pH 7.0) in a pre-chilled pestle and mortar and the homogenate were centrifuged at 10,000 rpm for min and the supernatant was used for analysis. The amount of total protein present in the different samples was quantified through the method of Bradford 2ml of binding dye 100 mg of Coomassie brilliant blue G 250 in 50 ml of 95% ethanol and then 100 ml of concentrated orthophosphoric acid and final volume made in to 200 ml by adding distilled water) was added in to 100 µl of each sample and blue colure developed within 30 minutes was read at 595 nm by using Spectrophotometer (Thermo Unicam UV 300).

Gel-electrophoresis of protein

The electrophoresis was carried out in 10% gel containing 1% SDS. The protein sample was cleaved in to polypeptides by adding equal volume of sample buffer (8 ml) containing 0.5M Tris HCl (pH 6.8)-mercaptoethanol (0.8M), glycerol (1.6 ml), 10% SDS (1.6 ml), 0.5% APS (0.4 ml) and water (2.6 ml) boiled for 3 and loaded about 100µl of protein. The electrophoresis was carried out at 180 volts in tank buffer containing 1% SDS for 4 hrs at room temperature. When the dye front reached near the edge of the glass plate, the current supply was discontinued and the separating gel portion of the gel was carefully taken out of the sandwich and stained in 50 ml staining solution overnight. The gel was distained with Coomassie destaining solution, until the background of the gel become colorless and the gel was photographed according to [18].

Statistical analysis

Data were statistically analyzed using Costat statistical package [4].

RESULTS

Effect of salinity on photosynthetic pigments content in presence or absence of Zn HEDTA foliar spray in leaves of two maize cultivars

The concentrations of photosynthetic pigment of maize cultivars grown in presence of NaCl 100mmol are shown in (Figure1a). In general, maize plants grown with NaCl showed a significant decrease in the amount of T-Chl contents when compared with maize plants grown in zero NaCl level. For example, T-Chl contents in single cross -10 and single cross -162 grown with 100 mmol NaCl were 0.970 and 0.625 mg/g^{-1} FW, respectively. Whereas these value was 2.75 and 2.325 mg g^{-1} FW, in plants grown without NaCl. Thus, chlorophyll degradation was dependent on salinity level. Foliar application of Zn -HEDTA foliar spray to both maize cultivars treated with 100NaCl led to significant increasing in the concentration of T-Chl, as compared with the values in plants treated with NaCl only. The T-Chl content was 1.89 and 1.23mg/g^{-1} FW in single cross -10 and single cross -162 respectively. While, in non-treated plants, these value was 0.970 and 0.625 mg g-1 FW. (Figure 1a). Thus, the level of photosynthetic pigment was found to be restored in treated maize plants with NaCl due to application of Zn HEDTA foliar spray. In general, the treated maize plants with Zn HEDTA were positive correlated with increasing of photosynthetic pigments restored in maize plants treated with NaCl. In other word, Zn HEDTA might improve the salt tolerance of single cross -10 plants by restoring the main photosynthetic pigments. The similar results were reported by [34], that chlorophylls in photosynthetic membranes could be protects the photosynthetic apparatus from excessive ROS by quenching of singlet oxygen and other radicals.

Effect of salinity on carbonic anhydrase activity in presence or absence of Zn-HEDTA foliar spray in leaves of two maize cultivars

The effect of NaCl on CAA for two cultivars of maize was presented in (Figure 1b). The photosynthetic carbon assimilation process in maize cultivars was found to be significantly depressed by 100 mmoL NaCl. Data presented in **(**Figure 1b**)** showed that Zn – HEDTA foliar spray stimulate the activities of CA in single cross -162 that were subjected to salinity stress while little response in the activities of CA in single cross -10 receiving both NaCl (100 mM) and Zn HEDTA.

Correlation coefficient of T-Chl and CAA in maize cultivars is given in (Figure 2a). Results showed that there is a linear and direct correlation between carbonic anhydrase activity and total chlorophyll of single cross -10 ($R2 = 0.9819$) Figure 2a, ($R2 = 0.7816$) single cross -162 cultivar (Figure 2b). The results indicated that the correlation of carbonic anhydrase and total chlorophyll was strong to moderate depending on the assay system used.

