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In vitro selection of salt tolerant cell lines in *Solanum tuberosum* L.

F. QUEIRÓS, F. FIDALGO*, I. SANTOS and R. SALEMA

Departamento de Botânica and Instituto de Biologia Molecular e Celular (IBMC), Universidade do Porto, Rua do Campo Alegre, 823, 4150-180 Porto, Portugal

Abstract

Cell lines able to grow on media containing 50, 100, 150 or 200 mM NaCl were established from potato callus cultures by direct recurrent selection or gradual selection. In callus subjected to direct selection only small clusters of cells survived on medium with 150 or 200 mM NaCl, whereas on 100 mM small cell portions appear necrotic. When cell lines were obtained by successive subcultures on media with increased concentrations of NaCl, salt-tolerant calli were more compact and developed a greenish colour free from necrotic areas. The response of calli lines grown on media with NaCl was compared to control line. The NaCl-tolerant calli showed a decrease in relative growth rate and water content, with higher reductions in the 150 mM tolerant callus. Lipid peroxidation was increased in 50 mM and 100 mM NaCl-tolerant calli, while in 150 mM tolerant callus remained similar to 100 mM values. There was a significant increase in ascorbic acid content in 100 mM and 150 mM NaCl-tolerant calli as compared to the 50 mM, that was two-fold the value found in the control. Also, the contents of soluble and insoluble proteins increased in salt-tolerant lines. SDS-PAGE of soluble proteins showed the synthesis of specific polypeptides in the presence of NaCl in culture medium and the synthesis of a new polypeptide.

Additional key words: ascorbic acid, callus tissue, lipid peroxidation, NaCl tolerance, potato, proteins, salt stress.

Introduction

Salinity is one of the most important abiotic factors in limiting plant productivity (Munns 2002). Salt stress results from a number of detrimental processes including an ion imbalance and toxicity, the impairment of mineral nutrition, a reduction in the water status of the plant tissues and oxidative stress, linked to the production of reactive oxygen species (ROS), which cause damage to lipids, proteins and nucleic acids (Hernández *et al*. 2000). Oxidative stress is considered to be one of the major damaging factors in plants and cells exposed to salinity (Gossett *et al*. 1994, Hernández *et al*. 1995, Khan and Panda 2002). To prevent the potential cytotoxic effects of ROS, plants have developed a highly efficient antioxidant defence system that is formed by non-enzymatic and enzymatic components, which normally maintain ROS balance within the cell (Apel and Hirt 2004).

The processes of salt stress response and tolerance have been studied at the whole-plant level, nevertheless the structural complexity of the whole-plant makes difficult to separate systemic from cellular salinity tolerance mechanisms (Leone *et al*. 1994a, Hawkins and Lips 1997). In recent years, tissue culture techniques have been used as a useful tool to elucidate the cellular mechanisms involved in salt tolerance by using as study system *in vitro* selected NaCl-tolerant cell lines (Davenport *et al*. 2003, Gu *et al*. 2004). Cell lines with enhanced tolerance to NaCl have been isolated from crop plants and various biochemical processes appear to contribute to the adaptation of cells to salinity (Gossett *et al*. 1996, Olmos and Hellín 1996, Rodríguez-Rosales *et al*. 1999, Davenport *et al*. 2003, Lutts *et al*. 2004). Besides the use of tissue culture in selection of salt-tolerant cell lines, these lines have been used to regenerate salt tolerant plants (Shankhdhar *et al.* 2000, Miki *et al*. 2001).

Although the production of salt-tolerant potato cell line and plants was described by Ochatt *et al.* (1999), no information was given about the mechanisms involved in salt adaptation either in the regenerated plants or in the selected cell line. Afterwards, Benavídes *et al.* (2000) using the plants regenerated from *in vitro* selected potato cell line (Ochatt *et al.* 1999) reported a relationship between salt tolerance and the antioxidant defence system. However, a better and deeper understanding

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Abbreviations: AsA - ascorbic acid; BA - benzylaminopurine; MDA - malondialdehyde; ROS - reactive oxygen species; SDS-PAGE - sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TCA - trichloroacetic acid.

