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The oxidative stress and antioxidant systems in cucumber cells during acclimation to salinity

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Abstract

In the present study, we measured the markers of oxidative stress as well as activity of antioxidative enzymes and content of α-tocopherol in the acclimated and non-acclimated cucumber (*Cucumis sativus* L.) cell suspension cultures subjected to 150 and 200 mM NaCl. The content of carbonyl groups and lipid peroxidation were lower in the acclimated cultures than in the non-acclimated ones as well as their increases after NaCl treatments. Both NaCl concentrations enhanced activity of glutathione peroxidase in the examined cultures whereas activity of glutathione-S-transferase rose only in the acclimated ones. The increase in content of α-tocopherol induced by NaCl was more pronounced in the acclimated cultures. NaCl caused high decline in cell vigour in the non-acclimated cultures up to 80 - 90 % at the end of the experiment. The presented data suggest that the acclimated cultures coped with the salt stress better than the non-acclimated ones.

Additional key words: carbonyl groups, *Cucumis sativus,* glutathione peroxidase, glutathione-S-transferase, α-tocopherol.

Introduction

On great areas, agricultural productivity is severely affected by soil salinity. Excess of salts causes many disturbances, such as drastic changes in the osmotic potential and accumulation of deleterious ions leading to membrane disorganization and oxidative stress (Flowers and Yeo 1995, Hassegava *et al*. 2000). Nowadays, different strategies are employed to obtain NaCl-tolerant plants: plant selection, genetic transformation, and acclimation. Acclimation consists in a pre-treatment with low, nonlethal levels of NaCl which increases subsequent plant tolerance to higher NaCl concentrations (Strognov 1964). Pre-exposure of seedlings and callus or cell suspension cultures to NaCl at a particular growth stage elevates plant salinity tolerance. The acclimated plants tolerate salinity better and grow at NaCl concentrations that are lethal to non-acclimated ones (Amzallag *et al*. 1990). Enhanced salinity resulting from NaCl pre-treatment was observed in bell pepper (Bethke and Drew 1992), jojoba (Ben Raïs *et al*. 1993), maize (González-Rodríguez *et al*. 1997), rice (Hassanein 2000), soybean (Umezawa *et al*. 2000), and cowpea (Silveira *et al.* 2001). Acclimation of seeds, seedlings, and callus and cell suspension cultures is an easy, cheap, and low-risk technique compared with genetic transformation.

Osmotic and ionic stresses caused by salinity promote oxidative stress (Parida and Das 2005) due to significantly increasing the content of reactive oxygen species (ROS), such as singlet oxygen, superoxide anion, hydrogen peroxide, and hydroxyl radicals (Mittler 2002). Reduction of the elevated ROS content by a cellular antioxidant system is one of the common defense responses against abiotic stresses (Apel and Hirt 2004). ROS detoxification system consists of enzymes, such as superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), glutathione peroxidase (GSH-PX, EC 1.11.1.9), and ascorbate peroxidas (APX, EC 1.11.1.1), and non-enzymatic antioxidants, such as ascorbate (Asc), glutathione (GSH), and tocopherols. Another enzyme which can play an important role in oxidative stress resistance is glutathione *S*-transferase (GST, EC 2.5.1.18; Light *et al*. 2005). GST is a family of multifunctional enzymes that play critical roles in the detoxification of

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Abbreviations: APX - ascorbate peroxidase; Asc - ascorbic acid; BAP - 6-benzylaminopurine; CAT - catalase; CO - carbonyl groups; 2,4-D - 2,4-dichlo-rophenoxyacetic acid; GSH - glutathione reduced; GSH-PX - glutathione peroxidase; GST - glutathione-S-transferase; MS - Murashige and Skoog; ROS - reactive oxygen species; SA - salicylic acid; TBARS - thiobarbituric acid reactive substances; TTC - 2.3.5-triphenyl tetrazolium chloride.

