In Vitro Selection of a Salt-tolerant Embryogenic Line in Habanero Pepper (*Capsicum chinense* Jacq.)

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Abstract. The effect of NaCl salinity on growth and development of somatic embryos of Habanero pepper was examined. Addition of 75 and 100 mM NaCl into the medium greatly increased the growth and development of somatic embryos and both of these concentrations favored the proliferation of somatic embryos. However, supplementation of 200 and 300 mM NaCl to the medium showed a negative effect on the growth and development of somatic embryos. Concentration increases of NaCl provoked a significant reduction of the embryos survival rate with the average lethal dose (46%) being registered in the treatment of 100 mM. Furthermore, a lower tolerance to salt stress (NaCl) was observed in deformed somatic embryos. Concentrations of 200 and 300 mM NaCl significantly delayed development in the surviving embryos in both treatments. These embryos remained at the globular stage throughout culture time. At 75 mM NaCl, most of the embryos were observed in the torpedo stage. However, the embryos exposed to 100 mM NaCl were observed mainly in globular and cotyledonar stages. It is quite likely that the transition from one intermediate stage of development to another occurs rapidly. With the exception of the concentration at 300 mM NaCl, salt stress stimulated embryonic germination, particularly at 100 mM NaCl. The content of proline in somatic embryos increased substantially in response to salinization. The results suggest that somatic embryos of *C. chinense* can tolerate concentrations of NaCl up to 100 mM without their development being affected. Moreover, they have sufficient cellular mechanisms to tolerate salinity at relatively higher levels.

Salinity has been recognized as a major factor limiting crop productivity, especially in irrigated areas. It is estimated that ≈400 million hectares of land, from the total of earth's surface, are affected by salinity (Flowers et al., 1977). The increase in salt concentration in the soil is one of the most serious environmental threats to plant survival.

Plants respond to salt stress at three different levels, i.e., cellular, tissue, and whole plant level (Borsani et al., 2003). The separate study of each level of response is the best way to correctly place the pieces to understand the whole picture of salt tolerance. However, as plant cells become specialized during ontogeny, it is clear that the

adaptive mechanisms to tolerate salt stress may be different. Reports confirm that salt stress can bring about physiological, biochemical, and genetic changes in plants (Dajic, 2006).

In vitro culture is a useful tool to evaluate the effect of salinity and to select salt-tolerant varieties in plant species (Davenport et al., 2003; Queirós et al., 2007). The method of selection under pressure has been used to obtain salt-tolerant somatic embryos in species such as *Vitis* (Lebrun et al., 1985), wheat (Galiba and Yamada, 1988), *Brassica juncea* (Kirti et al., 1991), wheat (Arzani and Mirodjagh, 1999), eggplant (Mukherjee, 2002), *Zea mays* (Urechean, 2003), *Triticum durum* (Zair et al., 2003), and sugarcane (Gandonou et al., 2005). Unnikrishnan et al. (1991) reported that somatic embryos of *S. trifoliatus* can tolerate high concentrations of NaCl without affecting growth. The aim of this study was to evaluate the behavior of somatic embryos of Habanero pepper undergoing different NaCl concentrations during in vitro development.

Preparation of plant material. Seeds of Habanero pepper cv. Mayan Balache were surface-sterilized with a solution of ethanol at 70% for 5 min, rinsed three times in sterile distilled water, soaked in a solution of commercial sodium hypochlorite at 13% for 15 min, and rinsed again three times (1 min) in sterile distilled water. The sterile seeds were cultured in glass jars with 20 mL germination medium composed of mineral salts recommended by Murashige and Skoog (1962) (MS) supplemented with 1.156 μM GA₃, 3% sucrose, and 0.2% Gelrite. The pH was adjusted to 5.7 before sterilization in an autoclave at 121 °C for 15 min. The cultures were incubated in darkness at 25 ± 2 °C for 7 d to accelerate germination. After the seeds had germinated, they were transferred to a photoperiod of 16 h lights (40–50 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) at 25 ± 2 °C. The hypocotyls were extracted from the dissection of plantlets at 15 d of germination.

