

Metabolic responses to salt stress in cell suspension cultures of sensitive and resistant *Citrus*

By A. L. FERREIRA* and M. E. LIMA-COSTA

Faculdade de Engenharia de Recursos Naturais, Universidade do Algarve,
Campus de Gambelas, 8005-139 Faro, Portugal
(e-mail: alferrei@ualg.pt)

(Accepted 26 July 2006)

SUMMARY

The acquisition of resistance to salt stress was investigated in cell suspension cultures of two *Citrus* cultivars, *Citrus* hybrid cv. 'Carvalho' and *Citrus sinensis* cv. 'Valencia late'. The metabolic responses in terms of scavenging enzyme activities (e.g., superoxide dismutase, catalase and guaiacol peroxidase), partitioning of Na⁺, Cl⁻ and K⁺ ions, and proline accumulation were investigated in relation to growth and salt damage at the cellular level. These two cell lines use different metabolic and cell biochemical strategies to tolerate salt stress. The 'Carvalho' cell line displayed a salt-resistant behaviour, even at high salt concentrations. This salt-resistance behaviour operated primarily by impeding the uptake of Na⁺ and Cl⁻ ions combined with intracellular proline accumulation and a high level of scavenging of reactive oxygen species (ROS). On the other hand, the cv. 'Valencia late' appeared to be salt-sensitive. Proline accumulation can be used as an indicator of the salt stress imposed on *Citrus* cell lines under the conditions used here.

Salinity limits plant production on almost 40% of agricultural land worldwide. In arid and semi-arid regions, salinity is a common problem, which affects plant growth, development and productivity. It is assumed that salt stress causes an imbalance in cellular ion levels, which may result in ion toxicity and osmotic stress (Greenway and Munns, 1980; Lima-Costa *et al.*, 2002). Studies of ion uptake by intact plants are complex, as they involve ion uptake by roots, the transport of ions to various organs and finally ion accumulation in the leaves. Cell cultures are a useful tool to elucidate mechanisms of salt-tolerance operating at the cellular level. In these simpler biological systems, ion uptake and intracellular accumulation occur in the same cell and no long-distance ion transport occurs.

Citrus is an economically important fruit crop in southern Portugal, and in the World. However, *Citrus* yield and growth are significantly affected by salinity (Storey and Walker, 1999). Because of the inherent sensitivity of *Citrus* plants to salt stress, there has been great interest in identifying cultivars that are resistant to salinity. Salt-tolerance mechanisms are complex and include osmotic adjustment through the accumulation of compatible solutes, lowering the concentrations of toxic ions in the cytoplasm by restriction the influx of Na⁺, its sequestration in the vacuole, and/or Na⁺ ion extrusion (Binzel *et al.*, 1987), and scavenging of reactive oxygen species (ROS; Mittler, 2002).

High salinity induces oxidative stress in plant tissues and many studies have shown that the acquisition of salt tolerance may be a consequence of improved resistance to oxidative stress (Dionísio-Sese and Tobita, 1998; Hernández *et al.*, 2000). The anti-oxidant enzymes, superoxide dismutase (SOD; EC 1.15.1.1), catalase (CAT; EC 1.11.1.6) and peroxidase (POX; EC 1.11.1.7)

constitute the main enzymatic mechanisms for the elimination of ROS in plant cells (Scandalios, 1993).

The present study aims to improve our understanding of the cellular and biochemical responses of *Citrus* cell lines to NaCl stress. This work also aims to identify the different metabolic abilities of cultivars and what determines their ability to tolerate salinity, and to investigate biochemical selection criteria for salinity tolerance.

MATERIALS AND METHODS

Cell culture

Cell suspensions of *Citrus* hybrid 'Carvalho' and *C. sinensis* cv. 'Valencia late' were maintained on a rotary shaker (140 rpm) at 24°C, in the dark, in basal liquid medium containing MS salts (Murashige and Skoog, 1962), supplemented with 100 mg l⁻¹ nicotinic acid, 400 mg l⁻¹ thiamine-HCl, 1 mg l⁻¹ kinetin, 0.5 g l⁻¹ malt extract and 50 g l⁻¹ sucrose, adjusted to pH 5.7. All media were autoclaved at 121°C at a pressure of 104 kPa for 20 min. Cultures were maintained in 500 ml Erlenmeyer flasks and sub-cultured every week with a 20% (v/v) inoculation. Erlenmeyer experiments were performed under the same cultivation conditions, and assays were run for 14 d. Addition of 50, 100, 150, 200, 300 or 400 mM NaCl was performed before the inoculation procedure. A control assay was done in the absence of NaCl. All experiments were performed in triplicate.

Biomass and determination of ion contents

Biomass was expressed as the gain in fresh weight (FW) between the initial phase (day-0) and the end of the exponential phase (day-14), and expressed as a percentage of control. FW was determined after filtration of 10 ml samples using Whatman N°1 filter paper. Dry weight (DW) measurements were made after

*Author for correspondence.

drying fresh cell material at 80°C to constant weight. The results are the means of three replicates.

