Differential responses of the antioxidant defence system and ultrastructure in a salt-adapted potato cell line

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A B S T R A C T

Changes in lipid peroxidation and ion content and the possible involvement of the antioxidant system in salt tolerance at the cellular level was studied in a potato (*Solanum tuberosum* L.) callus line grown on 150 mM NaCl (salt-adapted) and in a non-adapted line exposed to 150 mM NaCl (salt-stressed). Salinity reduced the growth rate and increased lipid peroxidation in salt-stressed line, which remained unaltered in the adapted line. Na^+ and Cl^- content increased due to salinity in both lines, but the adapted line displayed greater K^+/Na^+ ratio than the stressed one. Total superoxide dismutase (SOD, EC 1.15.1.1), ascorbate peroxidase (APX, EC 1.11.1.11), and glutathione reductase (GR, EC 1.6.4.2) activities decreased in both salt-exposed lines; catalase (CAT, EC 1.11.1.6) activity did not change in the adapted line, but decreased in the stressed cell line. Salinity caused the suppression of one GR isoform, while the isozyme patterns of SOD, APX, and CAT were not affected. Ascorbate and reduced glutathione increased in both salt-exposed calli lines. α-Tocopherol increased as a result of salt exposure, with higher levels found in adapted calli. Electron microscopy showed that neither the structural integrity of the cells nor membrane structure were affected by salinity, but plastids from adapted cells had higher starch content. The results suggest that the enzymic and non-enzymic components of the antioxidant system are differentially modulated by salt. Different concentrations of antioxidant metabolites are more relevant to the adaptive response to salinity in potato calli than the differences in activity of the antioxidant enzymes.

1. Introduction

Salinity is a major limitation of agricultural production worldwide. It is estimated that more than 6% of the arable land worldwide is affected by salinity and this is expected to increase rapidly in the next decades [1]. Salinity induces several alterations at the cell and whole-plant levels. High salt concentrations in the soil alter the osmotic water balance and increase the cellular concentration of Na^+ and Cl^-, leading to ion toxicity, nutritional imbalances, membrane disorganization, and disturbances on general metabolism, including the inhibition of photosynthesis [2]. At the whole-plant level, the most pronounced effect of high salt concentrations is growth inhibition and, eventually, plant death [3]. Adaptation of the plant cell to high salinity involves osmotic adjustment and compartmentalization of toxic ions and the scavenging of reactive oxygen species (ROS) by the antioxidant defence system [4,5].

Increased levels of ROS contribute to salt damage in plant cells [6,7]. Under normal conditions the production and destruction of ROS is well regulated in cell metabolism. However, disruption of the homeostasis under saline conditions results in the accumulation of ROS, and subsequent changes in normal cellular metabolism through oxidative damage to membranes, proteins, and nucleic acids [5].

The cell antioxidant system helps in the protection against the cytotoxic effects of ROS. Ascorbate (AsA) and reduced glutathione (GSH) appear to be the most important antioxidant metabolites,

Abbreviations: APX, ascorbate peroxidase; AsA, ascorbate; CAT, catalase; DHA, dehydroascorbate; DHAR, dehydroascorbate reductase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; H_2O_2, hydrogen peroxide; MDA, malondialdehyde; NADPH, reduced nicotinamide adenine dinucleotide phosphate; $^1O_2$, singlet oxygen; $O_2^{\cdot-}$, superoxide; $OH^{\cdot}$, hydroxyl radical; PAGE, polyacrylamide gel electrophoresis; ROS, reactive oxygen species; RGR, relative growth rate; SOD, superoxide dismutase.

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reacting directly with different ROS ($^1O_2$, $OH^{\bullet}$ and O_2^{-}) and participating in the ascorbate–glutathione cycle [8]. α-Tocopherol is also involved in antioxidative defence, cooperating with AsA and GSH to protect membranes from lipid peroxidation [9]. Additionally, the osmolyte proline has been reported to be involved in scavenging free radicals and in protecting enzymes against abiotic stresses [10]. The antioxidant metabolites act together with antioxidant enzymes, whose activities can be used to evaluate stress responses in plants [6,11]. Superoxide dismutases (SODs) are the first line of defence against ROS as it catalyzes the dismutation of O_2^{-} into H_2O_2 and molecular oxygen. H_2O_2 is scavenged by catalase (CAT) in peroxisomes, while in cytosol and chloroplasts the task is performed by the ascorbate–glutathione cycle [5].

Potato (*Solanum tuberosum* L.), the fourth most important food crop worldwide, has been classified as moderately salt-sensitive to moderately salt-tolerant [12]. However, NaCl at 50 mM is sufficient to reduce growth and to decrease tuber yield by about 50% and completely inhibits growth at 150 mM [13]. Moreover, the physiological and biochemical parameters that are negatively affected by NaCl [14] suggest that the salt sensitivity of potato plant is similar to that of glycophytic crops. Tissue culture techniques have been used in the selection of salt-tolerant cell lines and subsequent regeneration of whole plants with improved salt tolerance [15,16]. Ochatt et al. [15] have described the *in vitro* production of a salt-tolerant potato cell line, which was regenerated into whole plants, but no information was given about the mechanisms involved in salt adaptation either in the selected cell line or in the regenerated plants. Subsequent studies on these salt-tolerant potato plants revealed a relationship between salt tolerance and the antioxidant defence system, suggesting that the induction of antioxidant defence is one component of the tolerance mechanisms to NaCl exposure [17]. Despite the large body of literature on the effects of salinity on antioxidant activity, the characterization of antioxidant defence system in salt-tolerant callus tissue of potato remains unavailable. In particular, the partial contribution of antioxidant enzymes and antioxidant metabolites to the physiological adaptation remains unclear.

Two distinct calli cultures, non-adapted and adapted to 150 mM of NaCl, previously obtained from leaf explants of *S. tuberosum* L. [18], have been thoroughly characterized in the present study with the objective of clarifying the role of the enzymatic and non-enzymatic components of the antioxidant defence system in relation to acute salt stress and to salt tolerance of potato calli. Ultrastructural changes induced by salinity with a putative adaptive value for potato callus tissue are also reported.

2. Results

2.1. Relative growth rate and ion content

The physiological effect of NaCl can be documented by the growth rate and ion content of salt-adapted and salt-stressed cell lines (Table 1). Despite the ability of the salt-adapted calli to grow in the presence of 150 mM NaCl, the relative growth rate (RGR) was reduced by 64% in relation to the control. When control tissue was subjected to shock treatment with salt, i.e., subcultured directly onto the medium containing 150 mM of NaCl, it suffered a severe growth reduction (77% compared to control). No significant differences in relation to control tissue were observed when adapted calli were cultivated on NaCl-free medium for 28 d.

Na^+ and Cl^- increased in both adapted and stressed calli but accumulated to higher levels in the former (Table 2). Na^+ and Cl^- contents were also affected in the salt-adapted line grown on medium without salt, but the values were significantly higher compared to the control line. In contrast, K^+ and Ca^{2+} concentrations decreased in all situations under study when compared to control line. The lower level of these ions occurred in stressed tissue, which had the lowest K^+/Na^+ ratio (Table 2).

