BIOLOGIA PLANTARUM 48 (4): 613-615, 2004

BRIEF COMMUNICATION

Effect of NaCl on biomass and contents of sugars, proline and proteins in seedlings and leaf explants of *Nicotiana tabacum* grown *in vitro*

V. NIKNAM*, M. BAGHERZADEH, H. EBRAHIMZADEH and A. SOKHANSANJ

Department of Biology, Faculty of Sciences, University of Tehran, 14155 Tehran, I.R. Iran

Abstract

Effects of NaCl on growth *in vitro* and contents of sugars, free proline and proteins in the seedlings and leaf explants of *Nicotiana tabacum* cv. Virginia were investigated. The fresh and dry mass of the seedlings decreased under salinity. These growth parameters in leaf explants decreased at 50 mM NaCl and increased up to 150 mM NaCl and then decreased at higher level of salinity. Free proline content in both seedlings and leaf explants increased and polysaccharide content decreased continuously with increasing of NaCl concentration. Reducing sugars, oligosaccharides, soluble sugars and total sugars contents in both seedlings and leaf explants decreased up to 150 mM NaCl and then increased at higher concentrations of NaCl.

Additional key words: salt stress, tobacco, tissue culture.

Salinity is considered a major factor in limiting plant development and crop productivity, and salinization continues to increase, particularly in arid and semiarid regions (Shannon 1998). Cell cultures have served as a very useful tool in trying to elucidate mechanisms of salt tolerance operating at the cellular level. Plant cell and tissue culture are also relevant to crop improvement strategies because they offer a means of rapid selection on a mass scale and useful for the development of breeding techniques for salinity resistant crops (Cherian and Reddy 2003, Elavumoottil *et al.* 2003). Exposure of cells to salt stress causes a set of metabolic and developmental changes (*e.g.* Hasegawa *et al.* 2000). The identification of specific characteristics related to salt resistance such as compatible osmolytes (*e.g.* proline), sugars and proteins will provide potential biological markers useful in the identification and genetic manipulation of salt resistant plants and plant cells (Shonjani 2002).

Seedling establishment is often thought to be one of the most sensitive stages to salinity (Jones and Jones 1989). Seed germination and seedling growth are critical life stages often subject to high mortality rates. Understanding the responses of plants at these stages is particularly important for elucidating the mechanisms of salt resistance and sensitivity in plants and their survival (Mayer and Poljakoff-Mayber 1963).

The objective of this work was to investigate the merit of using *in vitro* cultures, such as seedlings and organogenic calli to provide a system for studying salinity (NaCl) induced changes in some metabolites of tobacco.

Tobacco (*Nicotiana tabacum* L. cv. Virginia) plants were germinated from seeds on Murashige and Skoog (1962, MS) medium. Seeds were surface sterilized with 10 % sodium hypochlorite solution and then washed several times with sterile distilled water. Seeds were germinated and maintained on MS media solidified with 8 % agar, 30 g dm^{-3} sucrose and containing 0, 50, 100, 150, 200 and 250 mM NaCl under 16-h photoperiod (white fluorescent lamps; irradiance of 46 μmol m^{-2} s^{-1}) and day/night temperature of 25/20 °C. Seedlings were raised on MS media after 3 d. Callus was established from surface-sterilized leaf explants obtained from 40-d-old potted tobacco plants on MS medium, supplemented with 0.1 mg dm^{-3} naphthalene acetic acid

Received 2 February 2004, *accepted* 15 July 2004.
Abbreviations: BA - benzyladenine; MS medium - Murashige and Skoog medium; NAA - naphthaleneacetic acid.
Acknowledgement: The financial support of this research was provided by Faculty of Science, University of Tehran.
* Corresponding author; fax: (+98) 21 6405141, e-mail: vniknam@khayam.ut.ac.ir

(NAA) and 2.5 mg dm^{-3} benzyladenine (BA). Seedlings and leaf explants were maintained in a growth chamber for 30 d under above mentioned conditions.

Germination rate was recorded on days 7, 14, and 30. Growth rate of both seedlings and explants as measured by fresh and dry mass was recorded in 30-d-old seedlings and explants. Three replicates containing about 20 seedlings and 4 leaf explants each were taken for measurements.

Free proline was extracted and determined according to Bates *et al*. (1973) using L-proline as a standard. High-speed centrifuge (*Beckman J2-21M*, USA) and UV-visible spectrophotometer (*Shimadzu UV-160* Japan) 10 mm matched quartz cells were used for centrifugation of the extracts and determination of the absorbance, respectively.

For determination of sugar content, 0.5 g of powder was extracted using 10 cm^3 of ethanol-distilled water (8:2 v/v), and supernatants were collected after twice centrifugation at 1480 *g*. The residue from ethanol extraction was subsequently used for polysaccharide extraction by boiling water (Seyyednejad *et al.* 2001). Total sugar contents were estimated by the method of Dubois *et al.* (1956). Reducing sugars were quantified according to Nelson (1944). Oligosaccharides content was obtained by difference between soluble and reducing sugars contents.

For determination of protein content, fresh seedlings and explants (0.1 g) were homogenized in a chilled (4 °C) mortar using a buffer containing: 500 mM Tris-HCl, pH 7.0. After centrifugation at 12 100 *g* for 1 h at 4 °C, the supernatant was filtered and then transferred to Eppendorf tubes and the samples kept on ice at 4 °C. Total protein contents were measured by the spectrophotometric method of (Lowry *et al*. 1951) using bovine serum albumin (BSA) as the standard.

The data determined in triplicate were analysed by analysis of variance (*ANOVA*) using *MSTAT-C* (*version 1.42*). The significance of differences was determined according to DMRT. P values < 0.05 are considered to be significant.