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xenobiotics, but also protect the tissues against oxidative damage by quenching reactive molecules. Some plant GST have also been shown to function as glutathione peroxidases directly detoxifying the products of oxidative stress. GSH-PX, GST, and APX function in the presence of reductants, GSH and Asc, respectively. Moreover, these compounds can directly quench ROS (Noctor and Foyer 1998, Roxas *et al*. 2000). Tocopherols are main lipophilic antioxidants in a cell (DellaPenna and Pogson 2006). Their antioxidant activity is dual, they act as scavengers of lipid peroxy radical (thus inhibiting propagation of lipid peroxidation) and as singlet oxygen quenchers (Munne-Bosch and Alegre 2002).

Ineffective ROS scavenging leads to accumulation of damaged lipids, proteins, and DNA. Protein oxidation induced by ROS and/or byproducts of oxidative stress is defined as a covalent modification of a protein. Most of these modifications are permanent but some of them are reversible (Moller *et al.* 2007). Carbonylation is a commonly occurring oxidative protein modification. Because of the large number of amino acids that can be oxidized in this way, carbonylation of protein is often used as a marker of oxidative stress (Moller *et al.* 2007, Sweetlove and Moller 2009). Many studies have shown that salinity causes increased accumulation of lipid peroxides. The increased content of lipid peroxides indicates qualitative changes of the cell membrane lipids leading to disturbance of membrane integrity. Thiobarbituric acid reagent substances (TBARS) derived from polyunsaturated fatty acids hydroperoxides are commonly used as biomarkers for lipid peroxidation (Moller *et al*. 2007).

To study the adaptive mechanisms of the antioxidant defense system in plants growing under high salinity, we generated NaCl-adapted suspension cell cultures. The aim of this study was to clarify the effect of acclimation to salinity on antioxidant enzyme activities, lipid peroxidation, carbonyl groups, and α-tocopherol content in cucumber cells treated with moderate or severe NaCl stresses in order to understand the physiological and biochemical mechanisms of salt acclimation in cucumber cell cultures.

Materials and methods

Cucumber (*Cucumis sativus* L.) cv. Cezar cell suspension cultures were grown on Murashige and Skoog (1962; MS) medium with 1.3 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D) and 0.1 mg dm^{-3} benzylaminopurine (BAP) on a rotary shaker (120 rpm) at temperature of 23 °C, a 16-h photoperiod, and irradiance of 350 μmol m^{-2} s^{-1}. The cell suspension cultures were transferred to a fresh MS medium every two weeks. The culture was initiated by placing freshly cut sections from cucumber hypocotyls of one-week old sterilized seedlings. The callus was separated from the parent explant and transferred to the fresh medium of the same composition. After three months, a very friable callus was formed which was transferred to the liquid medium giving a fine suspension.

To determine the effects of NaCl acclimation on subsequent salt stress tolerance, the cucumber cell culture was grown on the MS medium with 20 mM NaCl for 30 weeks (AC20). The non-acclimated cell culture (NAC) was grown without NaCl. Both the types of cultures were passaged to a fresh MS medium every 14 d.

The AC20 and NAC cultures were treated with 150 mM NaCl (moderate salt stress) or 200 mM NaCl (severe salt stress) in the linear growth phase, *i.e.*, 7 d after passage to the fresh medium. The following variants were used: NAC (non-stressed), control for the non-acclimated cultures, NAC stressed with 150 mM NaCl (NAC-150), NAC stressed with 200 mM NaCl (NAC-200), AC20 (non-stressed), control for the acclimated cultures, AC20 stressed with 150 mM NaCl (AC20-150), and AC20 stressed with 200 mM NaCl (AC20-200). All biochemical parameters and cell vigour (2,3,5-triphenyl tetrazolium chloride, TTC reduction) were examined after 24, 48, and 72 h of NaCl treatment. Additionally, TTC reduction was examined in 1st and 3rd hour after imposing the stress.

The cells were separated from the MS medium using a Büchner funnel. Fresh cells (500 mg) were homogenized at 4 °C in 5 cm^3 of the following medium: 100 mM sodium phosphate buffer (pH 7.5), 1 mM ascorbic acid, 1 mM Na-EDTA, 0.5 M NaCl, 1 % (m/v) polyvinylpyrrolidone (PVP). The homogenate was centrifuged at 15 000 *g* and 4 °C for 20 min. The supernatant was used for determinations of APX, CAT, GST, GSH-PX, TBARS, and protein content. The crude extracts were used for α-tocopherol determination.