* Corresponding author; fax: (+351) 226 099 157, e-mail: ffidalgo@ibmc.up.pt

of the biochemical mechanisms involved in the adaptative response in cultured cells needs to be evaluated. This knowledge is relevant for selecting plants to grow under saline conditions.

In this work, we report an efficient procedure to select potato cell lines tolerant to different concentrations of NaCl. The response of cell lines differing in their salt tolerance was analysed in order to contribute to better understanding of the mechanisms underlying salt tolerance.

Materials and methods

Callus induction and culture: Young leaves collected from 4 to 5-week-old plants (*Solanum tuberosum* L. cv. Désirée) were induced to form callus on Lam (1977; LM) medium supplemented with 1 mg dm^{-3} indole-3-acetic acid (IAA), 0.2 mg dm^{-3} α-naphthaleneacetic acid (NAA), 1 mg dm^{-3} kinetin, 0.5 mg dm^{-3} benzylamino-purine (BA), 0.4 mg dm^{-3} zeatin and 0.5 mg dm^{-3} gibberellic acid (GA_3). The medium was solidified with 0.6 g dm^{-3} agar and the pH adjusted to 5.7. The cultures were maintained at 27 °C under a 12-h photoperiod with a photosynthetic photon flux density of 35 µmol m^{-2} s^{-1} provided by cool fluorescent tubes. After 5 weeks, the resulting callus tissue was separated from the explants and transferred to a multiplication medium [LM with the same basic composition as the above except for growth regulators, which were 0.5 mg dm^{-3} BA and 2 mg dm^{-3} 2,4-dichlorophenoxyacetic acid (2,4-D)]. Callus tissue was subcultured to fresh medium every 4 to 5 weeks for 3 months for further proliferation.

Selection of tolerant lines: Two different procedures were followed to obtain NaCl-tolerant cell lines. Callus tissue grown on LM medium was directly exposed to different concentrations of NaCl (50, 100, 150 and 200 mM); thereafter subcultured onto LM medium without NaCl for 4 weeks. After this period the resulting calli were cultured again on media supplemented with the NaCl concentrations they had tolerated and were subcultured every 4 weeks, for 6 months.

NaCl-tolerant cell lines were also established by progressively growing callus tissue on media with increased concentrations of NaCl. Callus tissue was firstly subcultivated on medium containing 50 mM NaCl for 4 weeks. Then, a portion of the callus tissue was subcultured on medium supplemented with equal NaCl concentration (50 mM) and another portion of tissue was subcultured on medium supplemented with 100 mM NaCl on which it was grown for 4 weeks. After this, one part of the callus tissue was maintained at 100 mM and another part was transferred to medium with 150 mM NaCl. After 4 weeks, one part of the callus grown in the presence of 150 mM was subcultivated on medium with the same concentration of salt, while the remaining callus tissue was placed into culture medium containing 200 mM NaCl for 4 weeks. Cell lines tolerant to 50, 100, 150 or 200 mM were obtained after four successive subcultures on LM fresh medium supplemented with 50, 100, 150 or 200 mM NaCl, respectively. The NaCl-tolerant calli lines obtained through this selection procedure, except for callus tissue grown on 200 mM NaCl, were used for the evaluation of growth and biochemical parameters.

Assessment of growth and determination of water content: Callus tissue grown on medium supplemented with 50, 100 or 150 mM NaCl for four successive subcultures was used to evaluate growth and the water content. Petri dishes were inoculated with callus tissue of about 1 g on fresh mass. Thirty replicates per treatment were used (10 Petri dishes with 3 callus portions each). After 28 d of culture, the increase in fresh mass of the callus was determined. The relative growth rate of callus was calculated as the $(FM_f - FM_i)/FM_i$, where FM_f and FM_i were the final and the initial fresh masses, respectively. Afterwards a portion of the callus was oven-dried at 80 ºC for 48 h and the water content was calculated according to (FM - DM)/FM; where DM was dry mass. Samples of callus tissues were frozen in liquid nitrogen and stored at -80 ºC for further analyses.