Table 1
Relative growth rate (RGR), MDA and proline levels in control, salt-adapted, salt-stressed callus tissue and salt-adapted callus after transfer to medium without NaCl for one subculture. Values are mean ± SE ($n = 10$ for RGR, $n = 4$ for proline and MDA). Values in the same column followed by different letters are significantly different at $P < 0.05$.

Culture conditions	RGR (%)	MDA (nmol g^{-1} DW)	Proline (mg g^{-1} DW)
Control calli	232.22 ± 8.17[a]	171.19 ± 10.36[a]	4.19 ± 0.30[a]
Salt-adapted calli	82.71 ± 4.48[b]	162.60 ± 10.30[a]	39.54 ± 1.35[b]
Salt-stressed calli	53.84 ± 8.04[c]	182.88 ± 7.91[b]	41.97 ± 0.47[b]
Salt-adapted calli → Control condition	254.33 ± 11.39[a]	164.79 ± 3.83[a]	8.63 ± 0.24[c]

2.2. Lipid peroxidation and proline content

Lipid peroxidation was assessed by measuring the accumulation of MDA to verify the salt-induced oxidative stress. The highest lipid peroxidation was detected in callus tissue subject to shock treatment with salt, i.e., control tissue subcultured directly onto the 150 mM NaCl medium (Table 1) No differences in lipid peroxidation in relation to the control were observed in NaCl-adapted callus tissue or upon its transference to a salt-free medium for 28 d (Table 1).

All treatments induced an increase in proline content in relation to the control line (Table 1). Salt-adapted and salt-stressed calli accumulated about 10 times more proline than the control, and remained twice as high as control levels upon the transfer of adapted calli to medium containing no salt.

2.3. Activity of antioxidant enzymes

Salinity significantly affected total SOD activity in potato callus tissue (Table 3). SOD activity was lower in adapted calli in relation to control, and even more reduced in stressed calli. The transfer of the salt-adapted line to a medium without salt lead to a 13% increase in SOD activity, although its levels remained lower than those of the control line. The analysis of SOD polymorphism in native gels showed that salinity did not change the isozyme pattern; however, densitometric analysis of the zymograms revealed lower activity in the salt-adapted callus tissue (SA) than that in the control (C), in agreement with the data on total activity (Fig. 1A). SOD activity staining in gel revealed four bands, designated 1 to 4 from the least electronegative band (SOD-1) to the most electronegative band (SOD-4), with the same electrophoretic mobility in both control and adapted calli. In both gels, the upper band (SOD-1) was faint and SOD-2 appeared as the dominant form. Inhibition studies showed that all the bands are co-migration of two SOD types; bands 1, 3 and 4 consist of Cu/Zn-SOD and Fe-SOD, and band 2 contains Cu/Zn- and Mn-SOD isoforms (Fig. 1A). Comparison of the zymograms suggests that lower SOD activity in salt-adapted cell line was due to the decrease of SOD-2, SOD-3 and SOD-4 (Fig. 1A). The same SOD isoform pattern was observed when the adapted calli was transferred to medium without salt (SA → C) as well as on gels from salt-stressed cell line (SS), although SOD isoforms from the stressed line appeared with lower staining intensity when compared with those observed in control calli gel, due to lower activity (Fig. 1A).

NaCl in the culture medium did not alter CAT activity of adapted calli as compared with control levels (Table 3). CAT activity decreased when control tissue was subcultured directly onto

Table 2
Ion content and K^+/Na^+ ratio in control, salt-adapted, salt-stressed callus tissue and salt-adapted callus after transfer to medium without NaCl for one subculture. Values are mean ± SE from three independent experiments. Values in the same column followed by different letters are significantly different at $P < 0.05$.

Culture conditions	Ion content (mg g^{-1} DW)				K^+/Na^+
	Na^+	K^+	Ca^{2+}	Cl^-	
Control calli	6.87 ± 0.24[a]	60.25 ± 2.14[a]	4.12 ± 0.18[a]	18.69 ± 0.10[a]	8.81[a]
Salt-adapted calli	111.25 ± 2.16[b]	47.02 ± 1.32[b]	1.85 ± 0.06[b]	169.53 ± 6.47[b]	0.43[b]
Salt-stressed calli	90.83 ± 2.20[c]	30.25 ± 0.48[c]	1.60 ± 0.06[c]	112.40 ± 12.34[c]	0.33[c]
Salt-adapted calli → control condition	31.67 ± 0.44[d]	48.17 ± 1.17[b]	3.23 ± 0.13[d]	55.23 ± 1.93[d]	1.52[d]

150 mM NaCl. In contrast, CAT activity of adapted calli transferred to a salt-free medium was similar to that measured when cells were grown continuously in 150 mM NaCl. Only one CAT isoform was detected in the gels from the tissue subjected to all treatments (Fig. 1B). Protein gel blot analysis revealed that the CAT isoform present in potato calli corresponds to CAT2, since the antibody against CAT2 recognized a single band whereas no bands were observed on the blots with the anti-CAT1 antibody (data not shown). Densitometric analysis of activity gels revealed no significant differences in the band intensity of CAT2 of control and adapted calli, even upon the transfer of adapted cells to salt-free control conditions. CAT2 appeared with lower staining intensity only in stressed callus. On the other hand, densitometric analysis of the blots showed that the amount of CAT2 in the salt-adapted line was significantly lower than in the control line, a result that contrasts with the data on activity (Fig. 1C).

Plant cells contain multiple APX isozymes, some of which have been shown to be membrane-bound [7]. Therefore, APX activity was quantified in both soluble and membrane fractions of calli extracts. Total APX activity was lower in salt-adapted callus tissue than in control tissue. Both soluble and insoluble fractions, which were reduced by 21% and 17%, respectively, accounted for the difference (Table 3). While the transfer of adapted calli to medium without salt resulted in an increase of 28% in total APX activity, no significant difference was detected between stressed and adapted calli (Table 3). Native PAGE of the soluble fraction from both control and salt-adapted calli showed two bands with the same mobility (Fig. 1D). Despite the similar intensity of the lower band in both situations, APX staining activity of adapted callus tissue was lower than that of the control, in agreement with the spectrophotometric quantification of APX activity (Fig. 1D, d_1). Only one wide band was detected in gels loaded with protein from insoluble fraction, which had lower staining intensity in adapted callus tissue than its counterpart in the control (Fig. 1D, d_2). The changes in culture conditions of control and adapted calli did not alter the pattern of APX isozymes (Fig. 1D). The protein gel blot analysis performed with antibodies raised against cAPX and pAPX allowed the identification of the two APX isoforms detected in the soluble fraction, which corresponded to cytosolic isoform (Fig. 1E, e_1), while the single band detected in the insoluble fraction corresponded to the peroxisomal isoform (Fig. 1E, e_2). Densitometric analysis showed that the amount of cytosolic isoforms after immunoblotting of proteins from salt-adapted line was lower than that of the control line, whereas pAPX protein amount was not significantly affected by salt (Fig. 1E).