APX activity was measured as described by Nakano and Asada (1981). The activity was assayed following the oxidation of ascorbate to dehydroascorbate at 265 nm using a spectrophotometer (*Unicam UV 300*, Cambridge, UK). The 2 cm^3 of assay mixture contained 50 mM sodium phosphate buffer, pH 7.0, 0.25 mM ascorbic acid, 25 μM H_2O_2, and the enzyme extract, where hydrogen peroxide was used as a starter. Rates were corrected for the nonenzymatic oxidation of ascorbate by the inclusion of the reaction mixture without the enzyme extract. The activity was calculated using the coefficient of absorbance $\varepsilon = 13.7$ mM^{-1} cm^{-1}.

CAT activity was measured spectrophotometrically according to Dhindsa *et al*. (1981). The reaction mixture (2 cm^3) contained 50 mM sodium phosphate buffer, pH 7.0, 15 mM H_2O_2, and the enzyme extract. Decomposition of H_2O_2 was measured at 240 nm. The activity was calculated using $\varepsilon = 45.2$ mM^{-1} cm^{-1}.

GST activity was determined with 1-chloro-2,4-dinitrobenzene (CDNB) by the method of Habig *et al*. (1974). The reaction mixture (2 cm^3) contained 100 mM potassium phosphate buffer, pH 6.25, 0.75 mM CDNB,

30 mM GSH, and the enzyme extract. The activity was calculated from the increase in absorbance at 340 nm in 1 min due to CDNB-GSH conjugation ($\varepsilon = 9.6$ mM^{-1} cm^{-1}).

To detect GSH-PX activity, the method of Hopkins and Tudhope (1973) with *t*-butyl hydroperoxide as a substrate was used. The reaction mixture (1.6 cm^3) comprised 50 mM potassium phosphate buffer, pH 7.0, 2 mM Na-EDTA, 0.28 μM NADPH, 0.13 μM GSH, 0.16 U glutathione reductase, 0.073 μM *t*-butyl hydroperoxide, and the enzyme extract. Oxidation of NADPH was recorded at 340 nm for 6 min and the activity was calculated using $\varepsilon = 6.22$ mM^{-1} cm^{-1}.

Content of lipid peroxides was estimated spectrofluorometrically (model *F-2500*, *Hitachi*, Tokyo, Japan) according to Yagi (1982) by measuring the content of TBARS. The content of lipid peroxides was calculated in terms of 1,1,3,3-tetraethoxypropane which was used as a standard.

Carbonyl group (CO) content was estimated following the method of Levine *et al*. (1990). Cells were homogenized in 0.05 M potassium phosphate buffer (pH 7.5), containing 1 mM Na-EDTA, 2 mM dithiothretiol, 0.2 % (v/v) *Triton X-100*, and 1 mM phenylmethane sulphonyl fluoride (PMSF). The homogenate was centrifuged at 20 000 *g* for 20 min and the supernatant was used for the determination. A sample containing at least 0.5 mg of proteins was incubated with 1 % (m/v) streptomycin sulphate for 20 min to remove the nucleic acids. After centrifuging at 2 000 *g* for 15 min, the supernatant (0.2 cm^3) was mixed with 0.3 cm^3 of 10 mM 2,4-dinitrophenylhydrazine in 2 M HCl. The blank was incubated in 2 M HCl. After 1 h incubation at room temperature, proteins were precipitated with 10 % (m/v) trichloroacetic acid. Pellets were washed 3 times with ethanol:ethylacetate (1:1). Finally, the pellets were dissolved in 6 M guanidine hydrochloride in 20 mM potassium phosphate buffer (pH 2.3) at 37 °C. Absorbance was measured at 370 nm. Protein recovery was estimated for each sample by measuring the absorption at 280 nm. Carbonyl group content was calculated using the molar absorption coefficient for aliphatic hydrazone ($\varepsilon = 22$ mM^{-1} cm^{-1}).

