Effects of salt on lipid peroxidation and antioxidant enzyme activities of
Catharanthus roseus suspension cells

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Abstract

Cells of *Catharanthus roseus* (L.) G. Don were maintained and sub-cultured in the absence of NaCl (control) or were sub-cultured for 7 days in the presence of 50 (“50”) or 100 (“100”) mM NaCl, or were maintained and sub-cultured for 8 months in the continuous presence of 50 mM NaCl (“50T” or salt-adapted cells). Exposure to salt treatment reduced growth in a concentration-dependent manner. Salt-treated cells strongly accumulated Na^+ and, to a lesser extent, Cl^- . However, no significant change in Cl^- accumulation was observed in 50T cells.

Salt progressively increased superoxide dismutase (SOD) and ascorbate peroxidase (APX) activities whereas a strong increase in catalase (CAT) activity was only obtained in 50 and 50T cells. SOD activity was lower in 50T than in 50 cells, whereas the opposite was observed for APX activity.

After native polyacrylamide gel electrophoresis (PAGE) analysis, five SOD isoenzymes were detected and the increase in SOD activity observed in salt-treated cells seemed to be mainly due to isoenzymes (SOD 1, 2 and 3). Three APX isoenzymes (APX 1, 2 and 3) were detected markedly, especially in salt-adapted cells. Salt strongly induced CAT 2 isoenzyme in 50 cells and CAT 1 isoenzyme in 50T cells.

These data suggest that salt treatment provoked an oxidative stress in *C. roseus* cells, as shown by the increase in lipid peroxidation, in spite of the induction of antioxidant enzymes.

This increase in lipid peroxidation was paralleled by a rise in lipoxygenase (LOX) activity.

Increases in antioxidant activities could also be a response to the cellular damage provoked by NaCl. Probably, this increase could not stop the deleterious effects of salt, but reduced stress severity thus allowing cell growth to occur. On the other hand, and although no JA

measurements has been carried out, the antioxidant enzymes could be mediated, at least

partially, by an LOX-mediated JA increase in salt-treated *C. roseus* cells. SALEM: Please
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Keywords: Antioxidant enzymes, *Catharanthus roseus*, lipid peroxidation, lipoxygenase, Salt stress.

1. Introduction

High salinity levels in the external medium are known to affect many physiological and metabolic processes, leading to cell growth reduction [1]. The *in vitro* selection of cells able to be propagated in highly saline conditions opens the way for investigations of plant metabolism as well as mechanisms involved in salt tolerance at the cellular level [2-3].

Saline environments are generally correlated with changes in plant lipid metabolism [4]. Lipid peroxidation has been associated with damages provoked by a variety of environmental stresses [5-8]. Polyunsaturated fatty acids (PUFA) are the main membrane lipid components susceptible to peroxidation and degradation. Activated oxygen species (AOS), such as superoxide ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radicals ($\cdot OH$), can react with PUFA, forming conjugated dienes (CD) or trienes (CT), lipid peroxy radicals and lipid hydroperoxides [9].

Lipid peroxidation can also be initiated enzymatically through a sequential action of LOX, a ubiquitous plant enzyme which catalyses the hydroperoxidation of PUFA [10]. This enzyme incorporates molecular oxygen into linoleic and linolenic acids, to form lipid hydroperoxides.

The effects of various environmental stresses on plants are known to be mediated, at least partially, by an enhanced generation of AOS [11-13]. Plants contain a complex antioxidant system to eliminate AOS, including antioxidant enzymes such as superoxide dismutase (SOD, EC 1.15.1.1), catalase (CAT, EC 1.11.1.6), glutathione peroxidase (GPX, EC 1.11.1.9), peroxidases and the enzymes involved in the ascorbate-glutathione cycle (ASC-GSH cycle [14]: ascorbate peroxidase (APX, EC 1.11.1.1), dehydroascorbate reductase (DHAR, EC 1.8.5.1), monodehydroascorbate reductase (MDHAR, EC 1.6.5.4) and glutathione reductase (GR, EC 1.6.4.2).

