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BRIEF COMMUNICATION

Effect of NaCl and $CaCl_2$ on growth and contents of minerals, chlorophyll, proline and sugars in the apple rootstock M 4 cultured *in vitro*

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Abstract

The apple (*Malus domestica* Borkh) rootstock M 4 shoots were grown *in vitro* for 4 weeks on Murashige and Skoog (MS) medium containing three NaCl concentrations (35, 100 and 200 mM) in combination with two $CaCl_2$ concentrations (5 and 10 mM). Inclusion of 10 mM $CaCl_2$ in the medium, in the presence of 35 mM NaCl, significantly increased the number of shoots and the fresh mass compared to 5 mM $CaCl_2$. The number of shoots, length of shoots, and the fresh mass of cultures were very low in the presence of 100 and 200 mM NaCl, independently of $CaCl_2$ concentration of the medium. By increasing NaCl and $CaCl_2$ concentrations in the culture medium, contents of N, Na, Cl, proline and soluble sugars in plantlets increased, whereas K, Mg, B, Zn and chlorophyll content decreased in comparison to the control.

Additional key words: cell proliferation, ion toxicity, micropropagation.

The progressive salinization of agricultural land is considered as the major environmental factor limiting plant growth and productivity of the arid and semi-arid regions (Flowers *et al.* 1986). The effect of salinity on water relationships, nutritional imbalance and ion toxicity is well documented (McKensie and Leshen 1994). Salinity causes a range of deleterious effects such as inhibition of photosynthesis, pigment synthesis, damage to plasma membrane permeability and other metabolic disturbances (Salisbury and Ross 1992, Ashraf and Parveen 2002, Karimi *et al*. 2005). It is well known that Ca^{2+} is involved in cell elongation, cell division, structural stability and permeability of cell membranes, regulation of cation-anion balance and activation of enzymes (Marschner 1995). Rengel (1992) reported that the deleterious effects of Na^+ in plants may be ameliorated by increasing the Ca^{2+} external concentration. This amelioration has been attributed to maintenance of the plasma membrane integrity (Läuchli 1990) and alterations of K^+ and Na^+ transport (Subarrao *et al*. 1990). Among the organic solutes found in higher plants acting as osmoregulators, proline is conspicuous (Yoshiba *et al*. 1997). Mattioni *et al*. (1997) reported that proline accumulates in the cytosol in response to salt stress. The objective of the present research was to study the effect of increasing rates of NaCl and $CaCl_2$ on growth, and contents of minerals, chlorophyll, proline and sugars in the apple rootstock M 4 cultured *in vitro*.

The explants employed were shoots of the apple (*Malus domestica* Borkh) rootstock M 4 of about 25 mm in length, preserved from previous *in vitro* cultures and maintained in the growth room. Each explant was transferred and grown in a 15 × 100 mm glass test tube containing 3 cm^3 of the MS culture medium (Murashige and Skoog 1962). The culture medium was supplemented with 45 kg m^{-3} sorbitol, 1 g m^{-3} benzyladenine, and 1 g m^{-3} gibberellic acid. Three NaCl concentrations (35, 100 and 200 mM) were combined with two $CaCl_2$ concentrations (5 and 10 mM). MS medium was used as a control. The pH of the media was adjusted to 5.8 before autoclaving at 121 °C for 15 min. The tubes were maintained in the growth room at 22 ± 1 °C and 16-h photoperiod (cool white fluorescent tubes, irradiance of 45 μmol m^{-2} s^{-1}, 400-700 nm). After 4 weeks in culture, the number of

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Abbreviations: d.m. - dry mass; f.m. - fresh mass; MS medium - Murashige and Skoog medium.
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shoots, length of shoots, and fresh mass (f.m.) of cultures were measured. Leaf chlorophyll content was estimated in leaves by using a *SPAD* meter (*Minolta 502,* Osaka, Japan). Fresh leaves were cut into small pieces, placed in glass vials containing 10 cm^3 of 80 % (v/v) ethanol, and heated at 60 °C for 30 min. The extract was then filtered and diluted with 80 % (v/v) ethanol up to 20 cm^3 (Khan *et al*. 2000). Soluble sugars and proline were assayed following the acid ninhydrin reagent method and the anthrone method, respectively (Khan *et al*. 2000, Plummer 1987). For determination of the mineral composition, leaves and stems of each plantlet were harvested and rinsed twice with distilled water. These organs were then dried at 68 °C for 48 h, ground to pass a 30-mesh screen and dry ashed at 530 °C for 16 h. Potassium, Ca, Mg, Na, and Zn concentrations were determined by atomic absorption spectroscopy (*Perkin-Elmer 2380*, Wellesley, MA, USA). Nitrogen was determined by the Kjeldahl's procedure and boron by the azomethine-H method (Wolf 1974). Chloride was extracted from the dried tissue with distilled water and measured by titration with $AgNO_3$ (Richards 1954). Each treatment included fifteen replicates (tubes). The experiment was repeated twice, and the reported data are the means of the two experiments. The statistical design employed was the randomized complete block one. Differences between means were evaluated by using LSD test at $P < 0.05$. Furthermore, the standard error of the means was calculated.

