Cereal Research Communications 42(4), pp. 568–577 (2014)
DOI: 10.1556/CRC.2014.0005
First published online June 15, 2014

Oxidative Stress of Maize Roots Caused by a Combination of both Salt Stress and Manganese Deprivation

H.Q. ZHAO[1,2]**, L. WANG[3]**, J. HONG[1]**, X.Y. ZHAO[1]**, X.H. YU[1]**, L. SHENG[1], C.Z. HANG[1], Y. ZHAO[1], A.A. LIN[1], W.H. SI[4]* and F.S. HONG[1]*

[1]Medical College of Soochow University, Suzhou 215123, China
[2]College of Life Sciences, Anhui Agriculture University, Hefei 230036, China
[3]Library of Soochow University, Suzhou 215021, China
[4]Suzhou Polytechnical College of Agriculture, Suzhou 215008, China

(Received 9 December 2013; Accepted 22 January 2014;
Communicated by A. Pécsváradi)

Salt stress impaired Mn imbalance and resulted in accumulation of ROS, and caused oxidative stress to plants. However, very little is known about the oxidative damage of maize roots caused by exposure to a combination of both salt stress and Mn deprivation. Thus the main aim of this study was to determine the effects of a combination of salt stress and Mn deprivation on antioxidative defense system in maize roots. Maize plants were cultivated in Hoagland's media. They were subjected to 80 mM NaCl administered in the Mn-present Hoagland's or Mn-deficient Hoagland's media for 14 days. The findings indicated that the growth and root activity of maize seedlings cultivated in a combination of both salt stress and Mn deprivation were significantly inhibited; the compatible solute accumulation, malondialdehyde, carbonyl, 8-OHdG, and ROS were higher than those of the individual salt stress or Mn deprivation as expected. Nevertheless, the antioxidative enzymes such as superoxide dismutase, ascorbate peroxidase, glutathione reductase, glutathione-S-transferase and antioxidants such as ascorbic acid, glutathione and thiol were lower than those of the individual salt stress or Mn deprivation. In view of the fact that salt stress impaired Mn nutrition of maize seedlings, the findings suggested that Mn deprivation at the cellular level may be a contributory factor to salt-induced oxidative stress and related oxidative damage of maize roots.

Keywords: salt stress, maize, manganese deprivation, reactive oxygen species, antioxidant defense system, roots

Introduction

Salinization plays a major role in soil degradation. Approximately 7% of the global land surface is subjected to salinization (Ruiz-Lozano et al. 1996). Out of 1.5 billion ha cultivated land, about 77 million ha (5%) are affected by excess salt content mainly induced by irrigation with ground water of high salt content (Munns et al. 1999). As for in China,

* Corresponding authors; E-mails: swh8614@163.com, Hongfsh_cn@sina.com; Phone: +86 512 61117563; Fax: +86 512 65880103
** These authors have contributed equally to this work.

where it was estimated that about 3,693 ha land was saline (Zhao and Li 1999), which makes the prevention and improvement of salinization in China more and more urgent with the great growing population and reducing cultivated fields. It is well known that crop production is low in saline soil, mainly due to salt toxicity to plants leading to a decrease in plant water-holding capacity, the imbalance of nutrient uptake, and toxicity of ions towards plant antioxidant capacity (van Hoorn et al. 2001; Mittler 2002; Ashraf 2009; Gong et al. 2010, 2011; Noreen et al. 2010; Qu et al. 2011, 2012).

