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ANTIOXIDANT ENZYMES (CuZnSOD, GSH-Px) ACTIVITY AND LIPID PEROXIDES CONCENTRATION IN NaCl-STRESSED TOMATO LEAVES

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Effects of salinity on activities of superoxide dismutase (CuZnSOD), glutathione peroxidase (GSH-Px) and concentration of lipid peroxides (TBARS) were studied in tomato (*Lycopersicon esculentum* Mill. cv. "Perkoz") leaves. Compared to control, salt-stressed plants showed the greatest increase in CuZnSOD activity 3 hours after treatment by 166% and 185%, respectively, for 50 mM and 150 mM NaCl. GSH-Px activity in the case of 50 mM solution, was highest (134% of control) at the same time as CuZnSOD. After this time a progressive decrease in the activity of GSH-Px was observed up to 66% of the control value 5 days after treatment. The 150 mM NaCl solution treatment caused a 187% increase in GSH-Px activity in comparison to the control plants 1 day after stress. On the 3th day of experiment it was only 65% of the control value. TBARS concentration in tissues of plants stressed with 50 mM NaCl solution was increased above control (25–37%) on 3 h to 5th day. The higher concentration of NaCl solution caused an increase in TBARS level from the first day after treatment. The highest value of this parameter (230% of control) was observed on the 3th day after stress, in the same time when GSH-Px activity was the lowest. These results suggest that under mild saline stress (50 mM), the elevated levels of the antioxidant enzymes protect the leaf cells against activated oxygen species. However, severe saline stress treatment produced in tomato leaf tissue potentiated lipid peroxidation derived due to elevated levels of oxygen species and/or inhibited GSH-Px activity.

INTRODUCTION

Saline environments can induce a wide number of responses in plants, ranging from readjustment of transport and metabolic processes to growth inhibition (Yu & Rengel, 1999). The osmotic effects resulting from soil salinity might cause disturbances in the water balance of the plant reducing turgor and photosynthesis. The presence of toxic ions such as Na^+ and Cl^- determines absorption of essential nutrients, and additionally Na^+ can be toxic by competing with K^+ in biochemical processes (White, 1999).

The common denominator of many types of environmental stresses, of NaCl stress too, is the increased formation of reactive oxygen species (ROS) (Chowdhury & Choudhuri, 1985; Hernandez, Corpas, Gomez, Del Rio & Sevilla, 1993; Chen, Lin & Kao, 2000; Price, Atherton & Hendry, 1989; Rabinowitch & Fridovich, 1983). Overproduction of ROS damages macromolecules of cells such as proteins, nucleic acids and lipids. In order to prevent excessive accumulation of ROS plant species developed enzymatic and non-enzymatic antioxidative mechanisms. Non-enzymatic factors include among others glutathione, ascorbinian (vit C), tocoferol (vit E), flavonoids, β-carotene and cysteine. Key enzymes involved in detoxification are superoxide dismutase (SOD EC.1.15.1.1.), catalase (CAT EC.1.11.1.6) and peroxidases including glutathione peroxidase (GSH-Px EC.1.11.1.9).

Although the physiological and whole-plant responses to NaCl stress have been studied, the mechanisms which confer NaCl tolerance on non-halophytic plants are still poorly understood. Superoxide dismutases are a group of enzymes that catalyze the disproportionation of $O_2^{\cdot}$ radicals to H_2O_2 and O_2, and play an important role in protecting cells against superoxide-derived oxidative damage (Scandalios, 1993). However, activity of these enzymes in NaCl-stressed plant tissue is not clear, some authors found its increase (Comba, Benavides & Tomaro, 1998; Manchandia, Banks, Gossett, Bellaire, Lucas & Millhollon, 1999), but others a decrease (Liang, 1999; Savoure, Thorin, Davey, Xue-Jun Hua, Mauro, Inze & Verbruggen, 1999).

GSH-Px as one of the key enzymes involved in scavenging oxyradicals in animals has recently been identified in plants. Glutathione peroxidase catalyzes the reduction of H_2O_2 and other organic

and lipid hydroperoxides by glutathione to water or alcohols, respectively (Eshdat, Holland, Faltin & Ben-Hayyim, 1997).