Tocopherol content was assayed according to the method described by Taylor *et al*. (1976). After saponification of a sample with 10 N KOH in the presence of 1.42 M ascorbic acid, tocopherol was extracted to *n*-hexane. Fluorescence of the organic layer was measured at 280 nm (excitation) and 310 nm (emission) wavelengths. The α-tocopherol was used as a standard.

Protein content was measured as described by Bradford (1976) using bovine serum albumin as a standard.

The cell suspension culture vigour was determined using 2.3.5-triphenyl tetrazolium chloride (TTC) reduction method of De Block and De Brouwer (2002). Fresh cells (200 mg) were transferred to 50-cm^3 Falcon tubes and washed with 50 mM K-phosphate buffer, pH 7.4. Then the tubes were filled with the same reaction buffer containing 10 mM TTC. For the blank, no TTC was added. The cells were submerged but not vacuum infiltrated. The tubes were incubated upside down in the dark at 25 °C for 1 h. After that, the cell cultures were washed with distilled water and frozen at -70 °C for 30 min. After thawing, 50 cm^3 of technical ethanol was added. The reduced TTC-H was extracted by shaking probe for 1 - 1.5 h. The absorbance of the extract was measured at 485 nm.

All measurements were performed in duplicate with four (CO, proteins, and TCC reduction), six (enzyme activities), and eight (TBARS and α-tocopherol) independent samples. The significance of differences between means was determined by the Student *t*-test. Differences at $P < 0.05$ were considered as significant.

Results

The constitutive TBARS content in the control AC20 culture was significantly lower (about 40 %) than in the NAC one (Fig. 1*A*). Both NaCl concentrations led to significantly increased TBARS content in NAC and AC20 cultures. The moderate salt stress caused exposure-time dependent growth of the TBARS content in NAC-150 (by about 120 – 190 %). In NAC-200, content of TBARS increased by 220, 133 and 314 % after 24, 48 and 72 h, respectively. In AC20-150, the moderate stress caused growth of the TBARS content by about 90 % after 24 and 48 h. The maximum TBARS content in the AC20-150 culture was reached after 72 h and was higher by 150 % than in AC20. The content of TBARS in AC20-200 depended on time of exposure to stress factor. After 24 h TBARS concentration was higher than in AC20 by 150 % whereas after 48 and 72 h by 257 and 373 %, respectively (Fig. 1*A*).

Throughout the experiment, the constitutive content of CO was higher by 70 % in NAC than in AC20 (Fig. 1*B*). CO content significantly increased under 150 and 200 mM NaCl stress in all cell cultures. In NAC-150 culture greater NaCl stress triggered more pronounced changes in CO level, in NAC-150 it was 27 and 15 % above the control level after 48 and 72 h, respectively, while in NAC-200 it was 20 % above the control after 24 h and 44 - 46 % after 48 and 72 h. In AC20-150 and AC20-200 carbonyl groups content markedly increased mainly 72 h after stress, by 70 and 57 % than in AC20, respectively.

Both the NaCl concentrations caused a significant decrease in catalase activity in both types of cell cultures (Fig. 2*A*). In NAC-150, the CAT activity was lower by about 40 - 60 % after 48 and 72 h. Similarly in NAC-200, the CAT activity was reduced by 45 and 37 % after 48 and 72 h, respectively. In AC20-150 and AC20-200, the activities of this enzyme significantly decreased below the respective control by 24 and 18 or 32 and 23 % after 48 and 72 h, respectively.