To determine ion contents, samples of dried cells were ashed for 8 h at 500°C. The ash (500 mg) was boiled in 10 ml 50% (v/v) HNO₃ for 5 min, diluted in distilled water and filtered through 0.20 µm pore-size Whatman N° 1 filter paper. Na⁺ and K⁺ contents were quantified using an atomic emission spectrophotometer (Shimadzu AA-680, Kyoto, Japan). Chloride ion contents were determined using a selective electrode (Crison 15213 3000 Model GPL22, Barcelona, Spain).

Proline determination

Proline contents were determined by the method of Magné and Larher (1992). Fresh cells were extracted with 80% (v/v) ethanol at 80°C for 30 min. Then, 100 µl of the cell extract was mixed with 400 µl ninhydrin solution. After incubation for 1 h at 100°C, the tubes were cooled and 1 ml toluene was added per tube. The absorbance of the upper phase was determined at 520 nm and compared with a standard curve using proline.

Enzyme assays

Enzyme extraction: Fresh cells of both cell lines were collected on day-0 and after 14 d, and used for enzyme analysis. Cells were frozen in liquid nitrogen immediately after harvest and stored at -80°C. One g of cells was sonicated in 2 ml 100 mM sodium phosphate buffer, pH 7.8 supplemented with 1% (w/v) PVP (polyvinylpyrrolidone), 0.1 mM EDTA and 0.2% (v/v) Triton X-100 for 1 min at 120 W. The resulting supernatant was filtered through a Sephadex G-25 column (Amersham Pharmacia Biotech, Roosendaal, The Netherlands) for desalting. The extract was centrifuged for 5 min at 2,000 × g and used for enzyme activity and protein determinations. The soluble protein contents of the extracts were determined according to the method of Bradford (1976) with bovine serum albumin as a calibration standard.

Anti-oxidant enzyme activities: Determination of superoxide dismutase (SOD) activity was based on the method of Beauchamp and Fridovich (1971) which measures the percentage inhibition of photochemical reduction of nitroblue tetrazolium (NBT) caused by the presence of SOD. A 3 ml reaction mixture contained 100 mM sodium phosphate buffer, pH 7.8, supplemented with 1% (w/v) PVP, 0.1 mM EDTA, 1.5 mM NBT, 13 mM methionine, 0.12 mM riboflavin and 100 µl of crude enzyme extract. The tubes were shaken and placed 30 cm under two 15 W fluorescent lamps giving a light intensity of 300 µmoles m⁻² s⁻¹. The colour reaction was developed at 25°C for 10 min and the absorbance of the reaction mixture was then read at 560 nm. One unit of enzyme activity was defined as the amount of enzyme required to cause a 50% inhibition of the reduction of NBT, and the specific enzyme activity was expressed as enzyme units mg⁻¹ soluble protein.

Catalase activity was assayed by monitoring the decrease in absorbance at 240 nm due to hydrogen peroxide (H₂O₂) at 25°C (Beers and Sizer, 1952). The 3 ml reaction mixtures contained 100 mM sodium phosphate buffer, pH 7.8 supplemented with 1% (w/v) PVP, 0.1 mM EDTA and 30 µl enzyme extract. The

decrease in absorbance values was recorded for 3 min after addition of 40 mM H₂O₂. Specific catalase activity was expressed in enzyme units mg⁻¹ soluble protein.

Peroxidase activity was determined using the guaiacol oxidation method as described by Bajji *et al.* (1998). The increase in absorbance at 436 nm was measured 180 s after the addition of 40 mM H₂O₂ to each 3 ml reaction mixture which contained 100 mM sodium phosphate buffer pH 7.8 supplemented with 1% PVP (w/v), 0.1 mM EDTA, 0 – 30 µl crude enzyme extract and 0.1 mM guaiacol. One unit of peroxidase activity was expressed as the variation in absorbance min⁻¹, and the specific enzyme activity as units mg⁻¹ soluble protein. Reactions were conducted at 25°C.

Lipid peroxidation

Lipid peroxidation was determined by estimating the malondialdehyde (MDA) content in 1 g FW of cells according to Hodges *et al.* (1999). MDA is produced by lipid peroxidation through the thiobarbituric acid reaction. The concentration of MDA was measured as the absorbance at 532 nm and calculated by using an extinction coefficient of 1.55 × 10⁵ M⁻¹ cm⁻¹. A correction for non-specific compounds that absorb at 440 and 600 nm was done.

DNA content

A NucleoSpin Plant kit (Macherey-Nagel, GmbH, KG, Düren, Germany) was used for the extraction of genomic DNA. Frozen cells (0.1 g) were ground to a fine powder in a chilled mortar using liquid nitrogen and the DNA was extracted with lysis buffer [100 mM Tris-HCl, pH 8.0 supplemented with 1.4 M NaCl, 20 mM EDTA, 2% (w/v) hexadecyltrimethylammonium bromide (CTAB) and 0.2% (v/v) 2-mercaptoethanol]. In order to remove polysaccharides, phenolics, residual cellular debris and other contaminations, the mixture was cleared by centrifugation at 11,000 × g for 10 min at 25°C. The purity of the DNA was determined by calculating the ratio of absorbance at 260 nm to that at 280 nm using a UV-160A spectrophotometer (Shimadzu, Kyoto, Japan). The A₂₆₀/A₂₈₀ ratio was 1.80. Three independent DNA extractions were made from three different cell samples.