Effect of salinity on antioxidant enzyme activity (POD) in presence or absence of Zn HEDTA foliar spray in leaves of two maize cultivars

The change in antioxidant enzyme activity of POD enzyme activity in leaves of two maize cultivars was significantly ($P \leq 0.05$) affect by NaCl (Figure 1c) .The effect of salinity on activity of antioxidant enzyme (POD) was dependent on the enzyme type and plant cultivar. Data presented in (Figure 1c) showed that salinity led to a significant increase in POD activities in two cultivars of maize plants. The mechanisms that reduce oxidative stress are expected to play an important role in imparting tolerance in plants under saline conditions. The POD enzyme activity in single cross -10 and single cross-162 was increased in presence of 100 mmol NaCl level, with values of 4.02 and 4.14 U/g^{-1}FW , respectively. While, this value was 3.9 and 1.50 U/g^{-1}FW in plants grown in absence of NaCl. Treated NaCl 100mmol maize plant with Zn HEDTA had high POD activity with values 3.50 and 5.90 U/g^{-1}FW, respectively. Application of Zn HEDTA significantly increased ($P \leq 0.05$) the POD activity in both cultivars in presence or absence of NaCl.

The results of SDS-PAGE protein profile indicated that the differences in protein pattern were narrow among the two cultivars. While there was a qualitative and quantitative differences in polypeptide pattern noticed between salt sensitive and resistant cultivars under salinity conditions. The electrophoretically separated protein in NaCl-treated seedlings as compared with control revealed (i) quantitative decline in certain proteins, (II) rise in levels of other proteins, (III) some proteins remained unchanged, and(iv) induction of specific proteins. Proteins of molecular weights 72, 45, 34, 22and 15 kD in single cross -10; 45, 28, 24 and 15 kD in single cross -162 were detected by NaCl induced-stress. The results in the present study were confirmed by the earlier work that the proteins are specific to adaptation to salt stress [5]. Another 22 kDa protein is evident in salt tolerance single cross-162 under salinity stress while it was absent in salt sensitive single cross-10 cultivar. Application of Zn -HEDTA induced the synthesis and increased the intensity of the protein bands and caused the appearance of additional new bands. The results in (Figure 3) showed that Zn -HEDTA induced the synthesis of new protein bands and this might be attributed to the synthesis of polypeptides or derivative product of proteins due to the effect of hydrolytic enzymes on high molecular weight proteins.

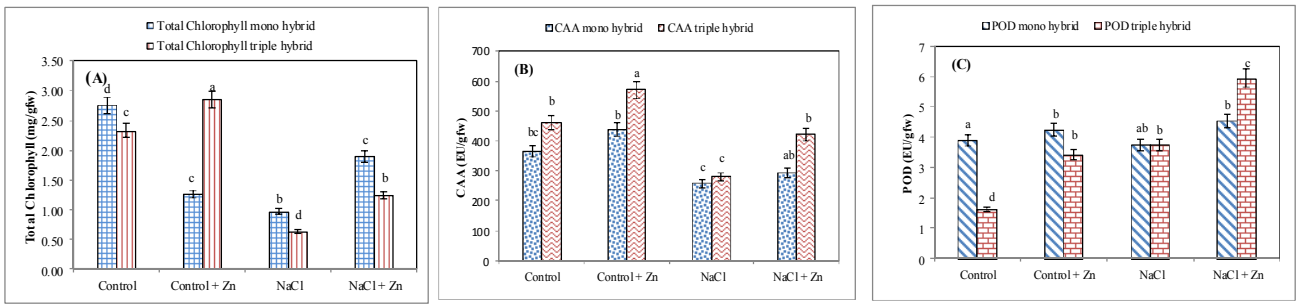

Figure 1. Effect of salinity 100 mmol NaCl on (A): total chlorophyll, (B): carbonic anhydrase activity (CAA) and (C): Peroxidases (POD) in leaves of two cultivars of maize single cross -10 and single cross-162. All values with the same letter are not significantly different at $p \leq 0.05$ Bars above the column are ± SD**.**