The presence of NaCl in the culture medium reduced total GR activity, an effect similar to that observed for SOD and APX. The level of GR activity was 23% lower in salt-adapted than in control calli; changes of similar magnitude occurred in salt-stressed callus tissue. In contrast, transfer of salt-adapted calli to medium without salt for one subculture restored GR activity to control levels. GR activity staining revealed the existence of three isoforms in control gel, but only two isoforms with similar electrophoretic mobility were observed in the gel of the adapted tissue, which corresponded to GR-1 and GR-2 from control (Fig. 1F). GR-1 and GR-2 were also present in stressed callus, and a new band with higher mobility (GR-3) appeared when adapted callus tissue was transferred to medium without salt. Therefore, salinity changed the pattern of GR isozymes causing the disappearance of one GR isoform. The disappearance of GR-3 accounts for the decline in total GR activity in response to salinity, as no significant changes regarding the staining intensity of the two other bands were observed.

2.4. Antioxidant metabolites

To investigate the contribution of antioxidant metabolites to salt tolerance, the levels of ascorbate, glutathione, and α-tocopherol were measured in potato calli. Calli accumulated significantly higher levels of AsA under salt condition than in the control situation (Table 4). AsA increased 30 and 41% in stressed and adapted calli, respectively, whereas the levels of DHA were not affected by salt conditions. Therefore, total ascorbate (tAsA) content also increased in salt-exposed lines, considering that the reduced form accounts for more than 50% of the total pool. The higher AsA/DHA ratio observed in calli subjected to salinity led to an increase in the redox status of AsA pool (Table 4). Although the amount of tAsA in the adapted calli grown in the absence of salt was lower than in the control, the redox AsA/DHA ratio was not affected.

In addition to AsA, glutathione can reduce most of the ROS and is also involved in AsA recycling from DHA [9], originating the oxidized form GSSG. In this study, no accumulation of GSSG was observed, probably because its levels were close to the detection

Table 3
Activity of superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (GR) in control, salt-adapted, salt-stressed callus tissue and salt-adapted callus after transfer to medium without NaCl for one subculture. Values are means ± SE from three independent experiments with five replicates per experiment. Values in the same column followed by different letters are significantly different at $P < 0.05$.

Culture conditions	SOD (U mg^{-1} protein)	CAT (nmol H_2O_2 min^{-1} mg^{-1} protein)	APX (μmol AsA min^{-1} mg^{-1} protein)		GR (μmol NADPH min^{-1} mg^{-1} protein)
			Soluble	Insoluble	
Control calli	46.83 ± 0.22[a]	496.29 ± 12.12[a]	7.78 ± 0.11[a]	5.15 ± 0.20[a]	0.35 ± 0.03[a]
Salt-adapted calli	36.88 ± 0.38[b]	476.79 ± 7.24[a,b]	6.14 ± 0.16[b]	4.28 ± 0.12[b]	0.27 ± 0.02[b]
Salt-stressed calli	30.36 ± 0.63[c]	364.26 ± 8.04[c]	6.62 ± 0.43[b]	4.58 ± 0.14[b]	0.27 ± 0.03[b]
Salt-adapted calli → control condition	41.66 ± 0.97[d]	451.32 ± 4.12[b]	7.36 ± 0.47[a]	5.94 ± 0.17[c]	0.32 ± 0.06[a]

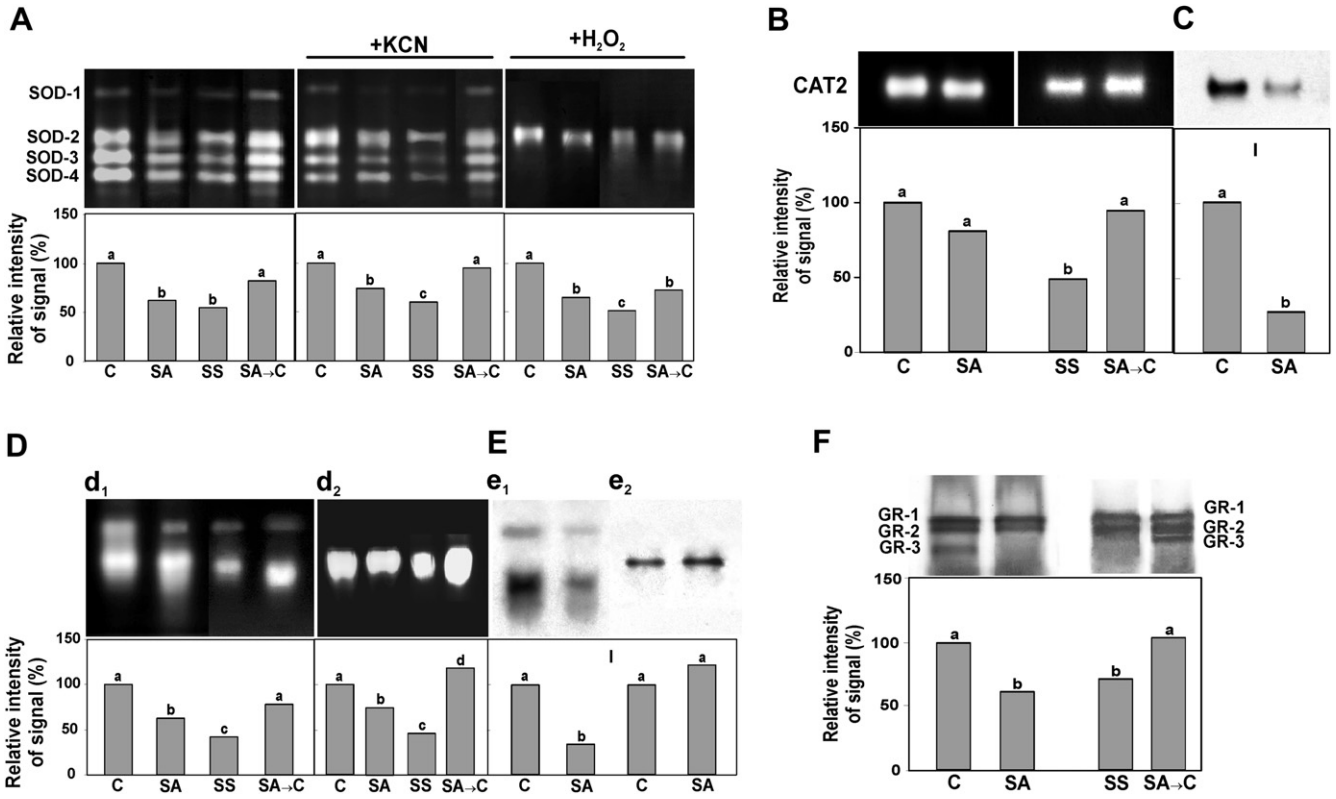

Fig. 1. SOD (A), CAT (B), APX (D) and GR (F) activities stained in native gels in which isoenzyme patterns are shown for control callus tissue cultivated in the absence of salt (C), salt-adapted (SA), salt-stressed callus tissue (SS) and salt-adapted callus after transfer to medium without NaCl for one subculture (SA → C). Discrimination between SOD isoforms was revealed by inhibition with KCN and H_2O_2 (A). APX isoform pattern was analysed by subjecting equal amounts of protein (25 μg) from soluble (d_1) and membrane (d_2) fractions to native gel (D). Gels were loaded with 30 μg of protein for SOD, 2 μg for CAT and with 40 μg of protein for GR. Immunoblot analysis of CAT (C) and APX (E) isoform protein levels was also performed using specific antibodies raised against CAT2, and against cAPX (e_1) and pAPX (e_2), respectively, in control (C) and salt-adapted (SA) callus tissue. Relative quantification of the enzymes activities in native gels and of the isoforms immunodetected in blot films (I) was determined by densitometric analysis in relation to control (100%). Results shown are representative of three independent experiments; vertical bars represent mean values and different letters at the top of each bar indicate significant differences at $P < 0.05$.

limit of the method (data not shown). The GSH content changed significantly in response to salinity. GSH increased 48% in adapted tissue, and 52% in stressed calli in relation to the control. On the other hand, the transfer of salt-adapted tissue to control conditions (0 mM NaCl) caused a decrease in GSH content to levels similar to those found in control callus (Table 4).