Statistics

All experiments were performed in triplicate. The data were subjected to analysis of variance (ANOVA) and mean values were compared using SPSS for Windows. Duncan Post-hoc tests were performed when significant differences occurred at the 5% level.

RESULTS

Influence of NaCl on biomass accumulation

The presence of different concentrations of NaCl in the growth medium caused distinct patterns of biomass (FW) accumulation in the two *Citrus* cell lines (cvs. 'Carvalho' and 'Valencia late'; Figure 1). Biomass accumulation in 'Valencia late' decreased with NaCl concentration (Figure 1 B). Biomass reductions at 50 mM and at 100 mM NaCl were 60% and 70%, respectively, compared with the NaCl-free treatment. In addition, at 150, 200 and 400 mM NaCl no increase in biomass could be observed (Figure 1 B). In contrast, the 'Carvalho' cell

line was less affected by the presence of NaCl, with similar levels of biomass accumulation at 50, 100, 150 or 200 mM NaCl. And, in the presence of high levels of salt (400 mM), the FW was reduced by 50% compared to the salt-free treatment (Figure 1 A).

Influence of NaCl on intracellular ion concentrations

Intracellular ion uptake at concentrations of NaCl lower than 100 mM showed a similar behaviour in the two cell lines ('Carvalho' and 'Valencia late'; Figure 2). However, for NaCl concentrations higher than 150 mM, the cells of 'Valencia late' showed a greater increase in intracellular Na^+ and Cl^- content (Figure 2 A,B).

After 14 d of growth, the K^+ level in 'Carvalho' decreased with increased NaCl in the growth medium (Figure 2 C). In contrast, the intracellular content of K^+ in the 'Valencia late' cell line did not change significantly under the different saline treatments assayed.

Influence of NaCl on intracellular proline contents

Proline levels increased with increasing NaCl concentrations in both cell suspensions (Figure 3). An analysis of the intracellular accumulation of proline during the 14 d growth cycle showed no significant

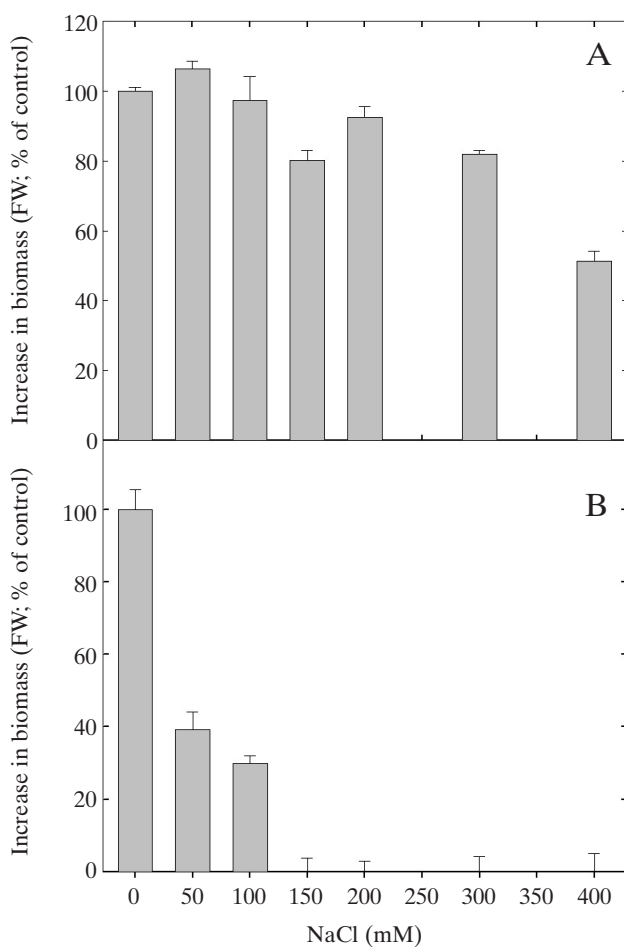


FIG. 1

Influence of NaCl concentration on the biomass (FW) of cell suspensions of 'Carvalho' (Panel A) and 'Valencia late' (Panel B). Results are expressed as percentages of the FW of the salt-free treatment (control), where 100% growth for 'Carvalho' and 'Valencia late' were $110 \pm 3.8 \text{ mg l}^{-1}$ and $92 \pm 6.7 \text{ mg l}^{-1}$, respectively. Bars represent standard errors and results are the means of three replicates.

differences between both cell lines, in the control and in the presence of low NaCl concentrations (50, 100 and 150 mM) in both cell lines. However, at 200 mM NaCl, the intracellular proline content was unchanged for the 'Valencia late' cell line, while in 'Carvalho', a significant increase in proline concentration was observed. Intracellular proline content was significantly higher for 'Carvalho' cells in the presence of high NaCl concentrations (300 and 400 mM) than in 'Valencia late'.