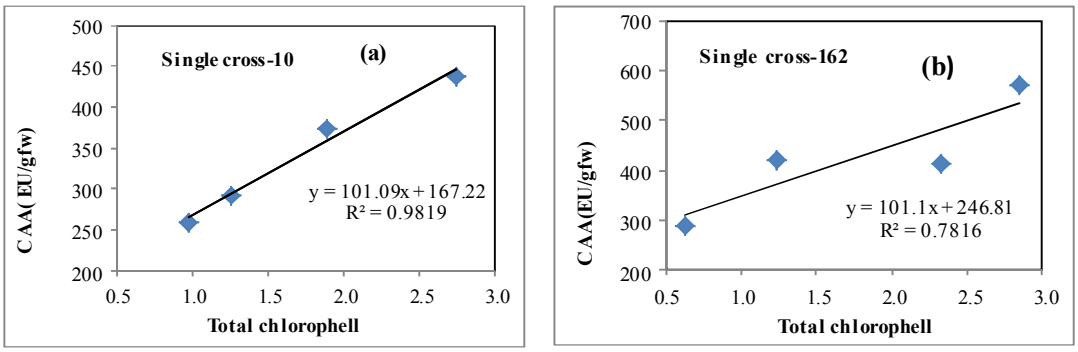

Figure 2. The relationship between carbonic anhydrase activity and total chlorophyll content in two maize cultivars single cross and triple cross

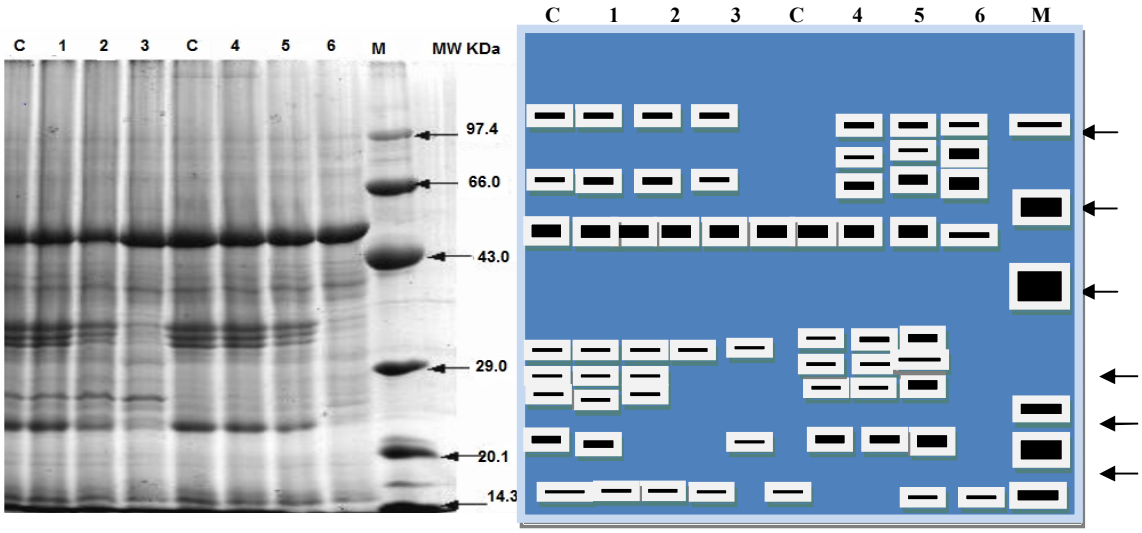

Figure 3. Effect of salinity on protein pattern in presence or absence of Zn HEDTA foliar spray in leaves of two maize cultivars. Control: control for single cross-10, Lane 1: C+ZnCh, Lane 2: NaCl, Lane 3: NaCl +Zn Ch, Control for single cross 162, Lane 4: C+ ZnCh, Lane 5: NaCl , Lane 6: NaCl + Zn-Ch.