Osmotic potential, water potential, and turgor potential determination. The somatic embryos to different concentrations 0, 75, 100, 200, and 300 were defrosted and they were used to determine the osmotic potential (ψ_s) using a vapor pressure osmometer (VAPRO-5520, Wescor Inc., Logan, UT). Water potential of the somatic embryos was assumed to be equal to the solute potential of the culture medium (ψ_s of the medium corresponds to the water potential of the tissue). Somatic embryos turgor potential was calculated as the difference between water potential (ψ) and ψ_s of the somatic embryos. Three samples of each treatment were used.

Induction of somatic embryogenesis. To induce somatic embryogenesis, the protocol reported by Zapata-Castillo et al. (2007) was used. Hypocotyl explants were cultured in the salts recommended by Murashige and Skoog (1962) and were always supplemented with 9.05 μM 2,4-D, 3% sucrose, 206.35 μM cysteine-HCl, 554.93 μM myo-inositol, and 29.64 μM thiamine-HCl. The media were solidified with 0.2% Gelrite and the pH was adjusted to 5.8 before they were sterilized in an autoclave (121 °C for 15 min). The cultures were incubated at 25 ± 2 °C under continuous light conditions (40–50 $\mu\text{mol}\cdot\text{m}^{-2}\cdot\text{s}^{-1}$) for 4 weeks.

Evaluation of the effect of NaCl on somatic embryo development. To evaluate the effect of NaCl on the development of somatic embryos, different NaCl concentrations (0, 75, 100, 250, and 300 mM) were added to the MS medium supplemented with 4.5 μM 2,4-D, 3% sucrose, 206.35 μM cysteine-HCl, 554.93 μM myo-inositol, and 29.64 μM thiamine-HCl. Eight hundred somatic embryos were used per treatment. After 4 weeks, all treatments were evaluated.

Normal, deformed, and dead embryos (%). All embryos in Erlenmeyer flasks (250 mL; Pyrex) were counted with a Nikon stereoscope (MilesCo Scientific) and were registered with photographic evidence.

Dry weight and water contents. After 30 d of salt culturing, fresh and dry weights were

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recorded. One hundred milligrams of somatic embryos were lyophilized and the water content was calculated according to (fresh weight – dry weight)/fresh weight, in which dry weight was dry mass. Three samples of each treatment were used.

Germination of somatic embryos (%). After pretreatment with NaCl, the somatic embryos were transferred to germination liquid medium (50 mL) composed of MS salts, 1.156 μM GA₃, and 3% sucrose. The cultures were incubated at $25 \pm 2^\circ\text{C}$ in darkness for 15 d and later at continuous light for another 15 d.

Proline determination. Free proline content was determined according to Bates et al. (1973). One hundred milligrams of dry weight of somatic embryos was homogenized in 3% aqueous sulphosalicylic acid and was filtered (Whatman No. 1 paper). The supernatant was mixed with acid ninhydrin and glacial acetic acid in a test tube and boiled at 100°C for 1 h. The reaction was stopped by cooling the tubes in an ice bath. The chromophore formed was extracted with toluene and the absorbance of the resulting organic layer was measured at 520 nm (Genesis 10uv). The concentration of proline was estimated by referring to a standard curve prepared using L-proline.

Determination of ion content. Oven-dried somatic embryos were digested in 200 mM HCl and 10 mM MgCl₂ for 12 h. After complete digestion of the sample, the final volume was adjusted to 50 mL with distilled water and the contents of Na⁺ and K⁺ were determined by inductively coupled plasma emission mass spectroscopy (Perkin-Elmer PE3100).

Protein extraction. Somatic embryos (100 mg) were homogenized at 4°C with 300 μL of extraction buffer [11 mM Tri-HCl pH 7.5, 0.45 mM polyvinylpyrrolidone (PVP-40), 0.75 mM sucrose, 0.042 mM ethylenediaminetetraacetic acid, 0.002 mM ascorbic acid, 0.005 mM bovine serum albumin, 10 mM MgCl₂, 1 mM CaCl₂]. The homogenate was vortexed for 5 min and centrifuged at 13,000 rpm for 10 min. The supernatant obtained was used for estimation of protein content (Bradford, 1976).