Influence of NaCl on anti-oxidant enzyme activities

Enzyme activities of superoxide dismutase, catalase and guaiacol peroxidase in 'Carvalho' and 'Valencia late' cell lines exposed to different NaCl concentrations (50, 100, 150, 200, 300 and 400 mM) are shown in Figure 4. In the 'Carvalho' cell line, a higher constitutive activity of superoxide dismutase, ($59.2 \text{ U mg protein}^{-1}$) was seen

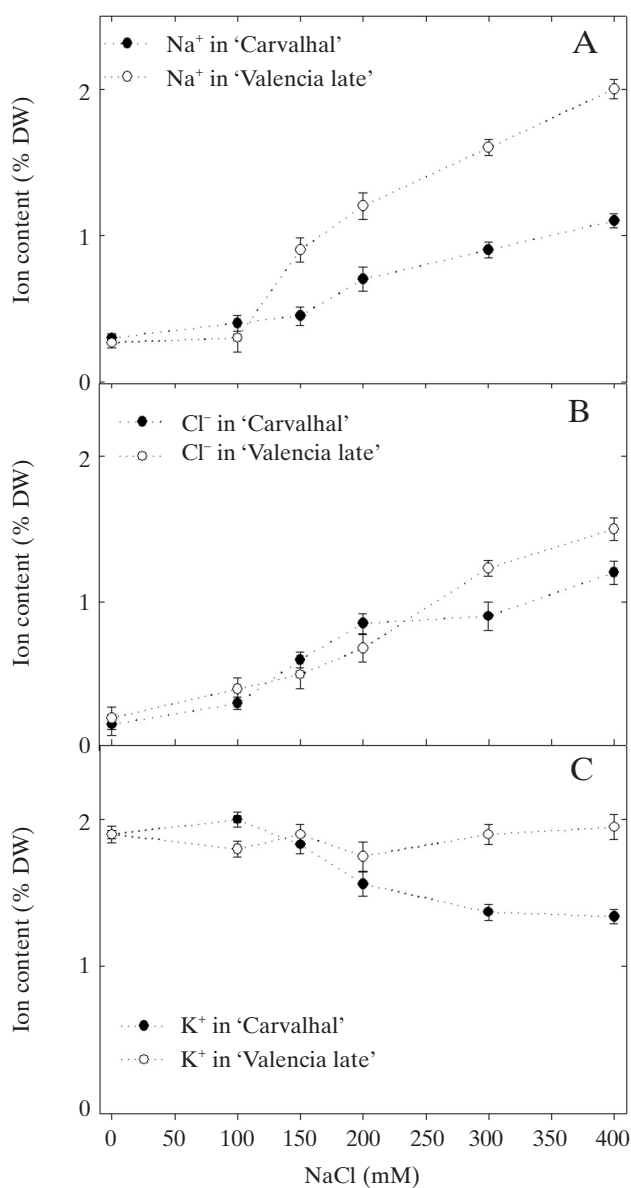


FIG. 2

Influence of NaCl concentration on intracellular concentrations of Na^+ (Panel A), Cl^- (Panel B) and K^+ (Panel C) ions, after 14 d, in cell suspension cultures of 'Carvalho' (closed circles) and 'Valencia late' (open circles). Results are expressed as percentages of DW. Bars represent standard errors and results are the means of three replicates.

than in the 'Valencia late' line (12.1 U mg protein⁻¹) (Figure 4 A). The 'Carvalho' cell line also showed a higher constitutive guaiacol peroxidase activity (60 U mg protein⁻¹) compared to 'Valencia late' (39 U mg protein⁻¹) (Figure 4 C).

In the absence of NaCl treatment, there was an increase in the anti-oxidant enzyme activities in both cell lines after 14 d of growth (Figure 4). The 'Carvalho' cell line, under mild NaCl levels (≤ 100 mM) did not show significant changes in superoxide dismutase activities after 14 d, but the presence of high NaCl concentrations (150, 200 and 400 mM) led to a significant induction of superoxide dismutase, catalase and guaiacol activities. In lower salt conditions (≤ 150 mM), 'Valencia late' showed higher specific activities of superoxide dismutase and catalase than 'Carvalho', with no significant differences ($P \leq 0.05$) between treatments in the superoxide dismutase activities (Figure 4 A, B). Under high NaCl concentrations (200, 300 and 400 mM), a significant decrease in anti-oxidant enzymatic activities was observed in 'Valencia late' cells (Figure 4).

Influence of NaCl on cellular damage

Membrane lipid peroxidation in the two *Citrus* cell lines was assessed by measuring the content of malondialdehyde (MDA) after exposure to NaCl. The effect of increasing salt concentration on MDA formation is shown in Figure 5. In both cell lines, an increase in MDA was observed after 14 d for all NaCl concentrations assayed. However, when exposed to 400 mM NaCl, this increase was higher in 'Valencia late' (72.6 nmol g⁻¹ FW; Figure 5 B) than in 'Carvalho' (46.2 nmol g⁻¹ FW; Figure 5 A).