DISCUSSION

The present study explore the role of Zn HEDTA foliar spray in regulation salt stress in maize plants. The decrease of photosynthesis ability under salt stress was due to stomata closure, inhibition of chlorophyll synthesis, a decrease of carboxylase due to high chlorophylase activity [6]. The changes in leaf chlorophyll content may have been due to reduce the biosynthesis or increased degradation of chlorophyll under saline conditions [1]. Furthermore, [22] suggested that in salt stressed plants, breakdown of ultra-structure of chloroplasts including plastid envelop, thylakoids and photosynthetic apparatus may result due to direct Na^+ toxicity or salt-induced oxidative damage. Adverse effect of salt stress on total chlorophyll was counteracted by Zn-HEDTA application. Zn HEDTA treatment increased the level of chlorophyll from (0.970 to 1.89 mg /gfw and from 0.625 to 1.235 mg/gfw) in single cross-10 and single cross-162 respectively, which is well supported by the earlier observations in wheat and/or mung bean seedlings under stress free conditions [23, 34].

The inhibition of CAA under salinity condition may be due to the sensitivity of CAA to chloride ions [20, 25]. Salt stress damaged the photosynthetic machinery at multiple levels, such as pigments, stomatal functioning and gaseous exchange, structure and function of thylakoid membrane, electron transport and enzymes [31]. Excess salt concentration cause the closure of stomata, decreasing the partial CO_2 pressure [7] as well as internal CO_2 concentration and consequently the activity of carbonic anhydrase was decreased. When NaCl exposure along with Zn foliar spray there was an additive effect on the inhibition of CAA. As has been found by [16] that Zn-HEDTA plays a key role in the activation of rubisco, PEP carboxylase and carbonic anhydrase under salt stress. The most appropriate to explain the (Zn-HEDTA) mediated elevation in the activity of CAA is that it corrects the stress mediated damage to the plasma membrane, as evident from an increase in the membrane stability [16]. Zn-HEDTA mediated elevation in CA activity may be related to increased membrane stability, because CA is present mainly in the cytosol and in the stoma of chloroplasts, so it is not a membrane protein, even if it can be associated to plasma membrane and to the thylakoid membranes. The results may rather be related to the role of Zn is stabilizing the catalytic site of CA through binding to histidine residues [9].

A consequence of salt-stress conditions is excess production of toxic oxygen derivatives. Under such conditions, plants possess defense systems for scavenging active oxygen species and protect cells from oxidative damages [8]. "hydrogen peroxide-scavenging enzyme activity", play a key role in the defense system of plant against oxidative stresses induced by salinity. A considerable increase in the peroxidase activity POD of single cross -10 and single cross 162 cultivars at 100 mmoL NaCl treatment. Over expression of the POD gene in plant has been reported to improve protection against oxidative stress [33]. These results are consistent with observations of many researchers who reported that POD activity plays a central protective roleduring salts tress [24]. These enzymes were also reported to be important in salt tolerance in mulberry [29, 30] and maize genotypes [24]. The activity of antioxidant enzymes has also been reported to increase under salinity in wheat shoot [21, 27] and pea [14]. A significant increase in the activity of POD was recorded in tolerant plant species, POD activity was found to be higher, enabling plants to protect themselves against the oxidative stress [28], whereas such activity was not observed in sensitive plants [26]. Our results are agreement with previously reported by [28] who found that the 250mM NaCl treatment resulted in two to four-fold increases above

the control level in the activity of POD, assuming that POD enables the plants to protect themselves against the oxidative stress.

The results of SDS-PAGE protein profile suggested that the appearance of new proteins under salinity stress may have a specific function to help the tolerance cultivar to alleviate the harmful effects of salinity. Also, based on the results of protein pattern with zinc chelates foliar spray it may be reasonable to assume that one of the multiple effects of zinc on stressed *Zea mays* plants is the *de novo* synthesis of a new proteins and the increased accumulation of certain existing proteins which may be involved in increasing the tolerance of maize plant [29]. Also, it has been found that Zn treatments induces a significant alterations in the enzymes related to protein metabolism this indicates that zinc might act as activators of protein synthesis [5].

Our results indicate that, Zn HEDTA foliar spray could be provide protection action against oxidative stress induce salt stress due to elevation "hydrogen peroxide-scavenging enzyme activity", which involved as mean one of the factor responsible for salt tolerance of maize plants and can be used as novel chelates to supply Zn and to increase tolerance to salt-stress of maize in salinized hydroponic nutrient solution.

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