Protein electrophoresis. The total protein equivalent to 4 μg was subjected to sodium dodecyl sulphate–poly acrylamide gel (12.5% polyacrylamide) at 24°C for 3 h at a constant current of 160 V (Laemmli, 1970). The protein gels were stained with silver and relative molecular weights were determined using a standard molecular weight marker mix (Invitrogen).

Data registered and statistical analysis. All experiments were repeated at least three times. Each treatment was photographed with a Kodak camera. The data obtained were analyzed by one-way analysis of variance with post hoc comparison of group means in the Tukey test. Significance was accepted with a 95% confidence level using SPSS 16.0 (SPSS Inc., Chicago, IL) for Windows as statistical software. The graphics were plotted with the SigmaPlot 11.0 program.

Results and Discussion

During the first week of the somatic embryogenesis induction, a slight increase in diameter of hypocotyl explants was observed. Two weeks later, the epidermis was broken spontaneously leaving a string of visible proembryos along the vascular bundles, confirming what was reported by Lopez-Puc et al. (2006) and Zapata-Castillo et al. (2007). At the fourth week of culture, the embryos were observed at early development stages (globular and heart-shaped).

Effect of NaCl on osmotic potential, water potential, and turgor potential in somatic embryos. Osmotic and water potential decreased

significantly with increasing concentration of NaCl in the culture medium (Table 1). Osmotic potential is one of the most important parameters often affected by abiotic stress. Under salinity stress, the ψ_s in tolerant plants is reduced with the increasing intensity of stress. This reduced ψ_s helps the plants uptake more water and maintain growth (Almansouri et al., 2000).

Effect of NaCl on the survival and development of somatic embryos. The results obtained from exposing Habanero pepper somatic embryos (SEs) to different concentrations of NaCl showed significant differences between treatments for all the variables evaluated in comparison with the control treatment

Table 1. Osmotic potential, water potential, and turgor potential in somatic embryos of *C. chinense* subjected to different concentrations of NaCl.

NaCl concn (mM)	Osmotic potential (–MPa)	Water potential (–MPa)	Turgor potential (MPa)
0	0.6259 + 0.0067 e	0.1375 + 0.0028 e	0.4884 + 0.0073 e
75	1.1200 + 0.0067 d	0.3200 + 0.0014 d	0.8 + 0.0649 d
100	1.2900 + 0.0599 c	0.3825 + 0.0052 c	0.9075 + 0.0614 c
200	2.2225 + 0.0190 b	0.5891 + 0.0106 b	1.6333 + 0.0297 b
300	2.7691 + 0.0528 a	0.9291 + 0.0491 a	1.8400 + 0.0534 a

Means $\pm$ SE, n = 3. Within each set of experiments, files with different letters on same treatment are significantly different ($P \leq 0.05$).

Fig. 1. Effect of different NaCl concentrations on somatic embryos of *Capsicum chinense*: (A) deformed, normal, and dead somatic embryos (SEs); (B) developmental stages. Error bars indicate SE (n = 3). Within each set of experiments, bars with different letters on same treatment are significantly different ($P \leq 0.05$).

(Fig. 1A). Embryo survival declined as the concentration of NaCl was increased in the culture medium. At 100 mM NaCl, the survival rate was 46%. Higher concentrations of NaCl provoked a drastic reduction in embryo survival. Deformed embryos showed greater sensitivity to salt stress in comparison with normal embryos and were unable to survive concentrations above 100 mM NaCl. Embryo development was also affected by the concentration of NaCl in the culture medium. Figure 1B shows that, at concentrations of 200 and 300 mM NaCl, most of the surviving SEs were at the globular stage, whereas at 75 mM NaCl, the most abundant embryos were at torpedo and cotyledonary stages. Similar results were reported by Rai (2010) working with somatic embryos of guava subjected to 150–200 mM NaCl in the culture medium. Figure 2Ia–e shows that 1 week after SEs were transferred to treatment with NaCl, morphologic changes were not observed compared with the control treatment (Fig. 2Ia), although it was apparent there was a slight reduction in the number of embryos surviving at higher concentrations of NaCl (200 and 300 mM) (Fig. 2Id–e). Three weeks later, SEs exposed to salt stress showed a significant size reduction, improving its appearance (shape and color) in treatments with lower NaCl concentrations (Fig. 2IIb–c). Probably these changes of SE appearance could be attributed to the reduction of endogenous water content of embryos. However, at this time, the number of surviving embryos had already been significantly reduced, particularly in treatments with higher concentrations of NaCl (Fig. 2IIa–e).