At the cellular level osmotic stress provides alterations in membrane lipid composition and properties. It has been postulated that at least part of the induced leakiness of membrane is caused by an uncontrolled increase in lipid peroxidation (Rodriquez-Rosales, Kerkeb, Bueno & Donaire, 1999).

The aim of this study was to determine the activities of CuZnSOD and GSH-Px, and concentration of lipid peroxidation products in cytosol fraction of tomato leaves from plants submitted to moderate and severe NaCl stress.

MATERIALS AND METHODS

Tomato plants (*Lycopersicon esculentum Mill*) cv „Perkoz" were grown from seeds in soil in a growth chamber under 16-h day and 8-h night cycles, with 350 μE m^{-2} s^{-2} light intensity, at 23°C. At the age of 6 weeks tomato plants were once treated with either 50 mM or 150 mM solutions of NaCl.

The second, third and the fourth leaves from the bottom were used for experiments 1 h, 3 h and 1, 3, 5 days after treatment. Leaves were harvested in the middle of the 16-h light period. Leaves were homogenized (1:5 w/v) in ice-cold mortar using 0.05 M phosphate buffer, pH 7.0, containing 1 mM EDTA. After centrifugation (10 000 $g \times 15$ min) the supernatant was used for determination of CuZnSOD and GSH-Px activities as well as TBARS content.

CuZnSOD activity was determined by the method of Minami and Yoshikawa (1979). The principle of this reaction is based on the measurement of the concentration of the reduced form of nitroblue tetrazolium at hte wavelength of 540 nm. The unit (corresponding to 50% inhibition) was established according to the definition of McCord and Fridovich (1969).

Lipid peroxides concentration as TBARS levels in the plasma was analyzed spectrophotometrically according to Yagi (1976) with 2-thiobarbituric acid. The concentrations of lipid peroxides were expressed in terms of TBARS with 1,1,3,3-tetraethoxypropane as standard, in μM.

GSH-Px activity was assayed according to the method of Hopkins and Tudhope (1973) using t-butyl hydroperoxide as a substrate. GSH-Px activity was expressed in units each corresponding to oxidation of 1 μmol NADPH per min per gram of protein under the assay condition used.

The obtained results represent the mean of six independent experiments. The significance of differences between mean values was determined by a non-parametric Mann-Whitney Rank Sum Test. Difference at $P < 0.05$ were considerate significant.

RESULTS

The obtained data show that already after one hour after treating tomato plants with 50 mM and 150 mM NaCl, CuZnSOD activity increased at both concentrations to 119% and 127%, respectively, as compared to control (Fig. 1). The greatest increase in this enzyme activity was observed in the 3rd hour after NaCl application when CuZnSOD activities were 166% at 50 mM and 185% at 150 mM of that of control. During the following days of the experiment CuZnSOD activity in leaves of tomato plants treated with the lower NaCl concentration came back to normal and was about 98 –111% of that of control while in those to which the higher dose was applied still exhibited an enhanced CuZnSOD activity as compared to control.

In plants watered with 50 mM NaCl GSH-Px activity, similarly to that of CuZnSOD, was increased already in the 1st hour of the experiment and maximum was observed in the 3rd hour (134% of that of control) (Fig. 2). Later a gradual decrease in the activity was observed being 111%, 105%, and 66% of that of control on the 1st, 2nd and 3rd days, respectively. In plants watered with 150 mM NaCl no significant changes in GSH-Px activity were observed up to the first day when an increase up to 87% above control was found. On the 3rd day a marked decrease in GSH-Px activity to 55% of that of control was observed and it persisted to the end of the experiment.

Lipid peroxides concentration in tomato leaves after treatment with 50 mM NaCl in the 1st hour dropped by 32% as compared to control while in the 3rd hour it was 137% of that of control which means an over 200% increase in relation to the level from the first hour (Fig. 3). During the following 3 days the value of this parameter did not change significantly and exceeded control by 25 –37%. In plants treated with the higher NaCl concentration TBARS level increased on the first day of the experiment (133% of that of control). Earlier measurements showed no changes while on the 3rd and 5th days their level was still increased reaching maximum (230% of that of control) on the 3rd day.