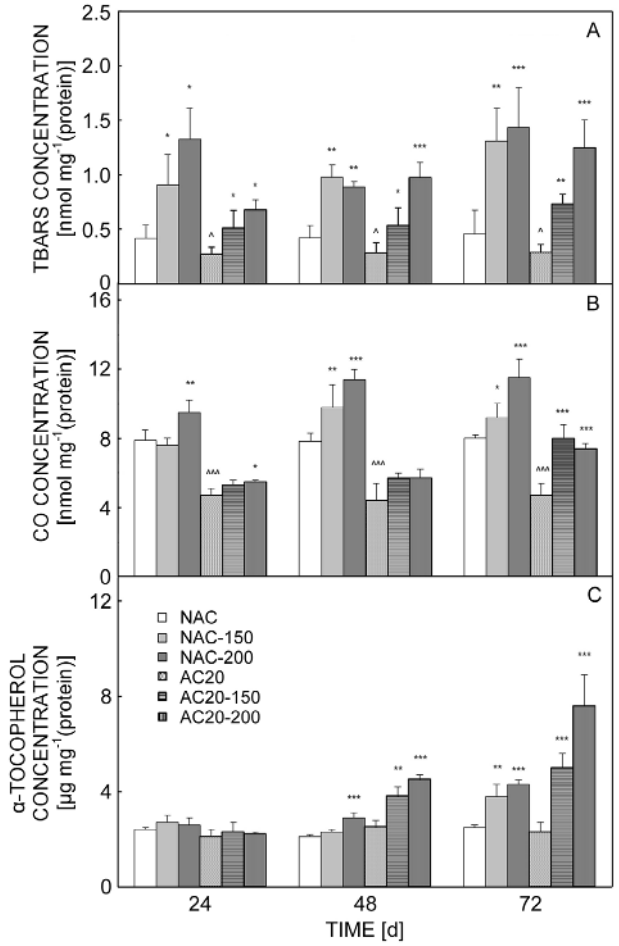

Fig. 1. Changes in content of TBARS (*A*), carbonyl groups (CO; *B*), and α-tocopherol (*C*) in the non-acclimated (NAC) and acclimated (AC20) cell suspension cultures in response to 150 and 200 mM NaCl. Means ± SD, $n = 8$; *, **, *** indicate values that differ significantly ($P < 0.05$, 0.01, and 0.001, respectively), from respective controls. ^, ^^^ indicate values that differ significantly ($P < 0.05$ and 0.001) between NAC and AC20.

Constitutive activity of APX was similar in NAC and AC20 cultures (Fig. 2*B*). Both types of salt stress caused a significant decrease in APX activity, in NAC-150 and NAC-200 by 30 and 50 %, respectively, after 48 h. The activity of APX in AC20-150 and AC20-200 cultures remained at the similar level.

GSH-PX constitutive activity was similar in the both types of cultures (Fig. 2*C*). In NAC-150, a significant increase (by about 50 %) in the GSH-PX activity after 48 and 72 h was observed. In NAC-200, this activity was much higher (111 and 144 %) after 48 and 72 h, respectively. In AC20-150, the activity of the GSH-PX decreased by about 30 % after 48 and 72 h, whereas in AC20-200, the respective values were higher by 36 and 54 % than those of the AC20.

In NAC, both NaCl concentrations did not cause significant changes in *S*-glutathione transferase activity (Fig. 2*D*), however, in AC-20, increases were observed, in AC20-150 by 40 - 50 % throughout the experiment and in AC20-200 by 36 % after 72 h.

The NaCl treatment significantly increased the α-tocopherol content in the all cultures (Fig. 1*C*). In NAC-150, an increase of the α-tocopherol content was observed only after 72 h (by 50 % above the control) whereas in NAC-200, it was higher by 38 and 72 % after 48 and 72 h, respectively. More pronounced effects were observed in AC20-150 where 52 and 117 % increases above the control level were noticed after 48 and 72 h, respectively. In AC20-200, the respective values were higher by 80 and 200 %.

Both NaCl concentrations caused declines in the TTC reduction, the marker of cell vigour (Fig. 3*A*,*B*). A stronger gradual decrease was observed in the NAC cultures starting from the first hour up to the end of experiments, in NAC-150 by 60 % below the control between the 1st and 3rd hour and by 85 % at the end of the experiment. Similarly, in NAC-200, the TTC reduction decreased by 50 % in the first hour and 90 % after 72 h. In contrast, the acclimated cultures were less sensitive. In AC20-150 and AC20-200, decreases by about 30 - 40 % below the control value were noted after 24 h of the NaCl stress.