By increasing NaCl and $CaCl_2$ concentrations of the culture medium, the number of shoots, length of shoots, fresh mass of plantlets and the chlorophyll content (SPAD units) of leaves were significantly decreased in comparison to the control (Table 1). Inclusion of 10 mM $CaCl_2$ in the medium, in the presence of 35 mM NaCl, significantly increased the number of shoots and the fresh mass, whereas it decreased the length of shoots compared to 5 mM $CaCl_2$. The number of shoots, length of shoots, and the fresh mass of plantlets were very low in the presence of 100 and 200 mM NaCl, independently of the $CaCl_2$ concentration of the medium. Proline and soluble sugar contents of leaves significantly increased in the presence of 100 and 200 mM NaCl in comparison to the control. At the end of the experiment, explants grown on media supplemented with 100 mM NaCl showed symptoms of leaf burn in restricted parts of the leaf blade, whereas those grown on media supplemented with 200 mM NaCl showed severe leaf burn symptoms.

Table 1. Effect of NaCl and $CaCl_2$ concentrations of the culture medium on the number of shoots per explant, length of shoots, fresh mass, chlorophyll content (SPAD units), proline and sugar concentrations of the apple rootstock M 4 grown *in vitro* for 4 weeks. Values are the means of 30 replications ± SE.

NaCl [mM]	$CaCl_2$ [mM]	Number of new shoots	Length of new shoots [mm]	Fresh mass [g $plant^{-1}$]	SPAD units	Proline [µmol g^{-1}(f.m.)]	Sugars [µmol g^{-1}(f.m.)]
0	0	3.5 ± 0.13	1.18 ± 0.05	0.36 ± 0.04	34.00 ± 3.67	18.1 ± 2.21	26.3 ± 2.91
35	5	1.9 ± 0.07	0.78 ± 0.04	0.24 ± 0.02	23.84 ± 2.89	22.4 ± 2.46	27.6 ± 2.86
35	10	2.8 ± 0.10	0.64 ± 0.04	0.28 ± 0.03	27.99 ± 2.96	21.2 ± 2.48	27.9 ± 2.79
100	5	0.9 ± 0.04	0.58 ± 0.03	0.17 ± 0.02	10.77 ± 1.87	39.2 ± 2.84	32.5 ± 2.96
100	10	0.6 ± 0.03	0.38 ± 0.02	0.18 ± 0.02	11.12 ± 1.92	37.8 ± 2.81	31.9 ± 2.92
200	5	0.4 ± 0.02	0.37 ± 0.02	0.10 ± 0.01	5.62 ± 0.34	47.6 ± 2.25	39.4 ± 2.86
200	10	0.3 ± 0.02	0.40 ± 0.03	0.14 ± 0.01	5.96 ± 0.36	43.8 ± 2.36	38.6 ± 2.95
$LSD_{0.05}$		0.3	0.06	0.03	1.98	3.12	3.32

Table 2. Effect of NaCl and $CaCl_2$ concentrations of the culture medium on N, K^+, Ca^{2+}, Mg^{2+}, Na^+, Cl^-, B and Zn^{2+} content in the apple rootstock M 4 grown *in vitro* for 4 weeks. Values are the means of 30 replications ± SE.

NaCl [mM]	$CaCl_2$ [mM]	N [mg g^{-1}(d.m.)]	K^+	Ca^{2+}	Mg^{2+}	Na^+	Cl^-	B [µg g^{-1}(d.m.)]	Zn^{2+}
0	0	2.96 ± 0.19	2.36 ± 0.13	0.47 ± 0.04	0.15 ± 0.02	0.67 ± 0.06	0.11 ± 0.01	44 ± 3.11	128 ± 6.11
35	5	3.11 ± 0.23	1.98 ± 0.12	0.79 ± 0.06	0.11 ± 0.01	1.89 ± 0.12	3.21 ± 0.25	40 ± 3.04	89 ± 5.21
35	10	3.14 ± 0.25	1.89 ± 0.11	0.85 ± 0.08	0.12 ± 0.01	1.92 ± 0.14	3.74 ± 0.28	41 ± 3.10	87 ± 5.19
100	5	3.88 ± 0.29	1.56 ± 0.10	0.54 ± 0.05	0.11 ± 0.01	2.84 ± 0.18	5.22 ± 0.41	38 ± 2.99	75 ± 5.04
100	10	3.90 ± 0.30	1.44 ± 0.09	0.72 ± 0.06	0.12 ± 0.02	3.12 ± 0.23	6.12 ± 0.42	39 ± 3.09	78 ± 5.11
200	5	4.59 ± 0.30	1.38 ± 0.09	0.52 ± 0.05	0.10 ± 0.01	3.38 ± 0.26	8.26 ± 0.47	37 ± 3.11	46 ± 4.92
200	10	4.56 ± 0.31	1.20 ± 0.07	0.60 ± 0.07	0.09 ± 0.01	3.81 ± 0.31	8.92 ± 0.45	39 ± 3.01	50 ± 4.86
$LSD_{0.05}$		0.11	0.17	0.04	0.02	0.09	0.43	2.75	5.2