Genomic DNA contents were quantified and, after 4 d of cell growth in the presence of NaCl, none of the cell lines showed significant differences (Figure 6). However, only in the 'Valencia late' cell line, a decrease in genomic DNA registered after 14 d of exposure to NaCl, particularly 200 mM and 400 mM NaCl (Figure 6 B).

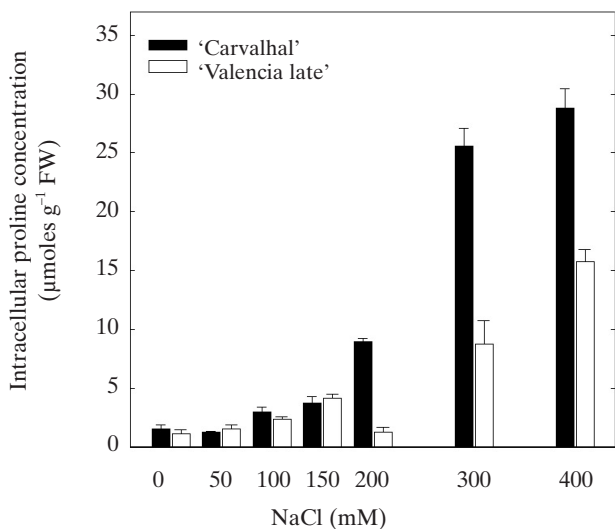


FIG. 3

Influence of NaCl concentration on intracellular proline accumulation in cell suspension cultures of 'Carvalho' (black bars) and 'Valencia late' (white bars), after 14 d. Results are expressed in $\mu\text{moles g}^{-1}$ FW of cells. Bars represent standard errors and results are the means of three replicates.

DISCUSSION

Biomass, ion and proline accumulation

The biomass profile of the cell line 'Carvalho' exposed to different NaCl concentrations was unaffected by moderate NaCl levels (50, 100 or 200 mM), and coped with high salt exposure (300 – 400 mM NaCl), suggesting that this is a salt-resistant cell culture. Ben-Hayyim and Kochba (1982) described a salt-resistant callus of *C. shamouti*. Salt-resistant species, such as beetroot, showed a reduction of 20% in DW, while moderately salt-resistant plants, such as cotton, showed a reduction of 60%. (Greenway and Munns, 1980). In sensitive species, such as soybean, 200 mM NaCl had a lethal effect (Greenway and Munns, 1980).

Avoidance of the uptake of Na⁺ and Cl⁻ ions into the

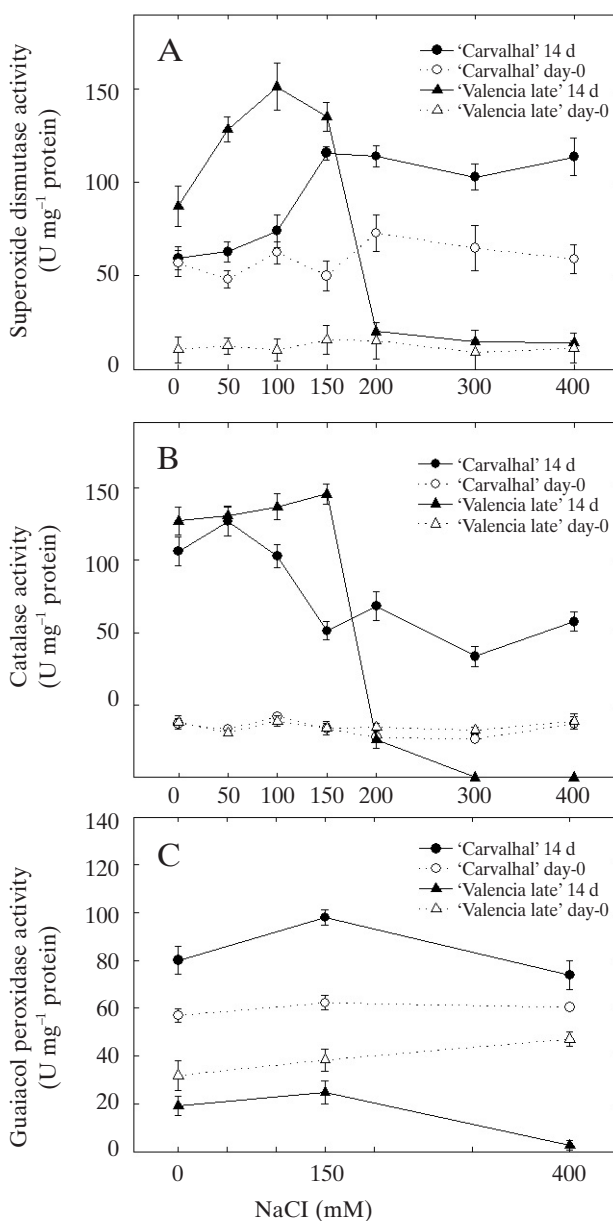


FIG. 4

Influence of NaCl concentration on the activities of superoxide dismutase (Panel A), catalase (Panel B) and guaiacol peroxidase (Panel C), determined on day-0 and after 14 d, in suspension cell cultures of 'Carvalho' (open and closed circles) and 'Valencia late' (open and closed triangles). Results are expressed in U mg⁻¹ protein. Bars represent standard errors and results are the means of three replicates.