Lipid peroxidation: Lipid peroxidation was determined as the amount of malondialdehyde (MDA) using the thiobarbi-turic acid reaction according to Wang *et al.* (1999). Frozen callus tissue was ground to a fine powder in liquid nitrogen with a mortar and pestle. Subsequently about 50 mg of powder was homogenized in 0.5 cm^3 of 5 % (m/v) trichloroacetic acid (TCA) and 2 % (m/v) methanolic butylated hydroxytoluene in an Eppendorf tube. The homogenate was heated at 95 °C for 30 min, then centrifuged at 12 000 *g*, for 20 min. To the aliquot of the supernatant, 0.67 % (m/v) TCA were added and the mixture was heated at 95 °C for another 30 min. After cooling the absorbance was measured at 532 nm using a Shimadzu UV-visible recording spectrophotometer and measurements were corrected for the non-specific absorption by subtracting the absorbance at 600 nm. The concentration of MDA was calculated using the coefficient of absorbance of 155 mM^{-1} cm^{-1}.

Determination of ascorbic acid: Ascorbic acid (AsA) was extracted in 5 % (m/v) metaphosphoric acid, with quartz sand, at 4 °C. The homogenate was then centrifuged at 3 000 *g* for 20 min at 4 °C. AsA content was quantified in the supernatant as described by Shukla *et al.* (1979). In brief, an aliquot of 1 cm^3 of the supernatant was mixed with 2.5 cm^3 of 1 % (v/v) freshly diluted Folin-Ciocalteu reagent. The reaction mixture was allowed to stand at room temperature for 40 min. The absorbance was recorded at 730 nm, using ascorbic acid as a standard.

Protein analysis: Samples of frozen callus tissue were ground in Tris-HCl (60 mM, pH 6.8) with quartz sand, at 4 °C, using a prechilled mortar and pestle. The extract was centrifuged (4 000 *g* for 15 min at 4 °C) and the supernatant was used for protein quantification (soluble protein) according to Peterson (1977). The pellet was re-suspended in the same buffer containing SDS (sodium dodecyl sulphate, 2.5 % m/v) in order to extract the insoluble proteins. The suspension was incubated for 3 h at 4 °C followed by 1 h at room temperature. Then, the suspension was centrifuged for 15 min at 4 000 *g* and insoluble proteins quantified by the same method. One-dimensional SDS-PAGE of proteins was carried out as described by Laemmli (1970) on 10 % polyacrylamide slab gels; the wells were loaded with 10 µg protein and run in parallel with the standard proteins. Gels were silver stained using the procedure of Dunbar (1988) and the relative intensity of the resulting polypeptide band densities was carried out by laser scanning (*GS 800* densitometer, *Bio-Rad*, Hercules, CA, USA) followed by analysis with *Quantity One* image software from *Bio-Rad*. Two gels were done in each independent experiment and the representative results from four gels obtained with material from the two experiments are presented.

Statistical analysis: The results presented are average over two independent experiments with four quantifications within each experiment. Data were processed with analysis of variance (*ANOVA*) and the means were compared using Student's two-tailed *t*-test at a level of significance of $P \leq 0.05$. The differences of variances were checked by Fisher's *F*-test.

Results

Establishment of salt tolerant cell lines: The NaCl-tolerant potato cell lines obtained in this study were selected from callus cultures produced from leaf explants (Fig. 1*A*) by using two procedures, the direct recurrent