D'Onofrio and Morini (2002) reported that the presence of $CaCl_2$ in the culture medium apparently mitigated the effects of salt stress. The previous researchers also reported that NaCl at 5 mM, in the presence of 0.3 or 1 mM $CaCl_2$ was favourable both to somatic embryo and root formation of *Cydonia oblonga* grown *in vitro*. Rengel (1992) demonstrated that Ca^{2+} ameliorates the Na-inhibition of growth for most plants but different species and different cultivars respond differently to supplemental Ca^{2+} when salinized. Furthermore, Bernstein *et al*. (1993) reported that supplemental Ca^{2+} can increase the length of the growth zones of salt-stressed sorghum plants. Furthermore, Na^+-Ca^{2+} interactions on photosynthesis have been observed. In *Vaccinium ashei*, supplemental Ca^{2+} increased photosynthetic rate in salt-stressed plants treated with Na_2SO_4 (Cramer 2002). Furthermore, Zhu *et al.* (1997) reported that an increase in cytosolic free Ca^{2+} concentration could act at a transcriptional level inducing osmotic regulated gene expression of enzymes involved in the biosynthetic pathway of proline. Chinnusamy *et al*. (2005) reported that proline serves as a storage sink for carbon and nitrogen and a free radical scavenger. It also stabilizes subcellular structures (membranes and proteins), and buffers cellular redox potential under stress.

By increasing NaCl and $CaCl_2$ concentrations of the culture medium, N, Na^+ and Cl^- concentrations of plantlets increased, whereas K^+, Mg^{2+}, B and Zn^{2+} concentrations decreased in comparison to the control (Table 2). Ca^{2+} concentration of plantlets of all treatments was significantly higher in comparison to the control. However, higher Ca^{2+} concentration was found at 35 mM NaCl in comparison to 100 and 200 mM. Salinity may reduce N accumulation in plants due to an antagonistic effect of Cl^- to NO_3^- uptake (Grattan and Grieve 1999). Mattioni *et al*. (1997) reported that proline accumulates in the cytosol in response to salt stress acting as osmoregulator. Furthermore, other soluble N-containing compounds such as other amino acids, polyamines and soluble proteins could protect plant tissues against osmotic stress (Kozlowski 1997, Rai 2002). In our experiment, the increased N concentration of cultures grown on media containing NaCl and $CaCl_2$ could be ascribed to accumulation of these N-containing compounds acting as osmoregulators. The decrease of K^+ concentration of plantlets in the presence of NaCl and $CaCl_2$ may be attributed to Na^+ and Ca^{2+} competition (Epstein 1998). Furthermore, salinity may increase energy consumption, required for osmotic regulation and competition of transported ions. This may subsequently lead to a reduction of metabolically important ions such as K^+ (Kwon *et al*. 1995). Nutrient imbalances may result from the effect of salinity on nutrient availability, competitive uptake, transport or partitioning within the plant or may be caused by physiological inactivation of a given nutrient resulting in an increase in the plant's internal requirement for that essential element (Grattan and Grieve 1999). Calcium is important nutrient element in the resistance of plants to salinity. The protective effect of Ca^{2+} in salinized plants is due to its role in maintaining membrane integrity. In the presence of adequate concentration of Ca^{2+}, plants exclude Na^+ and withstand the effects of relatively high NaCl concentration
(Al-Yassin 2004). The presence of Ca is necessary to maintain K^+/Na^+ selectivity and to prevent the harmful displacement of Ca^{2+} by Na^+ from the cell membranes and intercellular pools (Smith 1978). Sodium can enter cells through ion channels. In some cases these channels are more selective for Na^+ than K^+. Increase of the external Ca^{2+} concentration reduces Na^+ conductance through these channels (Roberts and Tester 1997).

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