Significantly higher amounts of α-tocopherol were found in NaCl-adapted cell line (ca. 70%) than in the control line. When control tissue was subjected to shock treatment with salt, α-tocopherol content also increased compared to control, but the final levels attained were lower to those of adapted tissue. Nevertheless, the transfer of this tissue to salt-free medium led to a decrease in the amount of α-tocopherol to the levels found in control callus (Table 4).

2.5. Ultrastructural changes

In comparison with control cells (Fig. 2) no changes were observed in the structural integrity of tolerant cells (Fig. 3), which is consistent with the macroscopic aspect of callus tissue grown under saline condition. Plasma membrane, endoplasmic reticulum, and mitochondria of tolerant cells were similar to those of control cells (Figs. 2 and 3A). However, the plastids from salt-adapted cell line appeared less differentiated, with a reduced membrane system, showing fewer grana with fewer thylakoids, than those in the control cells (Figs. 2 and 3B). These plastids were round instead of lobate, displayed a more transparent stroma with large starch grains (Fig. 3C). The stroma was partially occupied by starch grains, resembling the morphology of amylochloroplasts. Morphometric

Table 4
Levels of antioxidant metabolites, ascorbate (AsA), dehydroascorbate (DHA), reduced glutathione (GSH), and α-tocopherol in salt-adapted, salt-stressed callus tissue and salt-adapted callus after transfer to medium without NaCl for one subculture. Values are mean ± SE from three independent experiments. Values in the same column followed by different letters are significantly different at $P < 0.05$.

Culture conditions	Ascorbate (mg g^{-1} DW)			AsA/DHA	GSH (mg g^{-1} DW)	α-tocopherol (mg g^{-1} DW)
	AsA	DHA	tAsA			
Control calli	1.13 ± 0.09^a	0.73 ± 0.10^a	1.86 ± 0.07^a	1.54^a	0.31 ± 0.01^a	0.17 ± 0.01^a
Salt-adapted calli	1.59 ± 0.12^b	0.78 ± 0.06^a	2.37 ± 0.12^b	2.04^b	0.46 ± 0.01^b	0.29 ± 0.04^b
Salt-stressed calli	1.47 ± 0.09^b	0.79 ± 0.06^a	2.26 ± 0.03^b	1.86^b	0.47 ± 0.03^b	0.20 ± 0.01^c
Salt-adapted calli → control condition	0.72 ± 0.07^c	0.45 ± 0.08^b	1.17 ± 0.05^c	1.61^a	0.37 ± 0.03^a	0.14 ± 0.03^a

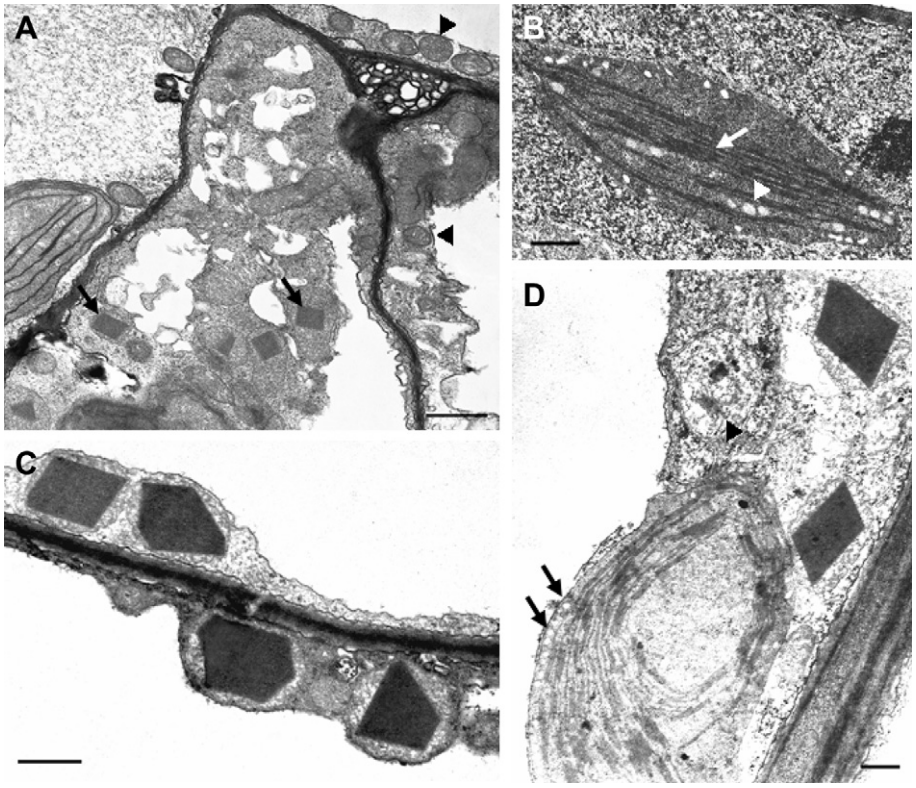

Fig. 2. Ultrastructural aspects of control callus tissue. (A) Normal organization and structure of control cells with numerous peroxisomes containing paracrystalline inclusion (arrows) and mitochondria (arrowheads) (bar: 1 μm). (B) Plastid showing developed grana (arrow) with some plastoglobuli associated (arrowhead) and the presence of vesicles at the periphery of the plastid (bar: 0.5 μm). (C) Detail showing four peroxisomes with paracrystalline inclusion (bar: 1 μm). (D) Region of control cell displaying a plastid devoided of starch but with grana well organized and various vesicles arranged along inside of the plastid envelope (arrows); peroxisomes with paracrystalline inclusion and rough endoplasmic reticulum (arrowhead) can also be seen (bar: 0.5 μm).