cytosol has been suggested as a primary cellular mechanism of salt resistance in glycophyte species. It appears that the 'Carvalho' cell line retained its viability due to partial inhibition of the entry and intracellular accumulation of the toxic Na^+ ion. The K^+/Na^+ selectivity ratio was greater in 'Carvalho' than in 'Valencia late' due to a higher efficiency of 'Carvalho' for excluding Na^+ from its tissues and replacement of Na^+ by K^+ ions. The 'Valencia late' cell line showed higher levels of accumulation of Cl^- and Na^+ ions, and a concomitant inhibition in biomass accumulation. Growth reduction and Na^+ and Cl^- accumulation have also been reported in other cell lines exposed to NaCl. For instance, in *Pisum sativum* calli adapted to 85 mM NaCl, a 65% reduction in DW occurred (Olmos *et al.*, 1994) and, in *Carantheus roseus* cell lines, cell growth was reduced by 67% (Elkahoui *et al.*, 2005). The toxicity of these ions can be a determinant in the metabolic processes that subsequently generate cell damage and necrosis.

Another metabolic response to cope with salinity is intracellular proline accumulation. Proline can act as a store of energy, which can be rapidly used when the plant is relieved of stress. It can also play an active role as an osmoprotectant of key enzymes and membranes and in scavenging free radicals. Our results showed increased accumulation of proline in 'Carvalho' cells adapted to high NaCl (Figure 3). Proline accumulation may also be essential for maintaining turgor and cell division, and

consequently cell viability implied by salt resistance. These results also corroborate a cellular strategy of accumulation of proline related to an osmoregulation mechanism in 'Carvalho' cells, as has been reported for salt-resistant plants (Jain *et al.*, 2001). In halophytic plants, proline functions as a cytoplasmic osmolyte, balancing the Na^+ ion concentration in cytoplasm. The increased proline concentration observed in the 'Valencia late' cell line, can be attributed to protein degradation during exposure to high saline concentrations (300 and 400 mM NaCl). Proline can also function as a molecular marker of salt-damage in salt-sensitive species, thus constituting a reliable indicator of the environmental stress imposed on plants (Claussen, 2005).

Anti-oxidant enzyme systems, peroxidation of lipids and DNA contents

Salt stress tolerance has been correlated with improved tolerance against oxidative stress in several crops (Ashraf and Harris, 2004). Our results suggest that the 'Carvalho' cell line is efficient, at high NaCl levels, in counteracting oxidative damage caused by NaCl due to its higher superoxide dismutase, catalase and guaiacol peroxidase activities than the 'Valencia late' cell suspension culture. 'Valencia late' cultures exposed to high NaCl concentrations (200–400 mM) showed no cell growth and, eventually, necrosis occurred. These results agree with those obtained by Scalet *et al.* (1995) and Davenport *et al.* (2003) in salt-resistant cells.

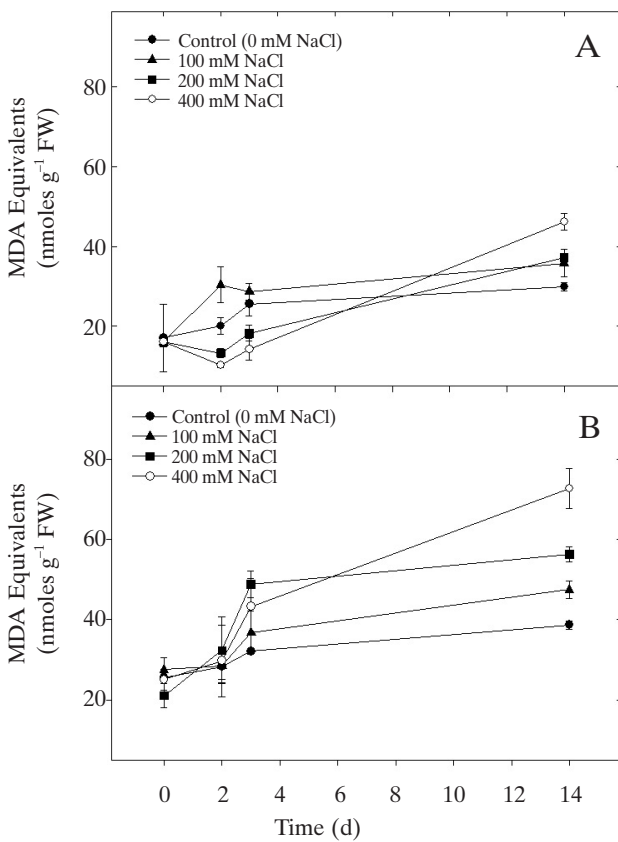


FIG. 5

Influence of NaCl concentration on the accumulation of MDA in cell suspension cultures of 'Carvalho' (Panel A) and 'Valencia late' (Panel B). Results are expressed as MDA equivalents (nmol g⁻¹ FW). Bars represent standard errors and results are the means of three replicates.