Numerous studies indicate that acquisition of salt tolerance may be a consequence of improving resistance to oxidative stress [6, 12, 13, 15-21]. Most show a positive correlation between resistance to salt stress and a more efficient antioxidative system. Salt tolerance is generally attributed either to high constitutive or upregulated activities of antioxidant enzymes [2, 3, 16, 20, 22]. For instance, data obtained in whole plants and cotton calli [18, 23] suggest that a more active ascorbate-glutathione cycle and a higher catalase activity are involved in the protection from salt stress-induced oxidative damage. Studies with *P. sativum* cell lines have also demonstrated an induction of two new Cu,Zn-SODs in salt-tolerant calli, as compared to sensitive cells [2]. Similarly, in salt-tolerant *C. limon* calli, the induction of a new Mn-SOD isoenzyme and an increase in peroxidase activity have been observed [3].

In the present work, the effects of salt treatment on lipid peroxidation as well as on the activities of some antioxidant enzymes (SOD, APX and CAT) of *C. roseus* cells were studied. Plant growth and Na⁺ and Cl⁻ contents were also analysed.

2. Material and methods

2.1. Cell culture

Cell suspensions of *Catharanthus roseus* (L.) G. Don (albino cell lines C₂₀) were propagated on a rotary shaker (100 rpm), at 24 °C in the dark, in B5 medium containing 58 mM sucrose and 4.5 µM 2,4-D, adjusted to pH 5.5 just before sterilisation by autoclaving [24]. Vitamins were sterilised by filtration (across 0.22 µm discs, Gilman) and added to media. They were sub-cultured weekly by 1:10 dilution in 250-ml Erlenmeyer flasks containing 50 ml suspension. For experimental purposes, cells were regularly sub-cultured in standard medium as:

0 (control): cells maintained and sub-cultured in the absence of NaCl.

50 and 100: cells sub-cultured for 7 days in the presence of 50 and 100 mM NaCl, respectively.

50T: cells maintained and sub-cultured for 8 months in the continuous presence of 50 mM NaCl (salt-adapted cells).

2.2. Cell growth and ions content determination

At the end of the experiment, cells were collected, dried at 65 °C for four days and weighed for growth determination. Na⁺ and Cl⁻ analyses were carried out after digestion with 0.5 % nitric acid and recovery of dry material as a fine powder. Na⁺ concentrations were determined by atomic absorption spectrophotometry (Corning). Chloride was measured colormetrically (Haake Büchler).

2.3. Enzymes extraction

All operations were done at 4 °C. For LOX activity, cells (500 mg) were homogenised in a mortar with 3 ml of 50 mM phosphate buffer, pH 7. Homogenates were filtered through two layers of Miracloth and the filtrate was centrifuged at 17000 g for 15 min, at 4 °C. The resulting supernatant was stored at -80 °C.

For antioxidant enzyme assays, frozen cell samples were ground to a fine powder with liquid nitrogen and extracted with ice-cold 0.1 mM phosphate buffer, pH 7.8, containing 1 mM ethylenediaminetetraaceticacid (EDTA), 1 mM phenylmethanesulphonylfluoride (PMSF) and 0.5 % polyvinylpyrrolidone (PVP). For APX analysis, extraction buffer contained 5 mM ascorbate. The homogenate was centrifuged at 14000 g for 20 min, at 4 °C, and the supernatant was used for enzyme activity and protein determinations.

2.4. Antioxidant and LOX activity assays

All spectrophotometric analyses were performed with a Varian Cary 100Conc (UV-visible) spectrophotometer.

LOX activity was measured spectrophotometrically, using linoleic acid [25] or linolenic acid [26] as substrate. Substrate solution was prepared by ultrasonic homogenisation of 1.3 mM linoleic or linolenic acid in 25 ml of 0.1 M tetraborate buffer, pH 10, containing 0.1 % Tween-20. Incubation tests had 100 μ l of substrate in 1.4 ml tetraborate buffer, to which 100 μ l of enzyme extract was added. The increase in absorbance at 234 nm was monitored. One unit of LOX activity was defined as an increase in absorbance of 0.001 unit per min at 234 nm.

CAT activity was assayed by measuring the initial rate of H₂O₂ disappearance at 240 nm [27]. The reaction mixture contained 33 mM H₂O₂ in 50 mM phosphate buffer, pH 7.0, and CAT activity was calculated using the extinction coefficient of 40 mM⁻¹ cm⁻¹ for H₂O₂.