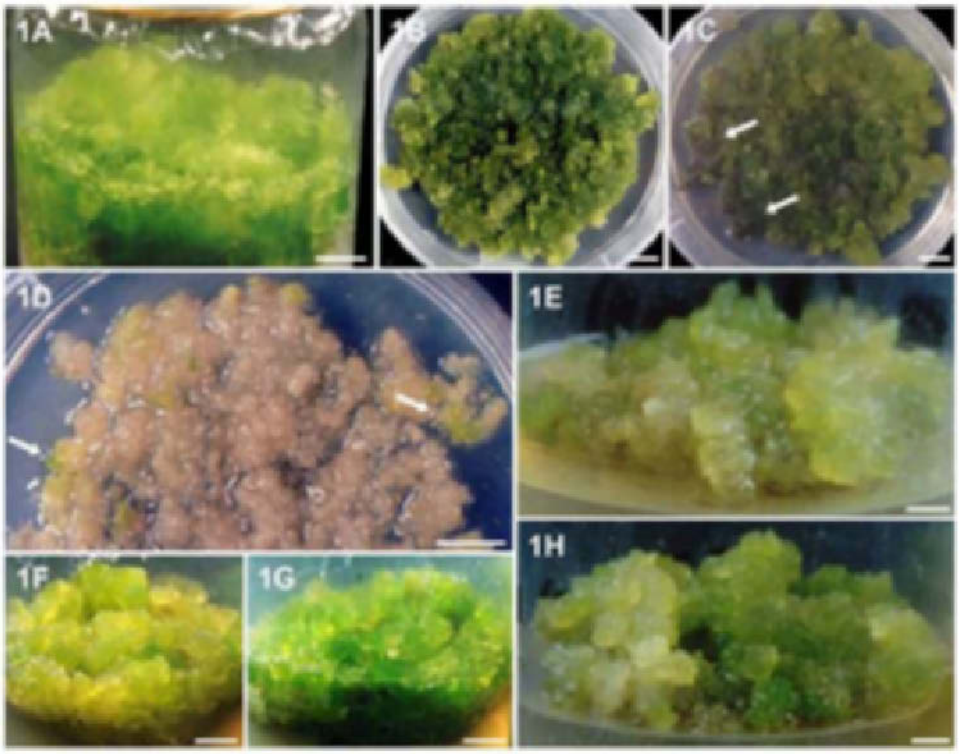

Fig. 1. Selection of NaCl-tolerant potato cell lines from established callus cultures by direct (*B-F*) and gradual (*G-H*) exposure to increased NaCl concentration. *A* - Callus tissue grown in LM medium used for tolerant cell lines selection without NaCl; *bar* = 0.83 cm. *B* - Callus tissue directly exposed to medium with 50 mM NaCl, after 4 weeks of culture; *bar* = 0.52 cm. *C* - Callus tissue directly exposed to 100 mM NaCl showing small necrotic portions (*arrows*); *bar* = 0.55 cm. *D* - Aspect of callus tissue when subcultured directly in medium containing 150 mM NaCl for 4 weeks; note only the small cell clusters that survived in the presence of salt and maintained greenish (*arrows*); *bar* = 1.0 cm. *E* - NaCl-tolerant callus tissue grown on medium with 200 mM NaCl after 6 months of culture; *bar* = 0.63 cm. *F* - 150 mM NaCl-tolerant callus line after 6 months of culture; *bar* = 0.62 cm. *G* - 150 mM NaCl-tolerant callus tissue obtained by gradual selection, after 6 months of culture; note that this salt-tolerant line was more compact and greenish than the corresponding callus obtained from direct selection procedure (compare with *F*); *bar* = 0.62 cm. *H* - 200 mM NaCl-tolerant callus tissue after 6 months of culture; note that this salt-tolerant line was more abundant and greenish than the corresponding callus obtained from direct selection procedure (compare with *E*); *bar* = 0.56 cm.

Table 1. Relative growth rate (RGR considering the value determined for the control as 100 %, n = 30), water content, MDA and ascorbic acid (AsA) contents, and amounts of soluble and insoluble proteins in control callus tissue (0 mM NaCl) and salt-tolerant calli grown on media containing 50 mM, 100 mM or 150 mM NaCl. Data are means ± SE. In the same column values denoted by different letter are significantly different by Student's t-test at 5 % level.