Azevedo Neto et al. (2006) demonstrated that in salt-stressed maize roots of the salt-tolerant genotype, superoxide dismutase (SOD) and catalase (CAT) activities decreased and ascorbate peroxidase (APX), guaiacol peroxidase (GPX) and glutathione reductase (GR) activities remained unchanged in comparison with the control, but in maize roots of the salt-sensitive genotype, salinity reduced the activity of all studied enzymes. Exposed tomato to salt stress for 2 weeks was suggested to induce up-regulation of the activities of SOD, CAT, APX and levels of the antioxidants of ascorbic acid and reduced glutathione in the tomato roots (Shalata et al. 2001). Salt stress led to elevating APX activity in the leaves, GPX activity in the roots, and GR activity both in the leaves and in the roots of maize seedlings (Szalai and Janda 2009), respectively. Importantly, interactions between salinity and manganese (Mn) availability have previously been reported in many species. For example, salinity could reduce Mn concentration in various plants including maize (Izzo et al. 1991). Cramer et al. (1991) also indicated that salt stress resulted in reduction of Mn uptake and concentration in the maize shoot and inhibition of growth, and added Mn could improve growth and photosynthesis of barely under salt-stresses (Cramer and Nowak 1992). It is common knowledge that Mn is one of the essential trace elements for plants' normal growth. In the north area of China, where is the major base of farm products and marketable food, Mn deprivation resulting from salt stress has restricted food production and agriculture development there (Zhao and Li 1999). While the root is a crucial plant organ that contributes to yield because root activity determines the ability of the plant to acquire soil resources (Costa et al. 2000), the reduction of crop growth and yield following exposure to Mn deprivation under salt stress may be associated with oxidative stress of crop roots. A major consequence of salt stress is the imbalance between generation and detoxification of reactive oxygen species (ROS), including superoxide anion radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2), hydroxyl radicals ($^{\cdot}OH$) and singlet oxygen ($O_1^{\cdot-}$). As we know, oxidative stress may be the mechanistic link between Mn deficiency, antioxidant capacity since Mn is necessary for the antioxidative enzyme associated with Mn-superoxide dismutase (Mn-SOD). Thus, a plausible chain of events is: salt stress induces a Mn deficiency, which in turn reduces the reduction of SOD activity and growth of plants. The question was raised whether oxidative stress of a combination of both salt-stress and Mn deficiency occur in roots of maize seedlings is the same with the two individual stresses.

In the present study, mazie seedlings were continuously exposed to a combination of both salt-stress and Mn^{2+} deficiency 14 days. Alterations in ROS production, lipids, proteins and DNA peroxidation, activities of the antioxidant enzymes and anoxidant contents in the roots of maize seedlings were investigated to determine oxidative stress of roots caused by a combination of both salt stress and Mn deprivation.

Materials and Methods

Material treatment and culture

Seeds of *Zea mays* (L. cv.) were planted in a quartz sand-containing pot and placed in porcelain dishes, to which 4 L each of the following Hoagland's nutrient solutions were added: 1. Mn-containing Hoagland's nutrient solution (Control); 2. Mn-containing Hoagland's nutrient solution+80 mmol/L NaCl(NaCl); 3. Mn-deprived Hoagland's nutrient solution(Mn-); 4. Mn-deprived Hoagland's nutrient solution+80 mmol/L NaCl(NaCl Mn-). Mn-containing Hoagland's nutrient solution and Mn-deprived Hoagland's nutrient solution were prepared as described in ref (Arnon and Hoagland 1940). Plants were grown at 25°C/18°C using a 16/8 h light/dark cycle in a growth chamber under 500 μmol m^{-2} s^{-1} of mercury-high-pressure lights for 2 weeks. The relative humidity was 70%. Each treatment was replicated for five times. The nutrient solution was renewed twice each week.

Plant growth measurement

The fresh weights of shoot and roots/per plant were weighted at 14^{th} day.

Assay of TTC-reducing activity of roots

Each root sample was mixed thoroughly and a subsample taken (0.5 g fresh weight) for root reducing activity analysis. Root vigor was measured according to the triphenyltetrazolium chloride (TTC) method. The root activity was expressed by the amount of TPF (triphenyl formazan) deoxidized by TTC (Steponkus and Lanphear 1967). While the active root absorption area were determined by the methylene blue adsorption method (Zou 1995).

Assay of soluble sugar and praline of roots

The soluble sugar content was determined by the absorbance of UV-3010 spectrophotometer at 620 nm, according to Yemm and Willis (1954) using glucose as standard. Free proline accumulation was determined using the method of Bates (1973).

Assay of oxidative stress and antioxidant capacity of roots

Superoxide ion ($O_2^{\cdot-}$) in the maize roots was measured by monitoring the reduction of 3'-{1-[(phenylamino) carbonyl] -3, 4-tetrazolium}-bis (4-methoxy-6-nitro) benzenesulfonic acid hydrate (XTT) in the presence of $O_2^{\cdot-}$, as described by Able et al. (1998). The detection of hydrogen peroxide (H_2O_2) contents in the maize roots was carried out by the xylenol orange assay (Nourooz-Zadeh et al. 1994). Lipid peroxidation of roots was determined as the concentration of malondialdehyde (MDA) generated by the thiobarbituric acid (TBA) reaction as described by Buege and Aust (1978). Protein oxidation of roots was investigated according to the method of Fagan et al. (1999) by determining the protein carbonyl (PC) content. DNA of roots was extracted using DNeasy Plant Mini Kit (Nanjing Jiancheng Bioengineering Institute, Jiangsu, China) as described by the manufacturer.