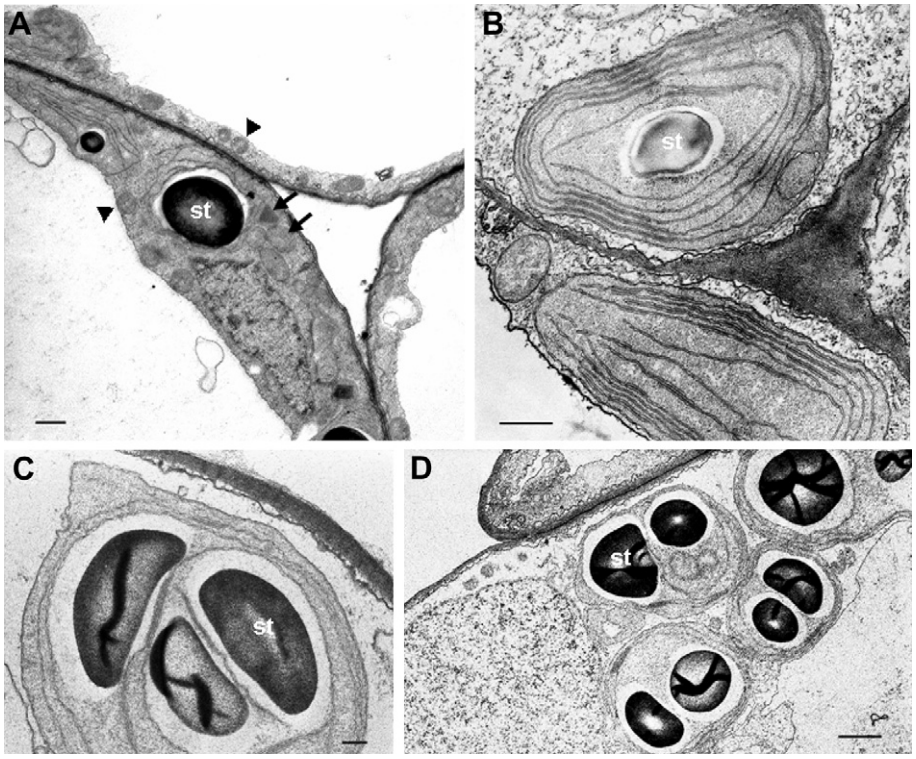

Fig. 3. Ultrastructural aspects of salt-adapted callus tissue. (A) Region of vacuolated cells showing well preserved cytoplasm and organelles such as plastids containing starch (st), mitochondria (arrowheads) and peroxisomes (arrows); no modifications in membranes structure were observed (bar: 1 μm). (B) Plastids displaying a well preserved structure, but tended to have a lower level of organization with a reduced lamellar system than those in control cells (compare with Fig. 2B) (bar: 1 μm). (C) Detail of plastid displaying a spherical shape with huge starch grains (bar: 0.5 μm). (D) Aspect of the plastids containing a high number of large starch grains (bar: 1 μm).

analysis of electron microscope images of plastid profiles from tolerant and control calli revealed similar plastid sizes (Table 5). However, the fractional volume of starch relative to plastid size was significantly higher in tolerant line, indicating higher starch accumulation. The average number of starch grains per plastid was higher in adapted cells than that observed in control (Table 5). Unlike control cells where plastids had small starch granules or were devoided of starch inclusions (Fig. 2D), more and larger starch grains can be seen in salt-exposed cells (Fig. 3D). In addition, the vesicles present in the stroma of the plastids of the control cells (Fig. 2D) were absent in plastids from adapted cells (Fig. 3B).

The screening of various electron micrographs also revealed fewer peroxisomes in salt-adapted callus than in the control. Peroxisomes containing paracrystalline inclusions were sporadically observed in salt-exposed cells (Figs. 2A,C–D and 3A). In these cells only 26% of the quantified peroxisomes had the inclusion, contrasting with a presence in 77% of the peroxisomes of control cells (Table 4).

3. Discussion

The development of salt-tolerant crops is arguably the most important strategy to mitigate the adverse effects of salinity. Success, however, has been limited, largely due to the genetic and physiological complexity of the salt tolerance trait. The understanding of the physiological and molecular processes involved in salt tolerance is essential to foster the improvement of salt tolerance in crops and to identify markers that might help devise agronomic strategies to improve crop yields in soils affected by salinity. As many features associated with salt tolerance are expressed at the cellular level [2], *in vitro* selection of salt stress tolerant material provide useful model systems to study the mechanisms of salt tolerance [17,19]. A potato callus line able to grow in the presence of 150 mM NaCl has been selected and characterized [18]. Subsequent studies on this cell line lead to the conclusion that Na^+ compartmentation into the vacuole contributed to salt tolerance of potato callus tissue [20]. In addition to the vacuolar transport of Na^+, other cellular and metabolic mechanisms are required to assure tolerance to salinity. The involvement of ROS in salinity stress [14,21] justifies the characterization of the antioxidant system as related to the tolerance mechanism.

The adapted callus line used in this study is characterized by slow growth, accumulation of Na^+ and Cl^-, and reduced levels of K^+ and Ca^{2+}. This behaviour was even more pronounced when control tissue was subjected to shock treatment with salt. Growth inhibition is a common response of plants to salinity and is one of the most important agricultural indices of salt stress tolerance [14,21]. Salt tolerance has also been assessed at the cellular level based on the reduction of the callus relative growth rate [19,22]. As in salt-tolerant calli lines of pea and tomato the relative growth rate was reduced by salinity [22,23], a reduction of about 64% was observed in our salt-adapted potato cell line (Table 1). Slower growth is an adaptive feature for plant survival under stress and the extent of salt tolerance often appears to be inversely related to growth rate [3]. Despite the slow growth, the salt-adapted callus line characterized herein was morphologically similar to control material in green colour and compactness. In contrast, stressed callus developed a light-green colour with necrotic areas and 77% growth inhibition (Table 1). These observations support the assumption that the selected callus line had acquired a salt-tolerant phenotype.

Excessive Na^+, a harmful mineral element not required by most glycophytes for normal growth, contributes to reduce plant growth. High Na^+ tissue content is often considered the most critical factor for salt toxicity in sensitive species [24]. Positive correlations between salt tolerance and Na^+ exclusion capacity have been documented [25]. However, Na^+ and Cl^- concentrations in adapted calli line were several folds higher than those found in control line, and remained higher when compared to levels in the stressed line (Table 2). This is consistent with the increased Na^+ and Cl^- concentrations in a number of systems, including salt adapted tobacco cells, NaCl-tolerant calli of pea and *Lycopersicon pennellii* [22,23,26]. Probably, the vacuolar Na^+ accumulation contributed to salt tolerance in our potato cell line [20]. The maintenance of high K^+ concentration and K^+/Na^+ ratio in the cytosol is also critical for salt tolerance [24]. Consistently, the lower K^+/Na^+ ratio was recorded in callus cultures exposed to NaCl, specifically in stressed line (Table 2). Interestingly, the transfer of adapted calli to the control medium did not alter K^+ content in relation to that observed when it was grown continuously on medium with NaCl, suggesting that salt-adapted cells were able to retain K^+. Calcium plays a relevant role in plant salt tolerance as a second messenger in stress signalling and in triggering a signalling cascade that activates Na^+/H^+ antiporters [27], and contributes to alleviate Na^+ toxicity by increasing plasma membrane selectivity for K^+ over Na^+ [25]. The observed decreases in Ca^{2+} (Table 2) are consistent with a report on *Populus euphratica* where adaptation to high salinity was associated with a decrease in Ca^{2+} levels to balance the osmotic pressure caused by excessive Na^+ accumulation [28].

Oxidative stress has been described as a consequence of salinity and oxidative damages are a possible cause of the growth reduction that accompanies salt stress [6,11,21]. Indeed, the non-adapted callus tissue subjected to shock treatment showed a significant increase in lipid peroxidation, as assessed by MDA (Table 1), the ultimate product of membrane peroxidation [6]. In contrast, salt-adapted tissue showed a similar behaviour to the control, and even when it was grown on salt-free medium, indicating that adapted cells have an effective ROS-scavenging system responsible for protection from the oxidative damage induced by salinity.