NaCl [mM]	RGR [% of control]	Water content [%]	MDA [nmol g^{-1}(f.m.)]	AsA [μg g^{-1}(f.m.)]	Soluble proteins [mg g^{-1}(f.m.)]	Insoluble proteins [mg g^{-1}(f.m.)]
0	100^a	98.00 ± 0.06^a	9.29 ± 0.27^a	117.60 ± 6.81^a	1.30 ± 0.04^a	1.54 ± 0.04^a
50	72^b	97.47 ± 0.22^a	10.98 ± 0.33^b	152.72 ± 2.79^b	1.81 ± 0.04^b	2.04 ± 0.07^b
100	59^c	96.52 ± 0.03^b	13.46 ± 0.46^c	243.35 ± 9.05^c	2.52 ± 0.14^c	2.35 ± 0.04^c
150	31^d	95.69 ± 0.07^c	14.68 ± 0.39^c	275.68 ± 10.04^c	2.34 ± 0.03^c	2.32 ± 0.07^c

selection (Fig. 1*B-F*) and gradual selection processes (Fig. 1*G-H*). Both procedures were successful in selection of potato cell lines exhibiting tolerance to 50, 100, 150 or 200 mM NaCl, however, the better results were obtained with the gradual selection.

Callus tissue cultured on medium containing 50 mM NaCl (Fig. 1*B*) showed a good cell proliferation and appeared morphologically similar to control callus (Fig. 1*A*), whereas callus tissue cultured on medium with 100 mM NaCl showed moderate cell proliferation and small portions displaying a brownish colour were observed indicating cell necrosis (Fig. 1*C*). When callus was cultured on medium supplemented with 150 and 200 mM NaCl, a great mass of cells did not show any sign of growth, and turned brown within 2 weeks of culture. However, small clusters of cells survived and persisted greenish (Fig. 1*D*). Subsequently, the cell clumps that proliferated in the presence of the salt were picked up and subcultured on NaCl-free medium (0 mM NaCl control) for 4 weeks. The salt-tolerant cell lines were again transferred onto a fresh media supplemented with 50, 100, 150 or 200 mM NaCl and proliferated into a light-green calli exhibiting a friable aspect (Fig. 1*E-F*).

The gradual selection process resulted in the establishment of NaCl-tolerant callus lines showing a good cell proliferation, in spite of callus growth rate decreased with the increase of salt concentration on culture medium (Fig. 1*G-H*). Compared to direct selection, callus lines selected by this process had in common a dark green colour and a high compactness. No necrotic portions were observed even when the callus tissue was subcultured on medium with the highest salt level tested (Fig. 1*H*). Take into account this result, we used NaCl-tolerant calli obtained by gradual selection to evaluate the effect of NaCl on different parameters. However, 200 mM NaCl-tolerant calli obtained either by direct or gradual selection processes did not sustain a regular growth on salt media. These calli suffered with time a decrease in cell proliferation and all callus tissue died when the cultivation was prolonged beyond a year.

Relative growth rate and water content: Salinity affected significantly the callus growth of NaCl-tolerant cell lines. In spite of the ability of the selected salt-tolerant lines to grow on the presence of NaCl, tolerant calli growth lower than control. RGRs were reduced by 28 and 41 % at 50 and 100 mM NaCl, respectively, whereas in calli grown on 150 mM a higher reduction in RGR was observed (69 %).

The presence of 50 mM on medium did not affected the water content of the callus tissue as no significant difference was found when compared to control tissue. On contrary, in callus tissue grown on the presence of 100 or 150 mM NaCl a significant decrease in water content was quantified (Table 1).

MDA content: MDA content increased with increasing NaCl concentration in the culture medium. Compared to control callus tissue (0 mM NaCl), an increase in the MDA content of about 18 % was observed in 50 mM NaCl-tolerant callus. MDA content was 45 % higher in callus tissue grown on 100 mM NaCl with respect to control one, indicating a significantly higher level of lipid peroxidation in comparison with the level found in tissue grown on media with 50 mM NaCl. The highest level was detected in 150 mM NaCl-tolerant callus tissue, however, this value was not significantly different from that found in callus tissue grown on medium with 100 mM (Table 1).