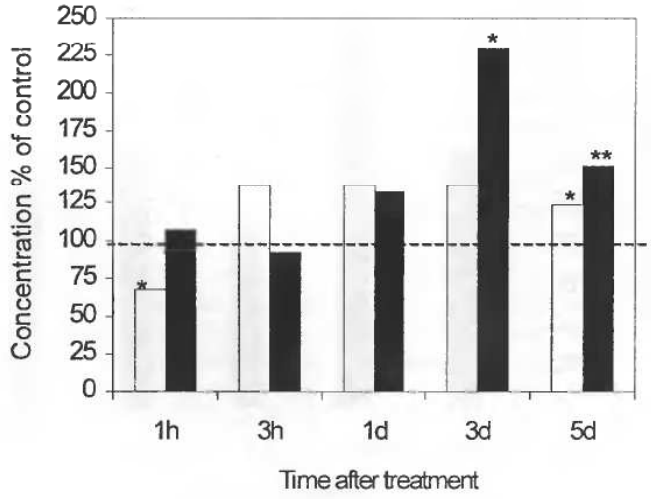

Fig. 1. Salinity stress effect on CuZnSOD activity in tomato leaf tissues; □ – 50 mM NaCl solution, ■ – 150 mM NaCl solution; *P < 0.05, **P < 0.01

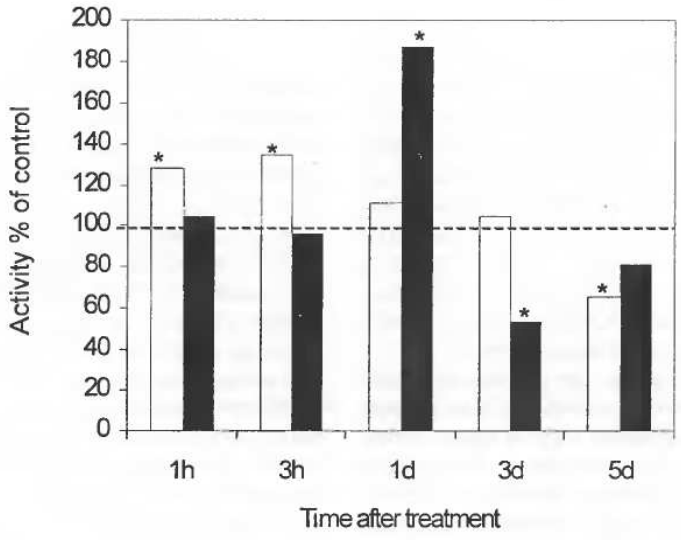

Fig. 2. Salinity stress effect on GSH-Px activity in tomato leaf tissues; □ – 50 mM NaCl solution, ■ – 150 mM NaCl solution; *P < 0.05

DISCUSSION

Numerous authors point out that salinity stress not only results in changes in cell metabolism connected with osmotic stress and toxic effect of Na^+ and Cl^- ions but also involves ROS activity especially reactions with O_2^* and H_2O_2 (Hernandez *et al.*, 1993; Singha & Chandhuri, 1990; White, 1999). It is suggested that these two compounds play an important role in secondary damaging mechanisms, connected with oxidative stress caused by salinity stress (Singha & Chandhuri, 1990). It has also been observed that species and cultivars of plants resistant to different abiotic stresses have an increased activity of antioxidative enzymes involved in detoxification of ROS among others SOD, CAT, and ascorbate peroxidase (APx E.C.1.11.1.11.) (Lin & Kao, 2000; Olmos, Hernandez, Sevilla & Hellin, 1994; Del Rio, Sevilla, Scandalio & Palma, 1991; Rabinowitch & Frido-