Formation of 8-OHdG was determined using the 8-OHdG ELISA kit (8-OHdG ELISA; Japan Institute for the Control of Aging, Haruoka, Japan).

The roots were homogenized in 10 mL of ice-cold 50 mM sodium phosphate (pH 7.0) that contained 1% polyvinyl polypyrrolidone (PVPP). The homogenate was centrifuged (30,000 *g* for 30 min) and the supernatant was used for assays of activities of SOD, glutathione-S-transferase (GST), and ascorbic acid peroxidase (APx), and and glutathione reductase (GR).

The activity of SOD was assayed by monitoring its ability to inhibit the photochemical reduction of nitroblue tetrazolium (NBT) (Beauchamp and Fridovich 1971). GST activity was measured following the method of Habig and Jakoby (1981). APx activity was assayed using the method described by Reuveni et al. (1992). GR activity was assayed as described by Foyer and Halliwell (1976). Glutathione (GSH) and oxidized glutathione (GSSG) contents in roots were estimated using the method of Hissin and Hilf (1976). Ascorbic acid (AsA) and dehydroascorbic acid (DHA) determination was determined as described by Kampfenkel et al. (1995). The content of the proteins was determined following the Lowry et al. (1951) method. Thiol and disulfide contents in roots were determined by the procedure described by Shiau and Yeh (2004).

Statistical analysis

All results are expressed as means ± SD. The Kolmogorov–Smirnov test with Dunn's post test was used to compare control and treated groups using SPSS 19 software (SPSS, Inc., Chicago, IL, USA). A *P*-value <0.05 was considered as statistically significant.

Results

Plant growth and root activity

It can be observed in Fig. S1* that the fresh weights of shoot and roots in the salt stress group and Mn^{2+}-deficient stress group were significantly decreased, as compared to control ($P < 0.05$ or 0.01); and the inhibition of both shoot and root growth caused by combined stresses was more significant than the each individual stress ($P < 0.05$).

The changes of root activity are presented in Fig. S2. Active absorption areas and appreciable TTC-reducing activity of the salt stressed roots, Mn^{2+}-deficient stressed roots and combined stressed roots were decreased significantly compared to the control ($P < 0.05$), but among the three stress-treated roots, the reduction of the combined stressed roots was the greatest, then that of the Mn-deficient stressed roots, followed by that of the salt stressed roots.

Compatible solute accumulation

The effects of various stresses on the contents of soluble sugar and proline in the roots are exhibited in Fig. S3. It can be observed that the compatible solute contents in the salt

* Further details about the Electronic Supplementary Material (ESM) can be found at the end of the article.

stressed roots or Mn^{2+}-deficiency stressed roots increased significantly ($p < 0.05$ or 0.01), but that of the combined stresses was higher than those of the each individual stress ($p < 0.05$).

ROS and macromolecule peroxidation levels

The effects of various stresses on the production rates of $O_2^{\cdot-}$ and H_2O_2 in the stressed roots are shown in Table 1. Compared to the control, ROS generating rates of all the three stressed groups rose sharply and that of the combined stresses was higher than individual stress, indicating that exposure to a combination of both salt stress and Mn^{2+}-deficiency caused much more strong oxidative stress in the roots, as compared to salt stress or Mn^{2+}-deficiency. To demonstrate the effects of stressed groups and control group on ROS generation, the levels of lipid peroxidation (MDA), protein peroxidation (PC) and DNA damage (8-OHdg) in the roots were examined and shown in Table 1. The increases of MDA, carbonyl and 8-OHdG in the treated roots were also observed, and those of the combined stresses were most significant (Table 1), indicating that ROS accumulation led to lipid, protein, and DNA peroxidation in the roots under various stresses.

Table 1. Effects of salt stress and/or Mn^{2+}-deficiency on oxidative stress in roots of maize seedlings

Oxidative stress	Control	NaCl	Mn-	NaCl Mn-
$O_2^{\cdot-}$ (nmol/mg prot. min)	34.56 ± 1.728	69.88 ± 3.494***	118.8 ± 5.938***	149.6 ± 7.482***
H_2O_2 (nmol/mg prot. min)	58.29 ± 2.915	128.2 ± 6.408***	110.3 ± 5.516***	179.4 ± 8.969***
MDA (μmol/ mg prot)	6.85 ± 0.34	12.4 ± 0.62***	9.71 ± 0.49**	16.7 ± 0.83***
PC (μmol/mg prot)	1.41 ± 0.07	2.16 ± 0.11**	1.81 ± 0.09*	2.98 ± 0.15***
8-OHdG (mg/g roots)	2.69 ± 0.13	4.58 ± 0.23**	3.22 ± 0.16*	6.49 ± 0.32***