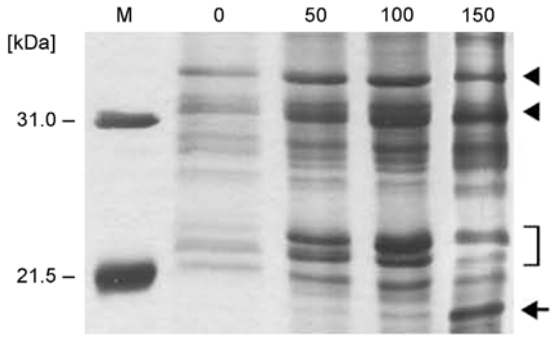

Fig. 2. SDS-PAGE of soluble proteins. In comparison to control callus (*lane* 0), in gels of NaCl-tolerant calli (*lane* 50 - 50 mM NaCl, *lane* 100 - 100 mM NaCl and *lane* 150 - 150 mM NaCl) some polypeptides appeared heavily stained (*arrow-heads* and *bracket*) and a new polypeptide with a molecular mass of about 17 kDa was detected, which appeared heavier stained in 150 mM NaCl-tolerant calli (*arrow*). Equal amount of protein were loaded on the gels. *Lane* M: protein markers.

Ascorbic acid and protein contents: AsA content was significantly greater in the NaCl-tolerant calli lines than in the control line grown on the 0 mM NaCl medium. An increase of about 30 % was found in callus tissue grown at the 50 mM NaCl, while in callus grown on medium supplemented with 100 mM NaCl the AsA content was two-fold that in the control and higher *ca.* 59 % than in tissue grown at 50 mM NaCl. However, no differences in AsA content among 100 and 150 mM NaCl-tolerant calli were detected (Table 1).

Callus tissue grown in the presence of NaCl showed an increase in both soluble and insoluble proteins (Table 1). This increase was higher in tissue grown on medium with 100 and 150 mM NaCl where the two-fold amount of soluble proteins with respect to control was found, whereas in the presence of 50 mM NaCl increase was 39 %. A similar increase was found in tissue grown on medium with 100 mM NaCl when compared with the content of soluble proteins observed in 50 mM-tolerant calli. The changes in insoluble proteins content of NaCl-tolerant calli showed similar tendency to those found in soluble proteins, although the increase in soluble proteins was more pronounced than the rise in insoluble proteins whatever the NaCl concentration in medium. The analysis of electrophoretic pattern of soluble proteins showed that NaCl induced the appearance of a new polypeptide with a molecular mass of about 17 kDa, whose synthesis was increased in 150 mM-tolerant calli (Fig. 2). Moreover, it was possible to identify a set of polypeptides whose synthesis was up-regulated in NaCl-tolerant calli. In this group, the synthesis of polypeptides with a molecular mass of about 32 and 34 kDa was found to be enhanced in calli lines grown on medium with NaCl. In addition, the synthesis of two polypeptides with a molecular mass of about 22 and 24 kDa appeared increased in 50 mM and 100 mM NaCl-tolerant calli compared to control, whereas the synthesis of 22 kDa polypeptide was not affected by the higher NaCl concentration (Fig. 2).

Discussion

We successfully selected potato cell lines that can grow on medium containing NaCl. The salt-tolerant cell lines were obtained by using two procedures: exposure of potato callus to a gradual stepwise increase in NaCl concentration or to a single step increase in NaCl concentration. When this later procedure was used the two higher concentrations of NaCl (150 and 200 mM) were lethal to a great population of cells, and only small clusters of cells survived. In contrast, the establishment of salt-tolerant cell lines by progressively growing callus tissue on media with gradual rises in NaCl concentrations was more successful. Salt-tolerant calli appeared more greenish, free from necrotic parts and showed a good cell proliferation. Thus the gradual selection process was more suitable and efficient than the direct one to establish salt tolerant potato calli. A similar response was described in salt-tolerant rice plants established *in vitro* by using the step up NaCl treatments procedure (Miki *et al.* 2001). There is evidence that gradual exposure to increasing intensity of stress-inducing factor allows physiological and biochemical adjustments, which are the basis for a new cellular homeostasis compatible with the imposed stress (Leone *et al*. 1994b). On the contrary, a single step exposure to a stress factor leads to irreversible cellular injuries, as previously demonstrated by Leone *et al.* (1994b) for potato cell-suspension cultures.