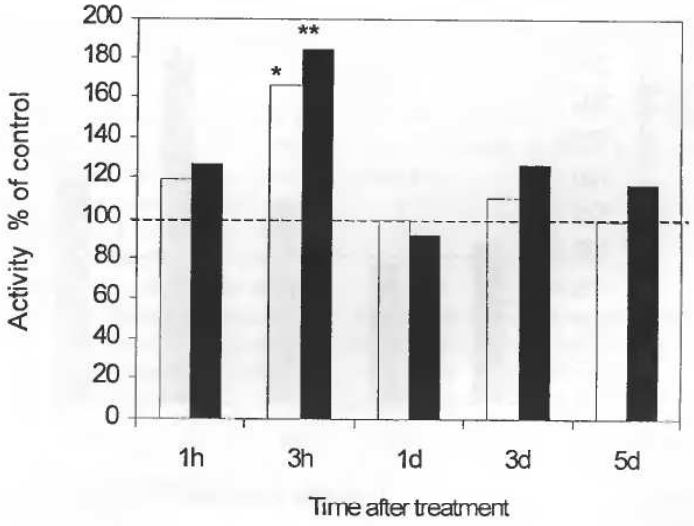

Fig. 3. Salinity stress effect on TBARS concentration; □ – 50 mM NaCl solution, ■ – 150 mM NaCl solution; *P < 0.05, **P < 0.01

vich, 1983). However, as far as SOD activity is concerned data are contradictory. Some authors (Liang, 1999; Sovoure *et al.*, 1999) observed a decrease in SOD activity in plant tissues resulting from salinity stress while others (Comba *et al.*, 1998; Manchandia *et al.*, 1999) an increase in this enzyme activity. However, papers in which authors described diverse reactions to this of different SOD isozymes to this kind of stress seem most interesting (Hernandez, Campillo, Jimenez, Alarcon, Sevilla, 1999; Yu & Rengel, 1999).

The data obtained in the present experiment show that CuZnSOD is an enzyme which not only takes part in antioxidative defense against oxidation caused by salinity stress but it is also one of the most quickly reacting elements of defense mechanisms since already 1 hour after stress application its activity rose and in the 3rd h after treatment with NaCl solutions (50 mM, 150 mM) it reached maximum. It was also observed that when stress was weaker (50 mM NaCl), CuZnSOD activity was at the same level as control already after 24 hours, which may suggest that after that period $O_2^{\cdot}$ concentration in the tested tissues was not above physiological level. However, contrary to these results, in plants treated with the higher NaCl concentration (150 mM), an enhanced CuZnSOD activity persisted, with the exception of the 1st day, to the end of the experiment i.e. till the 5th day, which might mean that peroxidation processes prevailed. Our results are in accordance with those presented by Yu and Rengel (1999) who reported an increased CuZnSOD activity (145%) while activity of others isozymes and total SOD activity remained unchanged in narrow-leafed lupins plants treated with 100 mM NaCl. Hernandez *et al.* (1999) have also showed that severe salinity stress (110–160 mM) results in a significant increase in both cytosol and chloroplasts CuZnSOD fractions activities as well as in total SOD activity in pea leaf tissues while 70 mM NaCl did not cause such changes. However, earlier experiments of Hernandez *et al.* (1993) on two pea cultivars,– sensitive and resistant to salinity stress, did not reveal CuZnSOD involvement in the defense system against ROS overproduction resulting from salinity stress. In resistant plants only MnSOD activity increased while in sensitive ones CuZnSOD activity decreased after treatment with 70 mM NaCl. An increase in CuZnSOD activity observed during the first hours of the experiment was followed by its decrease during the first day at both used concentrations may be related to synthesis of an appropriate H_2O_2 concentration, as this compound is a signaling molecule of an appearing stress (Hernandez-Ruiz, Rodriguez-Lopez, Garcia-Canovas, Acosta & Arnao, 2000).

Different degree of the salinity stress also caused varied changes in TBARS concentrations. Although Savoure *et al.* (1999) maintain that increase in their concentration is one of the first symptoms of oxidative stress, a significant increase was observed in tissues of plants submitted to severe salinity stress only after 3 days. It might

be connected with osmotic stress accompanying salinity stress as in experimental plants a decreased turgor potential was observed for prolonged time. In a tissue with a lowered amount of waters ROS-spread is limited so they can act only at the site of formation. Together with increase in hydratation of tissues ROS migrate more easily in a cell and react with other compounds which results among others in lipid peroxides formation (Halliwell & Gutteridge, 1988). This mechanism could be also supported by the fact that in plants treated with medium salinity stress TBARS concentration decreased in the 1st hour and then increased above control level in the 3rd hour. At high NaCl concentration the same kind of changes is observed later.