Behavior of dry weight and water content in somatic embryos. As is shown in Figure 3, water content fell significantly, whereas dry weight (DW) increased when SEs were exposed to increasing concentrations of NaCl in the culture medium. The enhanced ionic uptake, mainly Na^+ and Cl^- , and increased production of proline (Al-Khayri, 2002) play a vital role in DW increasing under NaCl-induced osmotic stress, and free amino acids, soluble proteins, and soluble carbohydrates also increased the DW (Al-Khayri, 2002). These results are consistent with those reported by Errabii et al. (2007) and Ahmad et al. (2007) who analyzed callus of *Saccharum* sp. and *Oryza sativa* L., respectively, after subjecting them to different concentrations of NaCl.

Germination of somatic embryos subjected to salt stress (NaCl). Except the treatment with 300 mM NaCl, the embryos from the other saline treatments showed better germination response, particularly in root emission, in comparison with embryos from the control treatment (Fig. 4A). Embryos subjected to 75 mM NaCl presented profuse rooting with very long disproportionate roots in relation to the size of the embryo (Fig. 4B), whereas the embryos treated with 100 and 200 mM NaCl formed well-proportioned roots and cotyledons (Fig. 4C–D). Conversion to plantlet was not observed. Embryos from the treatment with 300 mM NaCl did not germinate (Fig. 4E).

Figure 5 shows germinated embryos after treatment with 100 mM NaCl. These results allow us to infer that salt stress (NaCl) can substantially improve SE germination of a species (*C. chinense*), recognized as recalcitrant to germination and conversion to plants.

Behavior of proline content in somatic embryos. Proline content in SEs increased considerably in response to concentrations of NaCl in the culture medium. Highly significant differences were observed for the treatment (Fig. 6). The accumulation of proline

was greater ($196.11 \mu\text{mol proline/g DW}$) in the treatment with the higher concentration of NaCl (300 mM). In plants, a common response to osmotic stress is the accumulation of compatible osmolytes. Under stress conditions, monosaccharides, disaccharides, trisaccharides, and sugar alcohols accumulate in plants, and the gene expression levels of some of the relevant enzymes also increase accordingly. Engineering biosynthetic pathways for the production of osmolytes such as galactinol, fructans, trehalose, ononitol, proline, and

Fig. 2. Effect of different concentrations of NaCl on somatic embryos: (A) 0 mM; (B) 75 mM; (C) 100 mM; (D) 200 mM; (E) 300 mM. (I) One week of culture; (II) 3 weeks of culture.

Fig. 3. Water content and dry weight in somatic embryos of *C. chinense* subjected to different concentrations of NaCl. Error bars indicate SE ($n = 3$). Within each set of experiments, bars with different letters on same treatment are significantly different ($P \leq 0.05$).

Fig. 4. Germination of Habanero pepper somatic embryos after treatment with NaCl and transfer to germination medium (Murashige and Skoog + $1.156 \mu\text{M GA}_3$). (A) 0 mM NaCl; (B) 75 mM NaCl; (C) 100 mM NaCl; (D) 200 mM NaCl; and (E) 300 mM NaCl.