APX activity was determined by measuring the decrease in absorbance of the oxidised ascorbate at 290 nm, according to Nakano and Asada [28]. The reaction mixture contained 0.5 mM ascorbate, 0.1 mM EDTA and 1.2 mM H₂O₂ in 50 mM phosphate buffer, pH 7.0. Enzyme activity, expressed as μ mol min⁻¹ mg⁻¹ protein of oxidised ascorbate, was calculated using the extinction coefficient of 2.8 mM⁻¹ cm⁻¹ for ascorbate.

SOD activity was measured at 560 nm according to Beauchamp and Fridovich [29], based on the inhibition of the photochemical reduction of nitro blue tetrazolium (NBT). The reaction mixture contained 33 μ M NBT, 10 mM L-methionine, 0.66 mM EDTA and 3.3 μ M riboflavin in 50 mM phosphate buffer, pH 7.8. Reactions were carried out at 25 °C for 10

min, at a light intensity of about $300 \mu\text{mol}^{-1} \text{m}^{-2} \text{s}^{-1}$. One unit of enzyme activity was defined as the quantity of SOD required for 50 % inhibition of NBT reduction.

2.5 Detection of conjugated diene and triene lipids

Total lipids were extracted from cells using chloroform-methanol- H_2O (1:1:1, v/v/v) as described by Folch et al. [30]. The final lipid extract, concentrated under a nitrogen stream (oxygen-free), was immediately dissolved in chloroform-methanol (2:1) and used for CD or CT assay or inorganic phosphorous determination. All procedures were performed under dim light at 4°C to avoid lipid peroxidation.

CD or CT lipids were analysed according to Recknagel and Glend [31]. An aliquot of lipid extract was evaporated under an oxygen-free nitrogen stream, and dissolved in cyclohexane (spectrophotometric grade). The UV spectra of lipids were monitored with a Cary Varian 100 conc UV-visible spectrophotometer. The absorbances of CD and CT were measured at 232 nm and 274 nm, respectively. The intensity of CD and CT formation was expressed per μg of inorganic phosphorus (P_i), determined by the Bartlett method [32] with slight modifications, in relative units; $\text{RU} = \text{Abs}_{232\text{nm}} / \text{Abs}_{274\text{nm}} / \mu\text{g P}_i$.

2.6. Lipid peroxidation assay

Lipid peroxidation was determined by measuring malondialdehyde (MDA) formation, using the thiobarbituric acid method. Cells (2 g) were ground with liquid nitrogen and quartz sand, to give a fine powder. After thawing, 10 ml of 100 mM Tris-HCl buffer, pH 7.4, and 1.5 % insoluble PVP (w/v) were mixed with the sample and homogenised for 1 min. Samples were filtered and then centrifuged at 10000 g for 20 min. 1 ml of supernatant was mixed with 4 ml of 0.5 % (w/v) thiobarbituric acid in 20 % trichloroacetic acid (w/v) and the mixture was

heated at 90 °C, in a boiling water bath, for 30 min. After cooling and centrifugation at 10000 g for 20 min, the absorbance of the supernatant was measured at 532 nm and 600 nm. After subtracting the non-specific absorbance at 600 nm, the MDA concentration was determined by its extinction coefficient of $155 \text{ mM}^{-1} \text{ cm}^{-1}$ [33].

2.7. PAGE analysis of antioxidant enzymes

Samples containing equal amounts of protein were subjected to native PAGE as described by Laemmli [34]. Electrophoresis was conducted at 4 °C in a 10 % polyacrylamide gel for SOD and APX; 7 % gel for CAT activity. For APX activity determination, 2 mM ascorbate was added to the buffer. SOD activity was detected after staining with riboflavin and NBT [29]. APX activity was detected by following the procedure of Mittler and Zilinskas [35] and CAT activity was detected according to Woodbury [36].

2.8. Protein quantification

Total soluble protein contents were determined according to the method of Bradford [37], using the Bio-Rad assay kit with bovine serum albumin as a calibration standard.

3-Results

3.1. Effect of salt treatment on cell growth and ion accumulation

Salt inhibited the growth of *C. roseus* cells by 43 % and 67 % at 50 and 100 mM NaCl respectively compared to control (Table 1). Noteworthy that growth inhibition obtained in 50T cells is slightly lower than 50 cells (36 % instead of 43 %).