Control: Mn-containing Hoagland's nutrient solution; NaCl: Mn-containing Hoagland's nutrient solution+80 mmol/L NaCl; Mn-: Mn-deprived Hoagland's nutrient solution; NaCl Mn-: Mn-deprived Hoagland's nutrient solution+80 mmol/L NaCl. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Values represent means ± SD ($n = 5$).

Antioxidant defense

The activities of antioxidative enzymes, including SOD, APx, GR and GST, were examined (Table 2). These enzymes of seedling roots grown in three stresses were significantly decreased ($P < 0.05$), and those of the combined stresses were lower than the each individual stress. Among four enzymes, SOD decrease was greatest (Table 2) in the three stressed roots. To further explore the effects of various stresses on antioxidant capability, the redox states of GSH-GSSG, AsA-DAsA, and thiol-disulfide in the roots were examined and shown in Table 2. The decreases of GSH, AsA and thiol in the three stressed roots were also observed, and those of the combined stresses were most significant (Table 2). These results suggested that the combination of both salt stress and Mn^{2+}-deficiency decreased the capability of ROS removal in the roots more seriously than the each individual stress.

Table 2. Effects of salt stress and/or Mn^{2+}-deficiency on antioxidant capacity in roots of maize seedlings

Antioxidant capacity	Control	NaCl	Mn-	NaCl Mn-
SOD (Unit/mg prot. min)	59.55 ± 2.978	24.21 ± 1.211**	12.38 ± 0.619***	7.98 ± 0.41***
APx (Unit/mg prot. min)	70.46 ± 3.523	47.61 ± 2.381**	60.11 ± 3.016*	33.26 ± 1.665***
GR (Unit/mg prot. min)	62.57 ± 3.129	45.66 ± 2.283**	51.35 ± 2.568*	36.62 ± 1.831***
GS (Unit/mg prot. min)	37.96 ± 1.898	23.87 ± 1.195**	21.08 ± 1.054**	15.51 ± 0.776***
GSH/GSSG	8.16 ± 0.41	5.92 ± 0.31*	4.51 ± 0.25**	2.82 ± 0.14***
AsA/DHA	7.83 ± 0.39	5.08 ± 0.26*	3.58 ± 0.18**	2.24 ± 0.11***
Thiol/Disulfide	3.99 ± 0.21	2.18 ± 0.11**	1.92 ± 0.10**	1.88 ± 0.09***

Maize seedlings were treated as described in the legend to Table 1. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$. Values represent means ± SD ($n = 5$).

Discussion

Maize seedling growth was inhibited by salt stress, Mn^{2+}-deficiency and the combined stresses (Fig. S1), respectively, coupled with the decreases of active absorption areas and appreciable TTC-reducing activity of the roots (Fig. S2). However, among the three stress-treated seedlings, the reduction of plant growth (Fig. S1) and root activity (Fig. S2) in the combined stressed seedlings was the greatest, then that of the Mn-deficient stressed seedlings, followed by that of the salt stressed seedlings. The reduction caused by the combined stresses maybe related to severe oxidative stress of roots in maize seedlings.

Plants accumulate compatible solutes such as soluble sugar and proline to mitigate the damaging effects of salt stress. It had been demonstrated that increased levels of soluble sugar and proline accumulation in plant cells were associated with enhanced stress tolerance (Hasegawa et al. 2000; Ashraf and Foolad 2007; Gong et al. 2011). Our data suggested that the elevation of soluble sugar and proline accumulation in maize roots caused by exposure to a combination of saline and Mn^{2+}-deficiency was higher than those of individual salt stress or Mn^{2+} deficiency (Fig. S3), which was negatively consistent with plant growth and root activity (Figs S1, S2), suggesting that the concentrations of soluble sugar and proline in the roots are not high enough to adjust the osmotic potential in maize seedlings under stress conditions. In addition to their osmoprotective roles, compatible solutes may increase antioxidant defense mechanisms against stress damage (Hasegawa et al. 2000) and improve salt tolerance in plants (Khedr et al. 2003).