Fig. 5. Germination of Habanero pepper somatic embryos from the treatment containing 100 mM NaCl.

glycine betaine to modulate osmotic pressure have been shown to be an effective means of enhancing plant abiotic stress tolerance (Qin et al., 2011). According to Mademba et al. (2003), proline accumulation helps to stabilize proteins at high ionic strength or at low water conductivity. Several studies have shown that exposure to increasing sodium chloride concentrations caused an increase in proline content in *Hordeum marinum* and *Hordeum vulgare* (Garthwaite et al., 2005); *Phoenix dactylifera* callus (Al-Khayri, 2002); Carrizo citrange (Arbona et al., 2003); SEs of *Sapindus trifoliatus* L. (Unnikrishnan et al., 1991); *Saccharum* sp. callus (Errabii et al., 2007); *Oryza sativa* L. callus (Ahmad et al., 2007); and *Phaseolus vulgaris* L. callus (Stoeva and Kaymakanova, 2008).

Sodium and potassium content in somatic embryos under salt stress. With increasing concentrations of NaCl in the medium, the level of K^+ in somatic embryos declined, whereas Na^+ content increased significantly (Table 2). The results of the present study may be interpreted on the basis of an earlier assumption that the excess of Na^+ compensates for the loss of K^+ ions (Kumar et al., 2008). The analysis of K^+ content results shows that the treatments at 75, 100, and 200 mM NaCl differed significantly from the control treatment (0 mM NaCl). The Na^+ content in the treatments increased between seven and 25 times with respect to the control treatment (0 mM NaCl). The lowest K^+ content was registered in the treatment with the highest concentration of NaCl (300 mM). The highest Na^+ content was also detected at this concentration, differing significantly from those treatments, including the control.

Effect of saline stress on protein content and sodium dodecyl sulphate–polyacrylamide gel protein patterns of somatic embryos. The effect of saline stress on protein content of SEs subjected to different concentrations of NaCl during their development is shown in Table 3. A gradual increase in protein content was observed in response to the increment of NaCl concentration in the culture media, except in the treatment with 75 mM NaCl in which the

Fig. 6. Proline content in somatic embryos of *C. chinense* subjected to different concentrations (NaCl). Error bars indicate SE ($n = 3$). Within each set of experiments, bars with different letters on same treatment are significantly different ($P \leq 0.05$).

Table 2. Sodium and potassium content in somatic embryos of *C. chinense* subjected to different concentrations of NaCl.

NaCl concn (mM)	K^+ content ($\mu\text{g}\cdot\text{g}^{-1}$ DW)	Na^+ content ($\mu\text{g}\cdot\text{g}^{-1}$ DW)	Na^+/K^+ index
0	18 ± 0 a	2.9 ± 0.5 a	0.16
75	9.6 ± 0.3 b	14.54 ± 0.8 b	1.51
100	10 ± 0.3 b	47.6 ± 2.4 c	4.76
200	8 ± 2.5 b	50 ± 3 c	6.25
300	1.1 ± 0.9 c	60 ± 1.5 d	54.54

Means $\pm$ SE, $n = 3$. Within each set of experiments, files with different letters on same treatment are significantly different ($P \leq 0.05$).

DW = dry weight.

protein content showed a slight decrease compared with the control treatment. Protein content was observed in a range of 10.2–15.16 μg protein/mg fresh weight. A concentration of 300 mM NaCl provoked the higher protein content in SEs. Similar results were reported by Bekheet et al. (2000) on *Asparagus officinalis*. They found a positive correlation between protein content of callus cultures and salt stress level in culture medium. Poljakoff-Mayber (1982) reported that osmotic adaptation under salinity stress may be achieved by ion uptake or by internal synthesis and accumulation of organic solutes. Dubey (1994) reported that the marked increase in protein content in callus cultures grown on saline media may be the result of synthesis of new proteins (osmoprotectant protein) or inactivation of proteolytic enzymes. Under stress conditions, some proteins are induced in many plants, although both the expression and function of such proteins are unclear. It has been suggested that there is a relationship between some forms of plant adaptation and tolerance to stresses and the expression of stress induced proteins. In the sodium dodecyl sulphate–polyacrylamide gel analysis, 40 bands were observed in a range of molecular weights from 200 to 6 kDa (Fig. 7). Somatic embryos of *Capsicum chinense* exposed to salt stress

Table 3. Variation in the protein content of somatic embryos treated with different concentrations of NaCl.