Discussion

Numerous authors point out that salt stress induces prooxidative processes leading to disturbances in metabolism, such as modifications of lipids, proteins, or nucleic acids and simultaneously elevation of antioxidant enzyme activities (Hernandez *et al*. 1993, Moller *et al*. 2007). Formation of lipid peroxides and their accumulation have been shown in response to low, moderate, and severe salt stresses (Light *et al*. 2005, Stępień and Kłobus 2005) as well as in response to other abiotic stress factors, *e.g*., excess of cadmium, nickel, and copper (Verma *et al*. 2008, Gajewska and Skłodowska 2010, Shekhawat *et al*. 2010). Parallel to these observations, we found that TBARS content was enhanced in both the types of cell suspension cultures in response to moderate and severe NaCl stresses. An increase in lipid peroxidation may result from elevated ROS generation and/or decreased scavenging capacity of antioxidants. In our study, the applied conditions promoted both these processes because the enzymes involved in H_2O_2 degradation, CAT and APX, exhibited lower activities starting from 48 h after stress, whereas increases in TBARS and CO content continued. The decreased APX and CAT activities in the NAC cultures suggest that H_2O_2 accumulated in cytosol and other cellular compartments, whereas in the AC20 cultures the decreased CAT activity without any change in the APX activity might indicate great involvement of peroxisomes in peroxidation reactions. The observed decline of the CAT activity is in accord with other results

showing its dramatic decrease during the stress. Such a situation was observed, *e.g*., in wheat, rice, and cucumber leaves in response to 80 to 120 mM NaCl (Shim *et al*. 2003) or in rice roots treated with 50 to 150 mM NaCl (Tsai *et al*. 2004). Decreases of CAT activity due to increasing Cd concentration from 5 to 200 μM in calluses and seedlings of *Brassica juncea* were also noticed by Shekhawat *et al*. (2010). A decline of CAT activity may be due to its inactivation by salicylic acid (SA; Willekens *et al*. 1995). An increase in SA content in NaCl treated