The TBARS concentration increase observed at the end of the experiment is in accordance with results presented by Liang *et al.* (1999). They observed a 3.5-fold increase in malondialdehyde (MDA) content in tissues of 10-day old seedlings of sensitive barley and 0.5-fold increase in tissues of resistant seedlings grown on the medium supplemented with 120 mM NaCl. Similar increase in MDA concentration in mitochondria isolated from leaves of sensitive and resistant pea cultivars was observed by Hernandez *et al.* (1993) who measured it on the 14th day after salinity stress (70 mM).

However, in both cases TBARS concentration might result from GSH-Px activity, which is an enzyme directly involved in detoxification of lipid peroxides. An enhanced level of this enzyme was observed before TBARS increased over control level. However, while in medium stress increased GSH-Px activity coincided with maximum TBARS level for these plants, in severe stress such a coincidence was not observed. In that case enzyme activity declined significantly below control level when TBARS concentration reached maximum.

The obtained results support Willekens, van Camp, van Montaqu, Langebartles & Sanderman (1994) observations that GSH-Px may play an important role in the fast response of cell cytosol fraction in tissues of cells exposed to oxidative stress. However, there is also evidence suggesting that during salinity stress plastid defense system is stimulated more intensively than cytosol antioxidative enzymes such as CuZnSOD, GSH-Px or APx. Souvre *et al.* (1999) observed enhanced GSH-Px activity in leaves of plants exposed to salinity stress while in roots of the same plants no such changes were noticed. These results may suggest that GSH-Px might play a significant role in photosynthetising tissues, which are at greater risk of ROS formation and lesions caused by them. Navari-Izzo, Izzo and Quartacci (1998) have put forward another hypothesis suggesting direct toxic effect of Na^+ and Cl^- ions which, according to the authors, accelerated senescence of membrane structures which is connected with intensification of lipid peroxidation. This is in accordance with phenotypic changes related to senescence observed, especially in plants under severe salinity stress at the end of the experiment. These symptoms include leaf-yellowing, tissue withering and falling off of older leaves. These plants formed buds and started blooming earlier as compared to those under medium stress and to control. Structural changes were also observed manifested as characteristic corrugation. However, in accordance with the results of Meneguzzo, Navari-Izzo and Izzo (1999) who have showed that cultivars resistant to salinity stress were characterized by lower Na^+ and Cl^- ion concentrations and did not show symptoms of premature senescence the process of accelerated senescence observed in this experiment may be not only due to ROS concentration increase but also to same passive defense mechanisms involving rejection of organs which accumulated large amounts of Na^+ and Cl^- ions in order to protect younger tissues.

A significant growth inhibition was also observed in plants watered with 150 mM NaCl while at lower NaCl concentration changes in growth were only slight as compared to control. It indicates that the degree of the stress is the main growth-inhibiting factor. It also influences changes in CuZnSOD and GSH-Px activities as well as in TBARS concentration. Contrary to severe salinity stress the lower NaCl concentration caused an earlier increase in the TBARS level but it never exceeded 137% of that of control, which together with no significant phenotypic changes seems to be a lesser danger for normal plant development after disappearance of salinity stress. The obtained results seem to indicate that antioxidative defense system connected with CuZnSOD and GSH-Px effectively against overproduction of toxic lipid peroxides during the first period after stress regardless of its degree protects cells. Later however, differences between two groups of plants can be observed depending on the degree of stress.

Full elucidation of variations in antioxidative process on the 3rd day of the experiment in both tested groups requires testing other compounds involved in cell antioxidative defense.

CONCLUSION

The obtained results indicate that regardless of the degree of stress single application of the stressing factor causes changes in pro-anti-oxidative system.

Among the tested enzymes glutathione peroxidase seems to be most sensitive to salinity stress. Its activity in both tested groups was lower than in control at the end of the experiment and might be a cause of increased TBARS concentration observed at the same time.

It seems probable that increased CuZnSOD activity in plants treated with medium and high NaCl concentrations indicates involvement of this isozyme in defense mechanisms against salinity stress-induced ROS.

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