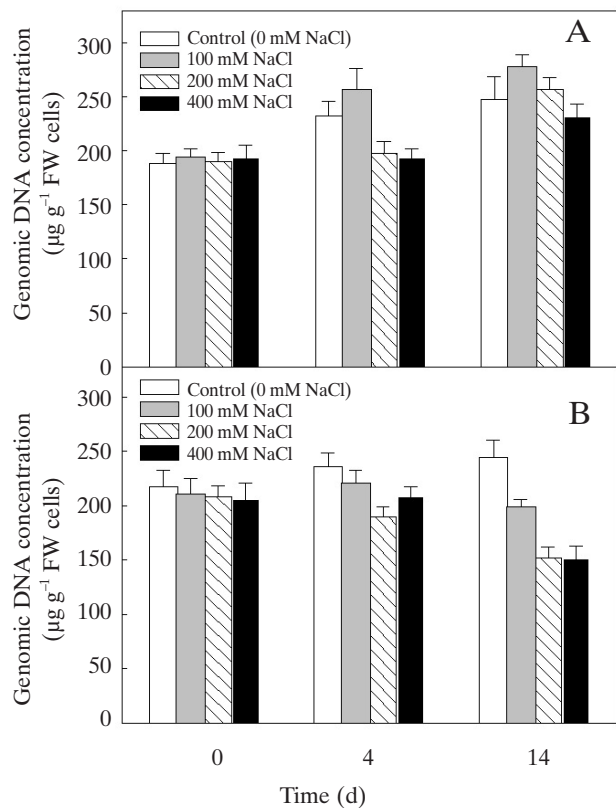


FIG. 6

Influence of NaCl concentration on the genomic DNA content of cell suspension cultures of 'Carvalho' (Panel A) and 'Valencia late' (Panel B). Results are expressed in µg g⁻¹ FW cells. Bars represent standard errors and results are the means of three replicates.

Lipid peroxidation occurs as a result of oxidative stress and has become an essential marker of the extent of cell damage caused by saline stress (Bor *et al.*, 2003). In accordance with our results, it is suggested that ROS that have not been eliminated by anti-oxidant enzymes could be responsible for the initiation of lipid peroxidation. Bandoğlu *et al.* (2004) also showed that lentil leaves exposed to high salinity, had an increased H₂O₂ content (4.4-fold) accompanied by a significant increase in lipid peroxidation and electrolyte leakage. Similar results have been reported for salt-tolerant species such as rice (Dionísio-Sese and Tobita, 1998) and beetroot (Bor *et al.*, 2003). Our results concerning lipid peroxidation indicate that the 'Carvalho' cell line had lower MDA accumulation than 'Valencia late', hence reaffirming its improved ability to resist cellular damage caused by salinity.

Genomic DNA measurements on 'Valencia late' also suggest that DNA damage after 14 d of growth could be caused by MDA generated by lipid peroxidation (Marnett, 1999). DNA destruction can be a slow and late oxidative process, as DNA is not an immediate target for ROS. This fact emphasises the salt-sensitivity of the 'Valencia late' cell

line. In 'Carvalho', DNA damage was not observed at any NaCl level (Figure 6), due to the better performance of its salt resistance strategy. This metabolic efficiency also resulted in lower membrane lipid peroxidation and lower DNA damage (Kononowicz *et al.*, 1990).

CONCLUSIONS

The metabolic responses of *Citrus* cell lines 'Carvalho' and 'Valencia late' to salt stress are distinct. Cell line 'Carvalho' showed a salt-resistant behaviour. This metabolic cell strategy seemed to be due to an efficient mechanism for reducing the cellular concentrations of Cl⁻ and Na⁺ ions, an increase in intracellular proline accumulation, and an increase in ROS-scavenging enzyme activities. In the presence of NaCl, higher levels of MDA accumulated and DNA damage was observed in 'Valencia late' which also confirmed its salt-sensitivity.

This work was supported by a Ph.D. fellowship (BD/21234/99, Praxis XXI) to Ana Luísa Ferreira from the Fundação para a Ciência e a Tecnologia.