Antioxidant metabolites and enzymes safeguard normal cellular functions under stressful oxidative condition [5]. SOD prevents the accumulation of O_2^- by converting it to H_2O_2, which can be eliminated via CAT and/or through the activity of the ascorbate–glutathione scavenging cycle. Several studies have documented a correlation between stress tolerance and increased activities of antioxidant enzymes [4–6,19]. In contrast, lower activities of SOD, APX and GR in salt-exposed calli lines than in control calli were observed in this study, even though SOD and CAT activities were higher in the adapted line as compared to the stressed one (Table 3). Only CAT activity was similar in adapted and control calli. Although salinity did not alter the isoenzyme pattern of SOD, CAT and APX, it was possible to relate the decrease in total APX activity with diminished cytosolic and peroxisomal isozyme activities,

Table 5
Fractional volume (Vv) of the plastids and starch, number of starch grains per plastid and peroxisomes containing paracrystaline inclusion in control and salt-adapted callus tissue. Values are mean ± SE. Values in the same column followed by different letters are significantly different at $P < 0.05$.

Culture conditions	Vv plastid (μm^2)	Vv starch/plastid	Number of starch grains/plastid	Peroxisomes with paracrystaline inclusion (%)
Control calli	12.65 ± 0.22^a	0.12 ± 0.01^a	1.02 ± 0.16^a	76.97 ± 5.04^a
Salt-adapted calli	11.19 ± 0.95^a	0.25 ± 0.02^b	2.41 ± 0.23^b	25.88 ± 2.35^b

whereas the decrease of all SOD isozymes contributed to the lowering of total SOD activity in salt-adapted calli, particularly the Cu/Zn-SOD isoform (Fig. 1). Our results contrast with previous observations of increased SOD activity in potato plants exposed to NaCl or UV-B radiation, in which Mn-SOD was not detected and the Cu/Zn-SOD isoform increased in response to the stress factors [14,29]. This discrepancy can be due to differences in experimental systems, since the regulation of SOD expression and activity by stress is developmentally regulated [29]. Other mechanisms are likely to play a role in the response of SOD to stress. In fact, *Arabidopsis thaliana* plants enhance the SOD transcript levels but decreased total SOD activity after a NaCl treatment, possibly due to enzyme inactivation by H_2O_2 [5]. Furthermore, the high Na^+ content found in callus cultures under NaCl may have a direct inhibitory effect on SOD [24]. The reduction of SOD activity could also be a consequence of reduced enzyme synthesis in salt-exposed calli [14]. Reduced protein synthesis is probably also the cause for the lower activity of cAPX isoenzymes found in NaCl-tolerant line, as salt led to lower levels of cAPX protein (Fig. 1). The amount of pAPX protein was not affected by salt condition (Fig. 1), suggesting that a post-translational regulation could be involved [7]. These results suggest a differential regulation of both APX isoforms upon exposure to salt stress, in which the sub-cellular distribution of the isoforms seems to play a role.

An increase in CAT activity in tolerant callus line was anticipated, since the total APX activity was negatively affected by salinity and peroxisomes are one of the major contributors to H_2O_2 production during abiotic stress [5]. The importance of the two enzymes in H_2O_2 scavenging has been demonstrated by several studies in which increased CAT and APX activities are correlated with the tolerance to salt and others environmental stresses [4,30,31]. Salt caused a decrease in CAT activity in stressed potato cell line, but did not affect CAT activity in adapted line, although CAT2 amount decreased in this cell line. CAT2 in potato leaves is localised in the paracrystalline inclusions in the peroxisomes and plays an important role in stress protection [30,32]. Such inclusions were only sporadically observed within peroxisomes in adapted callus tissue, the decreased levels of CAT2 protein in this tissue are consistent with ultrastructural observations. The low affinity of CAT for H_2O_2 makes it less sensitive to small changes in the amount of this ROS; in addition, CAT has a restricted distribution within the cell when compared with other antioxidant enzymes [5], which may explain CAT behaviour in NaCl-adapted calli. In contrast, CAT response in stressed callus are likely related to the fact that peroxisomal CAT protein is very sensitive to salt, suggesting that the enzyme structure was damaged and/or very low rates of *de novo* synthesis occurred [11].

GR is one of the three enzymes which catalyze reactions that maintain pools of GSH and AsA in the H_2O_2 scavenging pathway in chloroplasts, mitochondria and peroxisomes, playing an important role in protecting plants against oxidative stress [5]. Increased GR activity induced by stress can be due to new GR isozymes, as was observed on coffee cells grown in the presence of different metals (Cd, Ni, Se) [33,34]. In those cell cultures, a new isoenzyme of GR was induced in response to the metal stress, suggesting that it could be a marker for oxidative stress in coffee [34]. In contrast, the reduction of total GR activity in salt-adapted and -stressed potato calli was probably due to the disappearance of the GR-3 isoenzyme. The recovery of GR activity after transfer of the adapted line to a salt-free medium was concomitant with the appearance of GR-3 (Fig. 1), demonstrating its sensitivity to salt and the involvement of GR-1 and GR-2 in response to salt. Similarly, Martins et al. [35] found that specific isoenzymes of GR could be involved in the herbicide tolerance mechanism of soil bacteria.

High levels of Na^+ and Cl^- accumulated in adapted and, particularly, in stressed cells are likely to have a negative effect on the antioxidant enzyme activities while ionic homeostasis is re-established [24]. Arguably, vacuolar Na^+ compartmentation was the strategy used in adapted line to protect cytosol against Na^+ toxicity and to assure osmotic homeostasis [20].

Despite the negative effects of salinity on the activity of antioxidant enzymes, the pools of the antioxidant metabolites AsA and GSH increased significantly in both salt-adapted and -stressed lines. The ratio between the reduced and oxidised forms of the two major redox buffers, ascorbate and glutathione, is an indicator of the cell redox state and degree of oxidative stress [8]. This ratio is maintained by the enzymes of the AsA–GSH cycle, although increased biosynthesis of AsA and GSH may also occur in response to stress conditions [31,36]. The increase in tAsA and the high AsA/DHA ratio observed in both adapted and stressed calli are probably due to an increased rate of AsA biosynthesis rather than AsA recycling capability. Active AsA biosynthesis in callus tissue is not surprising, since both cell division and cell expansion require AsA [8], and these two processes are active in growing callus tissue. Moreover, the lower activities of APX and GR in tolerant callus tissue indicate an inability to regenerate AsA and GSH through the AsA–GSH cycle. The accumulation of GSH induced by salinity in potato callus tissue can be attributed to enhanced synthesis and to active GSSG removal, since GSSG did not accumulate in calli exposed to 150 mM NaCl. This hypothesis is supported by the significant reductions in GSH concentration and in the AsA/DHA ratio when the adapted cell line was transferred to a medium without salt. Induction of GSH synthesis by an oxidative stimulus is crucial in cellular protection against oxidative injury [36]. The role of these two metabolites in the regulation of ROS has been thoroughly reviewed, and studies in ascorbate-deficient mutants of *A. thaliana* (*vtc1*) support the important role of AsA in protection against varied environmental stresses, including salinity [8].