Salt also provoked a dose-dependent increase in Na⁺ and Cl⁻ accumulation, with a more pronounced effect on Na⁺ than on Cl⁻ ions. No significant change in Cl⁻ contents was observed in 50T cells when compared to control (Table 1).

3.2. Antioxidant enzymes

We report in Table 2 the effects of various salt concentrations on antioxidant enzyme activities such as SOD, APX and CAT. Salt increased, in a dose-dependent manner, SOD specific activity, reaching 5.7-fold the control level at 100 mM NaCl. However, in 50T cells, the increase in SOD activity was 2-fold lower than the levels presented in 50 cells. Salt also increased specific APX activity. The highest level was obtained in 50T cells (37-fold greater than control). CAT activity was highly increased at 50 mM NaCl (50 or 50T cells) and strongly decreased at 100 mM NaCl.

In native PAGE, five SOD isoenzymes were observed in control cells; called SOD 1 to SOD 5, ordered by increasing electrophoretic mobility in 10 % gel. Salt-induced SOD activity seemed to be mainly due to isoenzymes SOD 1, 2 and 3 (Fig. 1). APX activity was undetectable in control conditions and increased gradually with salt treatment. The highest APX level obtained, in 50T cells, reflects the presence of three isoenzymes (APX 1, 2 and 3)

(Fig. 2). Catalase activity increased significantly at low salt concentration (50 mM) but decreased at the higher level (100 mM). Salt strongly induced accumulation of the CAT 2 isoform in 50 cells and the CAT 1 isoform in 50T cells (Fig. 3).

3.3. Determination of lipid peroxidation

The data in Table 3 show the dose-related effects of salt on the MDA level, which increased 1.2- and 1.4-fold at 50 and 100 mM NaCl, respectively.

Lipid peroxidation was also quantified as CD and CT contents (Table 3). Salt increased progressively both CD (1.7-fold more than the control) and CT (3-fold more) levels up to 100 mM NaCl. It is noteworthy that CD and CT levels were similar in 50 and 50T cells.

3.4. Effect of salt treatment on LOX activity

In order to establish the involvement of LOX in the peroxidation process, we measured its activity in both control and salt-treated cells (Table 4). Salt treatment markedly enhanced LOX activity, especially at 100 mM NaCl. A higher increase in LOX activity was observed when linolenic acid was used as substrate instead of linoleic acid.

4. Discussion

Plant cell culture is a useful tool for the study of responses to environmental stress at the cellular level. We show here that salt provoked an inhibition of cell growth accompanied by an accumulation of Cl^- and, especially, Na^+ ions.

Growth reduction and Na^+ and/or Cl^- accumulation have been described also in other NaCl-treated cell lines. For instance, in *P. sativum* calli adapted to 85.5 mM NaCl, a 65 % reduction of dry weight was shown, compared with sensitive calli [2]. In NaCl-tolerant *Citrus* cell lines, 170 mM NaCl reduced growth by 5-fold when compared to the control [3].

Accumulation of Na^+ and/or Cl^- has been reported in other NaCl-tolerant cell lines. In salt-adapted *P. sativum* cells as well as in NaCl-tolerant *Citrus* cell lines, salinity provoked a strong increase in both Na^+ and Cl^- contents, with a higher increase for Cl^- , the opposite of what occurred in our present study.

NaCl induced changes in detoxifying enzyme activities of *C. roseus*. Salt provoked a dose-dependent increase in SOD activity, which could represent a defence mechanism against NaCl-induced $\text{O}_2^{\cdot -}$ generation. However, the increase observed in 50 mM NaCl-treated cells was 2-fold higher than in 50T cells, which could reflect a lower $\text{O}_2^{\cdot -}$ production or a higher capacity for elimination of $\text{O}_2^{\cdot -}$. In 50 and 50T cells, coordinated increases in the activities of SOD (a H_2O_2 -generating enzyme) and APX and CAT (H_2O_2 -scavenging enzymes) took place. However, in 100 mM NaCl-treated cells, the strong induction in SOD activity was not accompanied by an increase in CAT activity. Enhanced SOD activity without any accompanying increase in the ability to scavenge H_2O_2 can result in a metal-catalysed Haber-Weiss reaction, with the generation of hydroxyl radicals ($\cdot\text{OH}$) [38]. This could be related to the more drastic decrease in growth and higher lipid peroxidation found in 100 mM NaCl-treated cells. Native PAGE analysis revealed that, among the five SOD isoforms, salt

