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 Mirodjah, 1999), eggplant (Mukherjee, 2002), *Zea mays* (Urec Lana, 2003), *Tritricum durum* (Zair et al., 2003), and sugarcane (Gandonou et al., 2005). Unnikrishnan et al. (1991) reported that somatic embryos of *S. trifoliat us* 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.
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 GA₄, and 3% sucrose. The cultures were incubated at 25 ± 2 °C in darkness for 15 d and later at continuous light for another 15 d.

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 boiled at 100 °C for 1 h. The reaction was stopped by cooling the tubes in an ice bath. The chromatophore 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.

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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 mL 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 ψₛ helps the plants uptake more water and maintain growth (Almansouri et al., 2000).

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

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

Means ± se, n = 3. Within each set of experiments, files with different letters on same treatment are significantly different (P ≤ 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 ≤ 0.05).
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. At 100 mM they were observed mainly in the globular 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. 2Ia–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).
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 marinus and Hordeum vulgare (Garthwaite et al., 2005; Phoenix dactylifera callus (Al-Khayri, 2002); Carizzo 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 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 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.
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 kD, which could be related to a kinase protein of 42 kD; 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 Capsicum 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 the 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.

Literature Cited


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.