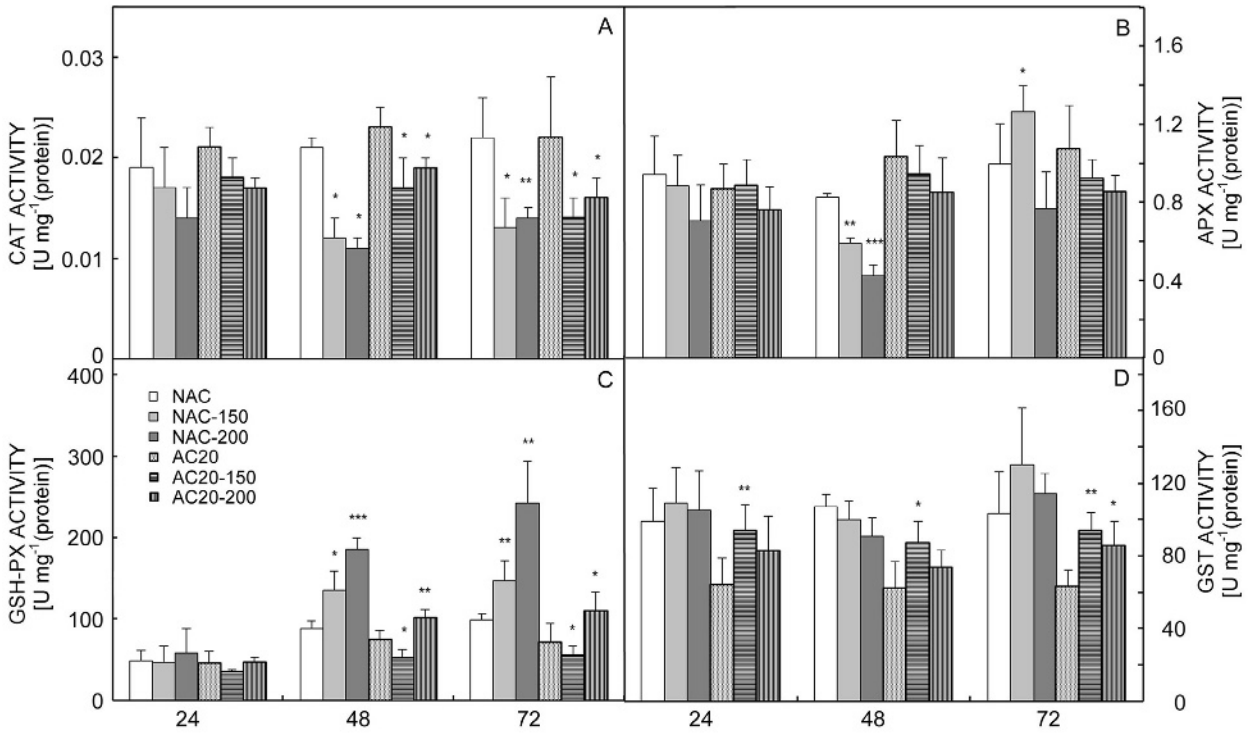

Fig. 2 Changes in catalase (CAT; *A*), ascorbate peroxidase (APX; *B*), glutathione peroxidase (GSH-PX; *C*), and glutathione S-transferase (GST; *D*) activities in in non-acclimated (NAC) and acclimated cell (AC20) suspension cultures in response to 150 and 200 mM NaCl. Means ± SD, $n = 6$; *, **, *** indicate values that differ significantly ($P < 0.05$, 0.01, and 0.001, respectively) from respective controls.

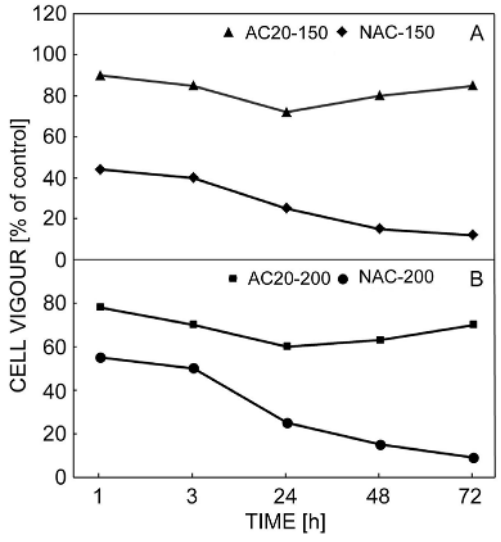

Fig. 3 Changes in cell vigour (TCC reduction) in the non-acclimated (NAC) and acclimated (AC20) cell suspension cultures in response to 150 (*A*) and 200 (*B*) mM NaCl ($n = 4$).

rice leaves was found by Shim *et al*. (2003). Another reason for a CAT activity decrease may be its carbonylation (Sweetlove and Moller 2009). Nguyen and Donaldson (2005) observed correlation between the protein carbonylation in oxidative stress conditions and loss of CAT activity in peroxisomes.

Based on our results for GST and GSH-PX activities, a hypothesis may be put forward that acclimated cell cultures can cope better with stress-induced disturbances.

Glutathione-dependent enzymatic antioxidants GSH-PX and GST are directly involved in limitation of TBARS content (Eshdat *et al*. 1997). In our study, the activities of CAT, APX, and of the above enzymes changed simultaneously (48 h) but differently depending on the culture type. In NAC, only GSH-PX exhibited the increased activity regardless of the NaCl concentration, whereas in AC20, the situation was more complicated. Under the moderate stress, complementary action of GSH-PX and GST was observed, manifested by simultaneous increases/decreases in the GST and GSH-PX activities, respectively. Under the severe stress, the content of both the enzymes grew, however, the GSH-PX

activity was lower than in NAC and it was accompanied with the increase in the GST activity.