specifically, and in a dose-dependent way, induced 3 isoenzymes (SOD 1, 2 and 3). APX activity, which increased with salt treatment, revealed the presence of 3 isoforms (APX 1, 2 and 3). At low salt concentration (50 mM), the increased CAT activity is mainly attributable to the presence of two different isoforms: CAT 2 in 50 cells and CAT 1 in 50T cells. It is worth noting that the higher salt concentration (100 mM) strongly inhibited CAT activity and isoenzymes amounts.

Indeed, it has been shown previously that salt treatment induced two CuZn-SOD isozymes and peroxidase activity in pea cells adapted to NaCl [2], and a new Mn-SOD as well as peroxidase activity in tolerant *Citrus* cells [3]. These previous studies suggest that SOD and peroxidase activity might play a role in the molecular mechanisms of plant cell tolerance to salt stress [2, 3].

Lipid peroxidation is often used as an indicator of salt-induced oxidative damage [6-8,13, 20]. NaCl induced an oxidative stress in *C. roseus* cells, as evidenced by the increase in MDA and lipid-conjugated CD and CT contents. MDA formation has been shown to be greater in NaCl-resistant than in sensitive pea calli, although resistant calli showed higher SOD and peroxidase activities [2]. Similarly, in cell suspensions of *Nicotiana tabacum* cv. BY2, lipid peroxidation was highly induced by NaCl treatment despite the observed increases in SOD, CAT and APX activities [39]. Recently, it has been shown that anger plants, resistant to 50 mM NaCl, had higher constitutive antioxidant enzyme levels than salt-sensitive loquat plants. However, in both cases, salinity produced an increase in lipid peroxidation, but it was higher in loquat than in anger plants [8].

Positive correlations between accumulation of ions, AOS production and lipid peroxidation have already been described [6, 13, 40, 41]. For instance, Sjölin and Møller [41] showed a cation-induced increase in electron transport of submitochondrial particles from potato, which led to enhanced $O_2^{\cdot -}$ production, as observed also in leaves of salt-treated pea

plants [16]. In these cases, the increase in AOS was also correlated with increases in Na^+ and Cl^- contents. In our case, salt treatment provoked increases in Na^+ and Cl^- accumulation which could have led to higher AOS production and lipid peroxidation.

Moreover, membrane peroxidation can also be initiated enzymatically by lipoxygenases, which convert 18:2 and 18:3 fatty acids to their corresponding hydroperoxides [38, 42]. In the present work, an increase in LOX activity was observed in salt-treated cells, mainly at 100 mM NaCl. LOX-derived fatty acid hydroperoxides could be converted into more stable compounds such as jasmonic acid (JA), which participates in the onset of defence reactions to biotic and abiotic stresses [43, 44] and is a mediator in antioxidant enzymes induction. Recently, Clarke et al. [45] reported that treatment of *Phaseolus vulgaris* plants with 2.5 nM JA greatly increased glutathione reductase and peroxidase activities but only slightly increased CAT and SOD.

In conclusion, our data indicate that salt induced an oxidative stress in *C. roseus* cells, despite the concomitant increase in antioxidant enzymes. This increase could reflect a defence response to the cellular damage provoked by NaCl treatment. Moreover, this increase in antioxidant activities, which was not strong enough to eliminate all the deleterious effects provoked by salt, only alleviated the impact of stress, thus allowing cell growth to occur. Further studies are necessary to assess the role of JA in defence mechanisms against salt stress, especially in antioxidant enzymes induction.