Oxidative stress, resulting from the generation of ROS, is a common phenomenon in many stress responses. Our data showed that the overproduction of ROS such as $O_2^{\cdot-}$, H_2O_2 occurred in three stressed maize roots, and that of maize roots by exposure to a combination of both salt stress and Mn^{2+}-deficiency was higher than the each individual stress (Table 1), implying that Mn^{2+} deprivation made more salinity-induced oxidative stress in the root cell, thus conducting to severe damages of roots.

As suggested by the present results, stress-induced overproduction of ROS in maize roots led to oxidative damages in biological macromolecules. Salt stress is known to result in extensive lipid peroxidation, which has often been used as an indicator of salt-induced oxidative membrane damages (Hernández and Almansa 2002). Our results showed that

MDA content significantly increased in the three stressed roots (Table 1). The level of carbonyl groups in proteins is widely used as a marker of oxidative protein damage (Stadtman and Levine 2003). Increased carbonyl formation has been shown in plant cells exposed to various oxidative stresses (Bartoli et al. 1999; Rodríguez-Serrano et al. 2006). In the present study, we observed an increased level of carbonyl content in three stressed roots (Table 1). As suggested by the present results, overproduction of ROS in cell cannot only lead to oxidative damages in lipid and protein, but also cause oxidative damage of DNA. DNA is oxidized to a number of damaged products. One of these products, 8-OHdG, has been suggested for a marker of oxidative damage in DNA *in vitro* and *in vivo* (Bidar et al. 2008). Accordingly, genomic alteration, 8-OHdG DNA adduct formation in the three stressed roots was significantly increased as compared to controls (Table 1). We can also observe that among the three stress-treated roots, the increases of MDA, carbonyl and 8-OHdG in the combined stressed roots was the greatest, then that of Mn-deficient stressed roots, followed by that of salt stressed roots (Table 1).

The production of oxidative stress in plant cell is because an unbalance between ROS over generation and their removal makes macromolecules and membranes damaged, resulting in the decrease of plant growth. The control plant possesses its own active antioxidant defense systems: antioxidative enzymes, such as SOD, APX (Asada 1999), GR (Crawford et al. 2000) and GST (John and Scandalios 1993; Noctor et al. 2002), as well as non-enzymatic antioxidants, including as AsA, GSH and thiol (Noctor and Foyer 1998; Bartoli et al. 1999; Shalata et al. 2001; Smirnoff 2005; Agrawal and Rathore 2007), through which overproduction and removal of ROS is in balance. It has been suggested that the reduction in the antioxidant system could increase protein carbonyl groups in pea plants exposed to cadmium or salt stress (Rodríguez-Serrano et al. 2006; Hoque et al. 2007a, b). Mn is reported to involve in the activation of many enzymes in plant systems, mostly in oxidation-reduction, decarboxylation and hydrolytic reactions hence may play a role in detoxification of ROS (Marschner 1995). Salt stress reduces the Mn uptake and induces many morphological and physiological disorders in plants. Our data suggested that Mn deficiency greatly inhibited the activities of SOD, APx, GR and GST, and decreased the ratios of GSH/GSSG, AsA/DHA, and thiol/disulfide under salt stress (Table 2), which were closely associated with increases in ROS, MDA, carbonyl and 8-OHdG (Table 1). It suggested that the combined stresses seriously impaired antioxidant defense systems of maize roots.

Exposed maize seedlings to a combination of both salt stress and Mn^{2+} deprivation caused an oxidative damage in cell monitored by an increase in ROS accumulation. The elevated levels of ROS, MDA, carbonyl and 8-OHdG in the maize roots following exposure to the combined stresses suggested an oxidative attack that was activated by reductions of SOD, APX GR and GST activities as well as AsA, GSH and thiol contents, leading to reductions of seedling weight and root activity.

Acknowledgements

This work was supported by A Project Funded by the Priority Academic Program Development of Jiangsu Higher Education Institutions and the National Natural Science Foundation of China (30470150, 20671067, 30800068).

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Electronic Supplementary Material (ESM)

Electronic Supplementary Material (ESM) associated with this article can be found at the website of CRC at http://www.akademiai.com/content/120427/

Electronic Supplementary *Figure S1*. Effects of salt stress and/or Mn^{2+}-deficiency on the fresh weights of shoot and roots of maize seedling

Electronic Supplementary *Figure S2*. Effects of salt stress and/or Mn^{2+}-deficiency on the roots activity of maize seedlings

Electronic Supplementary *Figure S3*. Effects of salt stress and/or Mn^{2+}-deficiency on the soluble sugar and proline accumulation in roots of maize seedlings