α-Tocopherol, in cooperation with AsA and GSH, plays a part in reducing ROS levels and limiting lipid peroxidation by scavenging the lipid peroxyl radicals [9]. α-Tocopherol levels change significantly in response to environmental constraints as a result of the altered expression of pathway-related genes, degradation and recycling [9,21]. Consistent with this role, salinity caused an increase in α-tocopherol in callus cultures exposed to NaCl and decreased when the adapted line was subcultured under control conditions (Table 4). The salt-adapted line is, therefore, able to protect membranes against the oxidative damage induced by salinity through the increased α-tocopherol levels (Table 4) as evidenced by the low MDA levels (Table 1). The parallel increase in α-tocopherol and AsA in adapted line is also indicative of the interplay between these two antioxidants in the control of ROS, as observed in water-stressed *vtc1* mutants of *A. thaliana* [9]. The overall behaviour of the antioxidant enzymes suggests that α-tocopherol, together with AsA and GSH, were important to the re-establishment of redox homeostasis in the salt-exposed cells.

Proline accumulation is a common response in plants under salt stress and increased levels of proline are generally correlated with enhanced stress tolerance [13]. The high proline content found in salt-adapted and -stressed calli (Table 1) is consistent with other reports on responses to stress [14]. Proline acts as an osmotic adjustment mediator, a free radical scavenger, and a redox potential buffer to protect cells from stressful conditions. Its capacity for quenching ROS helps cells to overcome oxidative damage caused by salt stress, protecting membrane integrity, and stabilizing enzymes [10].

Despite the usefulness of ultrastructural analysis in elucidating the cellular mechanisms of salt tolerance, there are few reports on the effects of salinity on cell ultrastructure for tissues cultured *in vitro*. Ultrastructural changes in cell walls and membranes, altered mitochondria and chloroplasts, and accumulation of rough

endoplasmatic reticulum have been observed in plants exposed to salt stress [14,37]. The structural integrity of the cells was maintained and no changes in membranes were evident in salt-adapted potato callus tissue (Fig. 3), suggesting that cellular homeostasis was maintained. Alterations observed in plastids are consistent with restricted organelle differentiation in the presence of salt. Under unfavourable growth conditions, the formation of grana is prevented because of inhibition of protein synthesis, as well as the formation of plastid ribosomes and chlorophyll accumulation [38]. The plastids from adapted cells contained a higher content of starch than those in control cells (Fig. 3), an observation compatible with the higher energy requirements for maintenance in this cell line [3]. The acclimation of plant cells grown under salinity requires not only the accumulation of osmotically active solutes of low molecular mass, but also the accumulation of starch [2]. Starch accumulation under salt conditions was also observed in NaCl-acclimated *Citrus* cell line [39] and it is tempting to speculate that starch synthesis plays a role in moderating the osmotic condition.

In conclusion, salinity had an overall negative effect on the activity of antioxidant enzymes, changed the pattern of GR isoenzymes, but increased the contents of non-enzymatic antioxidant metabolites. Nevertheless, the tolerance to salinity exhibited by adapted line can be attributed to the higher SOD and CAT activities accompanied by the enhanced levels of α-tocopherol. In addition, the accumulation of proline and the high reduction state of the ascorbate and glutathione pool detected in adapted callus were apparently able to mitigate oxidative damage caused by ROS allowing tissue growth in a saline environment. Starch accumulation in adapted cells may contribute to their ability to grow under salt conditions.

Based on the results obtained in this experiment, we suggest the possibility of using antioxidant metabolites as selection criteria for salinity tolerance in potato breeding programmes.

4. Methods

4.1. Callus culture

Induction and subculture of callus tissue, and the selection of a 150 mM NaCl-tolerant cell line from potato (*S. tuberosum* L. cv. Désirée) have been previously described [18]. The response of control line to NaCl stress was analysed by transferring control callus tissue to medium containing 150 mM NaCl for one subculture (NaCl-stressed calli). In parallel, NaCl-tolerant callus was cultured in control medium without NaCl added for one subculture. After 28 d of culture, calli were collected, frozen in liquid nitrogen and used for biochemical determinations.

4.2. Growth rate and ion content

Calli were weighed at the time of their transfer to fresh media (initial fresh weight, FW_i) and the increase in calli fresh weight was quantified after 28 d of culture (final fresh weight, FW_f). The relative growth rate (RGR) was calculated as the $(FW_f - FW_i)/FW_i$ ratio. Ten replicates per treatment were used. Ion content was determined in calli oven-dried at 80 °C for 48 h. Dried samples (1 g) were digested in a mixture of nitric and hydrochloric acids (1/1, v/v). Na^+ and K^+ were measured by flame photometry, Ca^{2+} was determined by atomic absorption spectrometry and Cl^- was quantified by titration using the Mohr method.

4.3. Lipid peroxidation and proline content

Lipid peroxidation was determined by estimating the malondialdehyde (MDA) content as described [4]. The concentration of MDA was calculated using the extinction coefficient of 155 mM^{-1} cm^{-1} and expressed as nmol MDA per gram of dry weight.

Proline was extracted from calli samples in 3% (w/v) sulphosalycylic acid and determined by spectrophotometry as reported by Olmos and Hellín [24].

4.4. Enzyme assays and protein gel blot analysis

Extracts for determination of enzyme activities were prepared from frozen calli (1 g) and homogenized as previously described [30] with the extraction medium supplemented with 5 mM ascorbate. To assay membrane-bound APX (EC 1.11.1.11) activity, the pellet obtained from soluble protein extraction was recovered and it was homogenized in the same extraction buffer used above containing 2% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS); homogenates were incubated for 30 min at 4 °C with gentle agitation. After centrifugation (35,000 × *g* for 18 min at 4 °C), the supernatant was recovered (insoluble protein extract) and the protein content of soluble and insoluble extracts was determined as reported elsewhere [4]. Extracts were then conditioned for spectrophotometric and electrophoretic assays. To the fraction to be used to assay SOD (EC 1.15.1.1) activity, sodium azide was added (10 μM final concentration) as well glycerol to a final concentration of 40% (v/v). For APX activity analysis, soluble and insoluble fractions were combined with 40% glycerol, and for CAT (EC 1.11.1.6) and GR (EC 1.6.4.2) assays, the extracts were also conditioned with glycerol (40%) and dithiothreitol (DTT) (10 mM final concentration). Spectrophotometric assays were carried out immediately after protein extraction. Samples for native gel analysis were stored at −80 °C, except for APX and GR gels which were run immediately after extraction.

Total SOD activity was assayed by the inhibition of the photochemical reduction of nitroblue tetrazolium (NBT) at 560 nm [4]. The enzyme activity was expressed as units mg^{-1} protein and one SOD unit was defined as the amount of enzyme that inhibited 50% of NBT reduction. For the separation of SOD isoenzymes, samples (30 μg protein per slot) were used for electrophoresis on non-denaturing 10% polyacrylamide gels with a 4% stacking gel at 4 °C. SOD activity was detected on these gels using the photochemical procedure described by Santos et al. [29]. The three types of SOD (Mn-, Cu,Zn-, and Fe-SOD) were identified by selective inhibition with 4 mM KCN and 5 mM H_2O_2 [29].