NaCl concn (mM)	Protein content ($\mu\text{g}\cdot\text{g}^{-1}$ FW)
0	11.06 ± 0.91 bc
75	10.2 ± 0.45 c
100	11.6 ± 0.59 bc
200	13.82 ± 0.68 ab
300	15.16 ± 0.36 a

Means $\pm$ SE, $n = 3$. Within each set of experiments, files with different letters on same treatment are significantly different ($P \leq 0.05$).

FW = fresh weight.

showed differences in the expression of peptides with molecular weights of 60.95, 56.44, and 7.5 kDa in response to increasing concentrations of NaCl in the medium. Similar behavior has been reported in *Jatropha curcas* (Kumar et al., 2008), *Trigonella* (Niknam et al., 2006), and *Solanum tuberosum* (Queirós et al., 2007). New bands were observed in somatic embryos exposed to 200 and 300 mM NaCl, respectively, with molecular weights of 43, 35.2, 34, 19.2, 17, and 7.5 kDa. Similar results were reported by Queirós et al. (2007), who also detected the presence of new polypeptides with molecular weights of 32.3 and 34 kDa in potato callus subjected to salt stress.

Fig. 7. Sodium dodecyl sulphate–polyacrylamide gel profiles of proteins in somatic embryos of *Capsicum chinense* under different concentrations of NaCl, carril: (1) 0 mM NaCl; (2) 75 mM NaCl; (3) 100 mM NaCl; (4) 200 mM NaCl; and (5) 300 mM NaCl.

These polypeptides are probably related to chloroplastic drought-induced stress proteins of 32 and 34 kDa identified in potato plants subjected to water stress (Pruvot et al., 1996a) and subsequently observed plants of the same species cultivated under salt stress conditions. It is possible to infer that these proteins might be associated with tolerance to osmotic stress (Pruvot et al., 1996b). Mikolajczyk et al. (2000) observed a band of 43.4 kDa, which could be related to a kinase protein of 42 kDa; this protein activates rapidly in response to hyperosmotic stress during the culture of tobacco cells. The late embryogenesis abundant-like proteins accumulate in the vegetative tissues of all plant species in response to osmotic stress, caused by drought salinity, or cold (Xiong and Zhu, 2002). Several salt-induced proteins have been identified in plant species and have been classified into two distinct groups (Ali et al., 1999; Mansour, 2000; Pareek et al., 1997): salt stress proteins, which accumulate only as a result of salt stress, and stress-associated proteins, which also accumulate in response to heat, cold, drought, waterlogging, and high and low mineral nutrients. Proteins that accumulate in plants grown under saline conditions may provide a storage form of nitrogen that is reused when stress is over (Singh et al., 1987) and may play a role in osmotic adjustment. Proteins may be synthesized de novo in response to salt stress or may be present constitutively at a low concentration and increase when plants are exposed to salt stress (Pareek et al., 1997).

Conclusions

In this study, we investigated the response of somatic embryos of Habanero pepper

to different NaCl concentrations evaluated through of the survival and development of SEs, germination of SEs, DW and water content, accumulation of proline, Na/K ratio, and protein content of embryogenic lines. The results show that somatic embryos of *C. chinense* can tolerate relatively high concentrations of salinity without affecting their growth and development. It was evident that the SEs of this plant are adapted to saline conditions through the Na^+ excess compensated by the loss of K^+ ions. These results also suggest that the embryos of *C. chinense* are able to maintain the functional integrity under stressed conditions. In addition, we could observe that lower concentrations of NaCl in the culture medium favored growth and germination of SEs. The results indicated that despite this being species recalcitrant, SEs showed a similar behavior in salinity as described in various species models. This opens new perspectives to establish selection system in this species and opens new routes for solutions to this phenomenon, which limits the application of biotechnological tools to the breeding and propagation of *Capsicum* genus. In conclusion, these results are important from a practical point of view to establish protocols that could be used for future genetic improvement of *C. chinense* by selection under pressure to obtain salt-tolerant genotypes in vitro.

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