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Table 1

Effect of salt on growth and Na⁺ and Cl⁻ contents of *C. roseus* suspension cells

NaCl (mM)	Growth (g ⁻¹ DW)	Na ⁺ (mmol g ⁻¹ DW)	Cl ⁻ (mmol g ⁻¹ DW)
0	0.67±0.04 ^a	0.17±0.03 ^c	0.37±0.02 ^b
50	0.39±0.05 ^b	1.15±0.03 ^b	0.45±0.01 ^{ab}
100	0.22±0.03 ^c	2.11±0.04 ^a	0.59±0.01 ^a
50T	0.43±0.03 ^b	1.30±0.03 ^b	0.34±0.02 ^b

Each value is the mean ± SE of three independent experiments with three replicates of cell extracts. Values followed by different superscript letters in each column differ significantly (Duncan's test, $P = 0.05$).

Table 2

Effect of salt on antioxidant enzyme activities of *C. roseus* suspension cells.

NaCl (mM)	SOD (U mg ⁻¹ protein)	APX (mmol mg ⁻¹ protein)	CAT (μmol mg ⁻¹ protein)
0	17.76 ± 2.76 ^d	0.93 ± 0.19 ^d	5.67 ± 1.19 ^c
50	60.52 ± 4.00 ^b	6.01 ± 1.23 ^c	135.45 ± 5.89 ^b
100	91.96 ± 5.79 ^a	12.75 ± 2.29 ^b	7.83 ± 2.08 ^c
50T	27.71 ± 2.39 ^c	34.32 ± 5.96 ^a	153.67 ± 7.35 ^a

Each value is the mean ± SE of three independent experiments with three replicates of cell extracts. Values followed by different superscript letters in each column differ significantly (Duncan's test, $P = 0.05$); SOD, superoxide dismutase; APX, ascorbate peroxidase, CAT, catalase.

Table 3

Effect of salt on lipid peroxidation products of *C. roseus* suspension cells: Malondialdehyde (MDA) and conjugated dienes and trienes,

NaCl (mM)	MDA (nmol g ⁻¹ DW)	Conjugated dienes (Abs _{233 nm} µg ⁻¹ Pi)	Conjugated trienes (Abs _{274 nm} µg ⁻¹ Pi)
0	531.52 ± 35.14 ^d	1.48 ± 0.15 ^c	0.43 ± 0.03 ^b
50	627.34 ± 23.38 ^c	1.93 ± 0.12 ^b	0.96 ± 0.09 ^a
100	753.95 ± 23.90 ^a	2.52 ± 0.21 ^a	1.30 ± 0.05 ^a
50T	687.24 ± 23.18 ^b	2.03 ± 0.16 ^b	0.94 ± 0.14 ^a

Each value is the mean ± SE of three independent experiments with three replicates of cell extracts. Values followed by different superscript letters in each column differ significantly (Duncan's test, $P = 0.05$); Pi, inorganic phosphorus.

Table 4Effect of salt on lipoxygenase activity (LOX) of *C. roseus* suspension cells

NaCl (mM)	LOX (U mg ⁻¹ protein)	
	18:2	18:3
0	0.26 ± 0.06 ^b	0.25 ± 0.02 ^c
50	0.28 ± 0.04 ^b	0.32 ± 0.03 ^b
100	0.38 ± 0.02 ^a	0.44 ± 0.03 ^a
50T	0.28 ± 0.03 ^b	0.32 ± 0.02 ^b

Each value is the mean ± SE of three independent experiments with three replicates of cell extracts. Values followed by different superscript letters in each column differ significantly (Duncan's test, $P = 0.05$); 18:2, linoleic acid; 18:3, linolenic acid.

Figure 1

Effect of salt on *C. roseus* SOD isoenzymes: Native PAGE analysis.

50 μ g of protein were loaded on 10 % gel. Lines 1, 2 and 3: 0, 50 and 100 mM NaCl-treated cells, respectively; line 4: 50T cells. Staining for activity was performed by the photochemical method, using NBT/riboflavin.

Figure 2

Effect of salt on *C. roseus* APX isoenzymes: Native PAGE analysis.

50 μ g of protein were loaded on 10 % gel. Lines 1, 2 and 3: 0, 50 and 100 mM NaCl-treated cells, respectively; line 4: 50T cells.

Figure 3

Effect of salt on *C. roseus* CAT isoenzymes: Native PAGE analysis.

50 μ g of protein were loaded on 7 % gel. Lines 1, 2 and 3: 0, 50 and 100 mM NaCl-treated cells, respectively; line 4: 50T cells.