CAT activity was measured by spectrophotometry by monitoring the decrease in absorbance at 240 nm due to H_2O_2 consumption, and the activity was calculated using the molar extinction coefficient of 39.4 mM^{-1} cm^{-1} and expressed as nmol H_2O_2 decomposed min^{-1} mg^{-1} protein [32]. CAT isoforms were analysed in 7% non-denaturing polyacrylamide gels with a constant current of 80 V. DTT to a final concentration of 60 mM was added to the sample loading buffer in order to maintain electrophoretic mobility [30]. Following electrophoretic separation, gels were stained according to the procedure described previously [30]. In order to identify the bands after CAT staining activity, a protein gel blot assay was performed, following the procedure described by Almeida et al. [32] and using specific antibodies raised against CAT1 and CAT2 of potato obtained as previously described [30,32].

APX activity was quantified by following the decrease in absorbance at 290 nm as ascorbate was oxidized, using the extinction coefficient of 2.8 mM^{-1} cm^{-1} and the activity was expressed as μmol oxidised ascorbate min^{-1} mg^{-1} protein [4]. Separation of APX isozymes was performed on non-denaturing 10% polyacrylamide gels at 4 °C. The electrophoresis buffer was supplemented with 2 mM ascorbate and the gels were pre-run for 30 min to allow ascorbate to enter the gel prior to samples application. Following electrophoretic separation, APX activity was

detected using the method reported by Santos et al. [30]. The identification of APX isozymes was done by protein gel blot analysis using polyclonal antibodies raised against peroxisomal and cytosolic APX. Soluble and membrane fractions were used in protein gel blot assays following the same procedure described to immunodetect CAT [32].

GR activity was determined by following the decrease in absorbance at 340 nm due to NADPH oxidation as described by Gomes-Junior et al. [33] by using the extinction coefficient of 6.2 mM^{-1} cm^{-1} and expressed as μmol oxidised NADPH min^{-1} mg^{-1} protein. GR isozymes were separated on native PAGE using 8% separating gels at 4 °C with a constant current of 80 V. After electrophoresis, gels staining was carried out as described previously [34]. Duplicate gels were also stained in the absence of GSSG to verify the identity of GR bands.

The quantification of total enzyme activities on the gels and relative levels of CAT and APX isoforms on the protein blot films were estimated by densitometric analysis using the software Quantity One (Bio-Rad, Hercules, CA). Data are given relative to the intensity of control samples defined as 100%.

4.5. Determination of ascorbate, glutathione and α-tocopherol contents

Frozen samples of calli (0.5 g) were homogenized at 4 °C in 3 ml of 5% (w/v) metaphosphoric acid and 1 mM ethylenediamine tetraacetic acid (EDTA), supplemented with 1% (w/v) insoluble polyvinylpolypyrrolidone (PVPP) just before use. After centrifugation at 19,000 × *g* for 15 min at 4 °C, the supernatant was filtered through a 0.2 μm nylon membrane filter (Schleicher & Schuell, Microscience) and used for ascorbate (AsA) analysis. An aliquot of 200 μl extract was combined with 20 ml of acetate buffer (200 mM, pH 4.5) and 100 μl of 2.8 mM *N*-bromosuccinimide. The mixture reacted for 5 min under N_2 before the addition of 5 ml of 115.6 mM *o*-phenylenediamine and total AsA (tAsA i.e. AsA and DHA) was analysed by polarography as described by Rodrigues et al. [40]. Dehydroascorbate (DHA) was determined using the same method except that the oxidation step with *N*-bromosuccinimide was omitted. AsA content was calculated by the difference between total and oxidized AsA.

Glutathione extraction was carried out following the same procedure described for AsA, except that 3% of metaphosphoric acid was used on the extraction. The concentrations of reduced (GSH) and oxidized (GSSG) glutathione in calli lines were directly determined by high-performance liquid chromatography coupled with electrospray ionization tandem mass spectrometry (HPLC—ESI/MS). Analysis was carried out by injecting 20 μl aliquots of filtered sample in a reverse phase C_{18} column (125 × 4.6 mm, 3 μm; Varian Inc., Palo Alto, USA) with the following linear gradient program from solvent A (water containing 0.1% formic acid) to B (acetonitrile): from 0 to 10% B over 25 min for the separation of the compound, followed by 100% B for 15 min to wash the column, at a flow rate of 0.3 ml min^{-1}. An ion-trap mass spectrometer (Finnigan LCQ Deca XP Plus, San Jose, CA, USA) equipped with an electrospray ionization (ESI) source in the positive ion mode and Xcalibur software version 1.4 (Finnigan, San Jose, USA) was used for data acquisition and processing. To optimize the MS signal, direct injection of standards solutions prepared in 0.1% formic acid were carried out using a Finnigan syringe pump operated at 3 μl min^{-1}. GSH and GSSG contents were determined using calibration curves measured with respective standards and expressed in mg per gram of dry weight.

For the extraction and analysis of α-tocopherol, frozen calli samples (0.6 g) were extracted with 6 ml of 100% methanol and sonicated for 5 min. The extract was centrifuged at 1500 × *g* for 15 min at 4 °C and the supernatant was transferred to a distillation flask. The pellet was re-extracted two times with 5 ml of hexane and the supernatants were collected in the flask. The combined supernatants were then passed through vacuum distillation to eliminate the solvents. The dried extract was resuspended in 500 μl of methanol and centrifuged at 1500 × *g* for 5 min at 4 °C. After filtration of the extract through a 0.2 μm nylon membrane filter (Schleicher & Schuell, Microscience), α-tocopherol was separated by HPLC system (Jasco Corporation, Tokyo, Japan) with a reverse phase C_{18} column (250 × 4.6 mm, 5 μm; Varian Inc., Palo Alto, USA), using methanol/acetonitrile (96/4, v/v) as an eluent at a flow rate of 1.6 ml min^{-1} 20 μl of the extract were injected onto the column and UV detection was carried out at 285 nm (diode array detector; model MD-1510 UV/vis multiwavelength detector) for 10 min. Identification of peak corresponding to α-tocopherol was achieved by comparison of retention time and spectra with commercially available standard (Sigma). α-tocopherol content was calculated using a calibration curve prepared with standard solutions and expressed in mg per gram of dry weight. Peak purity was checked to exclude any contribution from interfering peaks.

4.6. Electron microscopy and morphometric evaluation

Callus tissue were fixed in 2.5% (v/v) glutaraldehyde followed by 2% (w/v) osmium tetroxide, using Na-piperazine-N,N′-bis(2-ethanesulfonic acid) (Na-PIPES) buffer (pH 7.2), dehydrated in acetone and embedded in Epon 812. Ultra thin sections were cut and contrasted with uranyl acetate and lead citrate, and viewed using a Zeiss EM C10 transmission electron microscope (Zeiss, Göttingen, Germany).

Quantification of cellular structural changes induced by the salt was performed. To assure random pictures for morphometric studies, five blocks were chosen from control and NaCl-adapted calli and 250 micrographs were taken at random for each situation and magnified 12,000 × . Morphometric determinations of fractional volume (Vv) of starch/chloroplast were performed by measuring the area of the starch and chloroplasts using the ImageJ software from NIH Image. The same micrographs were used for quantification of average number of starch grains, and of peroxisomes with and devoid of paracrystalline inclusions.

4.7. Statistical analysis

Data were subjected to one-way analysis of variance (*ANOVA*) and the means compared with Duncan's multiple range test at $\alpha = 0.05$. The results presented are averages of three independent experiments with at least five replicate assays within each experiment.

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