Cell lines exhibiting tolerance to NaCl have been selected from a range of species (Gossett *et al*. 1996, Olmos and Hellín 1996, Ochatt *et al*. 1999, Shankhdhar *et al.* 2000, Davenport *et al*. 2003). Once selected, salt tolerant cell lines could be transferred to a regeneration medium (Ochatt *et al.* 1999) or maintained in salinity conditions to study the biochemical changes induced by NaCl (Olmos and Hellín 1996, Rodríguez-Rosales *et al*. 1999, Davenport *et al.* 2003). In our study, the increase in NaCl concentration in media reduced growth rate of potato callus lines cultivated in the presence of NaCl. In fact, the reduction in growth is a common phenomenon of salt stressed plants (Benavídes *et al*. 2000, Fidalgo *et al.* 2004, Sotiropoulos *et al.* 2006), which has also been observed in cultured cells on medium supplemented with NaCl (Rus *et al*. 1999, Shankhdhar *et al.* 2000, Gu *et al.* 2004, Lutts *et al*. 2004, Niknam *et al*. 2006). As reported by Zhu (2001), 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.

A general response to high salinity seems to be the alteration in the water status at the cellular level (Hasegawa *et al.* 2000). The water content was decreased when potato callus tissue was grown on 100 or 150 mM NaCl. A study in cell lines of pea also showed that the water content was decreased in the adapted line growing on medium with NaCl (Olmos and Hellín 1996). As referred by Dracup (1991), the reduction in the water content observed may be due to the high osmotic pressure of the culture medium with high salt concentration. Thus, the salt tolerance at the cellular level seems to be related to the capacity of callus to resist dehydration (Binzel *et al.* 1985).

Salt stress is known to result in extensive lipid peroxidation (Hernández *et al*. 2000). Indeed, calli lines subjected to NaCl showed an increase in lipid peroxidation, which means that an oxidative stress is induced by salt conditions. Several studies also showed a correlation between an accumulation of lipid peroxidation products and high salinity (Khan and Panda 2002, Davenport *et al.* 2003, Özdemir *et al*. 2004).

Another metabolic response of many plants to salinity is the increased synthesis of ascorbic acid. Our results showed that ascorbic acid increased in calli lines in

response to NaCl. A positive correlation between ascorbic acid content and salinity was also observed in NaCl-tolerant cell lines of other species (Gossett *et al.* 1996, Olmos and Hellín 1996), as well as in salt-tolerant plants (Gossett *et al*. 1994, Hernández *et al*. 1995, Benavídes *et al*. 2000). This antioxidant compound is one of the most effective free radical scavengers implicated in the adaptation of plants to environmental stresses (Smirnoff and Wheeler 2000). As emphasised by Shigeoka *et al.* (2002), a high content of ascorbic acid is essential for protection against oxidative stress.

It is known that the adaptation to salt stress also involves changes in plant gene expression (Borsani *et al*. 2003). We found that in callus cultures exposed to salt the amount of both soluble and insoluble proteins increased. The pattern of soluble proteins revealed several polypeptides (22, 24, 32 and 34 kDa) whose synthesis was increased in calli lines grown in the presence of NaCl, and the appearance of a novel 17 kDa polypeptide, that was over-expressed in 150 mM NaCl-tolerant calli. To be noted that 32 and 34 kDa polypeptides have molecular mass similar to CDSP 32 and CDSP 34 proteins identified in potato plants subjected to water deficit (Pruvot *et al*. 1996a). These drought-induced stress proteins were also observed in salt-stressed plants, and it was suggested that they are associated with the tolerance to osmotic stress (Pruvot *et al.* 1996b). As it is well known, plant responses to salt and water stress have much in common (Munns 2002). In addition, the polypeptide of apparent molecular mass of 24 kDa may be related with osmotin, a protein which accumulates in tobacco cells adapted to NaCl (Singh *et al.* 1987) and also in cultured potato cells adapted to low water potential (Leone *et al.* 1994a,b).

In conclusion, the results reported in this paper suggest that the gradual selection is the most efficient procedure for the establishment of salt-tolerant potato cell lines. NaCl-tolerant cell lines can be a useful model to improve the understanding of the biochemical mechanisms of salt tolerance.

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