REFERENCES

- ASHRAF, M. and HARRIS, P. J. C. (2004). Potential biochemical indicators of salinity tolerance in plants. *Plant Science*, **166**, 3–16.
- BAJJI, M., KINET, J. M. and LUTTS, S. (1998). Salt stress effects on roots and leaves of *Atriplex halimus* L. and their corresponding callus cultures. *Plant Science*, **137**, 131–142.
- BANDEOĞLU, E., EYIDOĞAN, F., YÜCEL, M. and ÖKTEM, H. A. (2004). Antioxidant responses of shoots and roots of lentil to NaCl-salinity stress. *Plant Growth Regulation*, **42**, 69–77.
- BEAUCHAMP, C. and FRIDOVIC, I. (1971). Superoxide dismutase: improved assays and an assay applicable to acrylamide gels. *Analytical Biochemistry*, **44**, 276–287.
- BEERS, R. F. and SIZER, I. A. (1952). Spectrophotometric method for measuring the breakdown of hydrogen peroxide by catalase. *Journal of Biological Chemistry*, **195**, 133–140.
- BEN-HAYYIM, G. and KOCHBA, J. (1982). Growth characteristics and stability of tolerance of *Citrus* callus cells subjected to NaCl stress. *Plant Science Letters*, **27**, 87–94.
- BINZEL, M. L., HASEGAWA, P. M., RHODES, D., HANDA, S., HANDA, A. K. and BRESSAN, R. A. (1987). Solute accumulation in tobacco cells adapted to NaCl. *Plant Physiology*, **84**, 1408–1415.
- BOR, M., OZDEMIR, F. and TURKAN, I. (2003). The effect of salt stress on lipid peroxidation and antioxidants in leaves of sugar beet *Beta vulgaris* L. and wild beet *Beta maritima* L. *Plant Science*, **164**, 77–84.
- BRADFORD, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, **72**, 248–254.
- CLAUSSEN, W. (2005). Proline as a measure of stress in tomato plants. *Plant Science*, **168**, 241–248.
- DAVENPORT, S. B., GALLEGO, S. M., BENAVIDES, M. P. and TOMARO, M. L. (2003). Behaviour of antioxidant defense system in the adaptative response to salt stress in *Helianthus annuus* L. cells. *Plant Growth Regulation*, **40**, 81–88.
- DIONÍSIO-SESE, M. L. and TOBITA, S. (1998). Antioxidant responses of rice seedlings to salinity stress. *Plant Science*, **135**, 1–9.
- ELKAHOUI, S., CARVAJAL, M., GHRIR, R. and LIMAM, F. (2005). Study of the involvement of osmotic adjustment and H⁺-ATPase activity in the resistance of *Catharanthus roseus* suspension cells to salt stress. *Plant Cell, Tissue and Organ Culture*, **80**, 287–294.
- GREENWAY, H. and MUNNS, R. (1980). Mechanisms of salt tolerance in nonhalophytes. *Annual Review of Plant Physiology*, **31**, 149–190.
- HERNÁNDEZ, J. A., JIMÉNEZ, A., MULLINEAUX, P. M. and SEVILLA, F. (2000). Tolerance of pea (*Pisum sativum* L.) to long-term salt stress is associated with induction of antioxidant defenses. *Plant Cell and Environment*, **23**, 853–862.
- HODGES, D. M., DELONG, J. M., FORNEY, C. F. and PRANGE, R. K. (1999). Improving the thiobarbituric acid-reactive-substances assay for estimating lipid peroxidation in plant tissues containing anthocyanin and other interfering compounds. *Planta*, **207**, 604–611.
- JAIN, M., MATHUR, G., KOUL, S. and SARIN, N. B. (2001). Ameliorative effects of proline on salt stress-induced lipid peroxidation in cell lines of groundnut (*Arachis hypogaea* L.). *Plant Cell Reports*, **20**, 463–468.
- KONONOWICZ, A. K., FLORYNOWICZCZEKALSKA, K., CLITHERO, J., MEYERS, A. and HASEGAWA, P. M. (1990). Chromosome-number and DNA content of tobacco cells adapted to NaCl. *Plant Cell Reports*, **8**, 672–675.
- LIMA-COSTA, M. E., FERREIRA, A. L., DUARTE, A. and BELTRÃO, J. (2002). Saline stress and cell toxicity evaluation, using suspended plant cell cultures of horticultural crops grown in a bioreactor. *Acta Horticulturae*, **573**, 219–225.
- MAGNÉ, C. and LARHER, F. (1992). High sugar content of extracts interferes with colorimetric determination of amino acids and free proline. *Analytical Biochemistry*, **200**, 115–118.
- MARNETT, L. J. (1999). Lipid peroxidation - DNA damage by malondialdehyde. *Mutation Research*, **424**, 83–95.
- MITTLER, R. (2002). Oxidative stress, antioxidants and stress tolerance. *Trends in Plant Science*, **7**, 405–410.
- MURASHIGE, T. and SKOOG, F. (1962). A revised medium for rapid growth and bioassay with tobacco tissue cultures. *Physiologia Plantarum*, **12**, 473–497.
- OLMOS, E., HERNÁNDEZ, J. A., SEVILLA, F. and HELLÍN, E. (1994). Induction of several antioxidant enzymes in the selection of a salt-tolerant cell line of *Pisum sativum*. *Journal of Plant Physiology*, **144**, 594–598.
- SCALET, M., FEDERICO, R., GUIDO, M. C. and MANES, F. (1995). Peroxidase activity and polyamine changes in response to ozone and simulated acid rain in aleppo pine needles. *Environmental and Experimental Botany*, **35**, 417–425.
- SCANDALIOS, J. G. (1993). Oxygen stress and superoxide dismutases. *Plant Physiology*, **101**, 7–12.
- STOREY, R. and WALKER, R. R. (1999). *Citrus* and salinity. *Scientia Horticulturae*, **78**, 39–81.