Gosset *et al*. (1996) indicated that GST activity is involved in alleviation of salt stress in a cotton callus. Moreover, Roxas *et al*. (1997, 2000) showed that over-expression of GSH-PX/GST in tobacco transgenic seedlings provide enhanced capacity to scavenge ROS and lead to enhanced seed germination and growth under salt stress. These seedlings were characterized by reduced amount of lipid peroxides and higher metabolic activity after stress exposure (Roxas *et al*. 2000). No changes in GST activity observed in the NAC-150 and NAC-200 cultures may have resulted from the inhibitory effect of GSH-conjugates on this enzyme activity (Marrs 1996) or from the stress-induced decrease in *mRNA GST* level in the cytosol as well as from silencing the activated defence response (Roxas *et al*. 2000). Different character of changes of the glutathione-dependent enzyme activities in the acclimated and non-acclimated cultures under the stress conditions might indicate that GSH was prefe-rentially targeted to GSH-PX mainly in the NAC-150, NAC-200, and AC20-200 cultures whereas GST in the AC20-150 one. Additionally, these different defence reactions involving glutathione could be connected with the protein glutathionylation process (Dixon *et al*. 1998) or with the direct reaction of glutathione with ROS (Noctor *et al*. 2002). The increase in α-tocopherol content in both the types of cell suspension cultures did not cause a decrease in the TBARS content in spite of the fact that the principal role of tocopherol is to scavenge lipid peroxy radicals which are responsible for lipid peroxi-dation propagation (Munne-Bosch 2005). The ineffective reduction in lipid peroxy radicals (by α-tocopherol) in both the types of examined cultures might be connected with an ineffective process of its regeneration at the early phase of the experiment which depends on the reduced form of ascorbate and glutathione. An increase in the α-tocopherol content around the second day mainly in the acclimated cultures and the third in the non-acclimated ones under both the NaCl concentrations may indicate that in the suspension cell cultures, this compound was synthesized *de novo* at an early phase of the NaCl stress. Brem *et al*. (2004) showed that during *in vitro* study, α-tocopherol achieved a plateau in its antioxidant activity after 90 min which seems to prove the above suggestion. The late growth of α-tocopherol content in tomato chloroplasts under NaCl stress was observed by Skłodowska *et al*. (2008). Generally, an increase in α-tocopherol is associated with stress tolerance and a decrease with stress susceptibility (Munne-Bosch and Alegre 2002). Growth of α-tocopherol content in salt stressed plant was observed by Gosset *et al*. (1994). They showed that cotton cultivars with 3- to 4-fold higher content of α-tocopherol tolerate this stress better than those with lower content of this compound. Earlier appearance of the higher α-tocopherol content in both the stressed AC20 cultures than in NAC ones observed by us may indicate a more efficient process of its synthesis and its participation not only in membrane stabilization mechanisms. These mechanisms may be connected with higher vigour of the acclimated cells under the stress conditions. The values of TTC index demonstrate that about 24 h after the stress, the decrease in vigour of cells in both the types of cultures occurred, however, the acclimated cultures regained their vigour, whereas the non-acclimated did not. It seems possible that significant changes in metabolism trigged the repair mechanisms which were effective only in the acclimated cultures.

Protein oxidation, besides lipid peroxidation, is widespread and often used as a diagnostic marker of oxidative stress (Stadtman and Levine 2003). The increase in protein CO formation correlated with enhanced ROS has been shown in response to 200 mM NaCl in strawberry leaves (Tanou *et al*. 2009) and in a tobacco cell suspension culture (BY-2) (Hoque *et al*. 2007). Both types of examined cultures, NAC and AC20, show the increased CO content in response to the NaCl stress, however, the CO content in the non-acclimated cultures was increased throughout when in the acclimated ones only after 72 h. A hypothesis can be put forward that the later increase in CO in AC-20 resulted from adaptive processes evoked by their pre-treatment with a low dose of NaCl. It may have resulted from effective degradation of oxidized proteins started by acclimation, whereas in the NAC cultures, this process could be activated only after the stress appearance. It is well established that oxidatively modified proteins are preferred substrates for proteolytic degradation (Moller *et al*. 2007) and increase of protease activities in leaves of cashew in response to salt stress was found (Gomes Silveira *et al*. 2003).

The lower constitutive content of CO and TBARS accompanied by lack of changes in the constitutive amounts of the examined antioxidants in the AC20 cultures in comparison with the NAC ones suggests a modification of oxygen metabolism in both types of cultures, or existence of other mechanisms triggered by the acclimation influencing the oxidative reactions.

The data obtained for glutathione-dependent enzymes indicate their crucial role in the limitation of oxidative processes. The AC20 cell cultures coped with the NaCl stress better than NAC which was reflected by less pronounced changes in the TBARS and CO levels, the increased activities of GSH-PX and GST and in the α-tocopherol content, as well as by higher vigour of the former.

Summing up, the acclimated cultures tolerated the salt stress conditions better than the non-acclimated ones but the range of this tolerance is associated with stress severity or NaCl concentration used for acclimation.

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