The seed germination decreased with increasing salt

Table 1. Fresh mass, dry mass and the contents of free proline, proteins, reducing sugars (RS), polysaccharides (PS), oligosaccharides (OS), soluble sugars (SS = PS + OS) and total sugars (TS = PS + SS) in seedlings and leaf explants of *Nicotiana tabacum* under NaCl stress. Values are means ± SE of 3 determinations. Data were analysed by Duncan's multiple range test and means followed by identical letters were not statistically different within the columns ($P < 0.05$). In 250 mM NaCl no germination occurred.

NaCl [mM]		Fresh mass [g]	Dry mass [mg]	Free proline [%(d.m.)]	Protein [%(d.m.)]
0	seedlings	0.18 ± 0.00a	7 ± 0.3a	4.2 ± 0.16d	18.9 ± 0.21a
	explants	3.87 ± 0.34b	180 ± 12.0b	2.7 ± 0.48e	13.6 ± 0.20d
50	seedlings	0.17 ± 0.00a	5 ± 0.4b	7.0 ± 0.25c	12.1 ± 0.26c
	explants	3.67 ± 0.27b	170 ± 6.0b	4.4 ± 0.27e	26.8 ± 0.60a
100	seedlings	0.14 ± 0.00b	2 ± 0.4cd	10.7 ± 0.90b	14.6 ± 0.19b
	explants	4.73 ± 0.13a	210 ± 6.0a	6.9 ± 0.25d	17.9 ± 0.70b
150	seedlings	0.10 ± 0.01c	3 ± 0.2c	11.1 ± 0.13b	19.0 ± 0.52a
	explants	4.56 ± 0.07a	180 ± 9.0b	9.4 ± 0.90c	17.2 ± 0.70b
200	seedlings	0.04 ± 0.00d	1 ± 0.3d	15.1 ± 0.28a	14.1 ± 0.34b
	explants	1.33 ± 0.14c	110 ± 9.0c	13.3 ± 0.29b	15.8 ± 0.84c
250	explants	0.59 ± 0.48d	70 ± 3.0d	18.2 ± 1.46a	11.8 ± 0.08e

NaCl [mM]		RS [%(d.m.)]	PS [%(d.m.)]	OS [%(d.m.)]	SS [%(d.m.)]	TS [%(d.m.)]
0	seedlings	8.2 ± 0.29b	16.7 ± 0.39a	26.7 ± 2.16b	34.9 ± 1.88b	51.7 ± 2.25b
	explants	5.2 ± 0.29b	14.3 ± 0.73a	22.2 ± 0.81b	27.4 ± 0.64b	41.8 ± 1.23b
50	seedlings	6.0 ± 0.38c	14.7 ± 0.30b	24.0 ± 0.30b	29.7 ± 0.92c	44.7 ± 0.86c
	explants	2.9 ± 0.25d	11.9 ± 0.01b	20.3 ± 0.53c	23.1 ± 0.20c	35.0 ± 0.15c
100	seedlings	2.8 ± 0.21d	10.6 ± 0.18c	13.4 ± 0.25d	16.1 ± 0.43d	26.7 ± 0.27d
	explants	2.3 ± 0.09d	10.8 ± 0.21b	14.6 ± 0.35d	16.9 ± 0.51d	27.7 ± 0.72d
150	seedlings	2.3 ± 0.09d	9.6 ± 0.16d	15.4 ± 0.22c	17.7 ± 0.14d	27.3 ± 0.23d
	explants	2.0 ± 0.18d	10.4 ± 0.15c	5.6 ± 0.20e	7.6 ± 0.35e	18.0 ± 0.23e
200	seedlings	12.2 ± 0.74a	8.3 ± 0.33e	43.1 ± 0.50a	55.3 ± 0.37a	63.6 ± 0.51a
	explants	3.9 ± 0.30c	9.8 ± 0.20c	13.1 ± 0.36e	17.0 ± 0.15d	29.6 ± 0.34d
250	explants	8.7 ± 0.59a	8.4 ± 0.40d	26.7 ± 0.36a	35.5 ± 0.90a	43.9 ± 0.50a

concentration up to 200 mM NaCl. In 250 mM NaCl no germination was occurred. Salinity is a major environmental stress, which unfavourably affects germination of seeds (Dubey 1984). Seedling as well as explant growth decreased slightly at 50 mM NaCl. Further increase up to 200 mM NaCl diminished seedlings growth significantly ($P < 0.05$). Contrary to growth of seedlings, further increase up to 100 mM NaCl increased explants growth significantly, and then at the levels of 150 to 250 mM NaCl the growth decreased (Table 1). Thus, growth pattern of explants under salinity are different from that of seedlings.

The proline content of seedlings and explants increased up to 200 mM NaCl significantly ($P < 0.05$) and almost the same pattern was obtained in seedlings and explants (Table 1).

The salinity induced changes in protein content of seedlings and explants had different pattern. Protein content in seedlings diminished at 50 mM NaCl ($P < 0.05$) but further increased up to 150 mM NaCl and at 200 mM the content returned to that of 100 mM NaCl. Contrary to that, 50 mM NaCl increased protein content in explants and further increase in NaCl concentration up to 250 mM NaCl decreased the protein content significantly ($P < 0.05$) (Table 1).

Polysaccharides content decreased under salinity in both of seedlings and explants continuously with increasing of NaCl concentration. Reducing sugars, oligosaccharides, soluble sugars and total sugars content in both seedlings and leaf explants decreased up to 150 mM NaCl and then increased at higher concentrations of NaCl (Table 1).

Accumulation of osmolytes such as proline, reducing sugars and polysaccharides is a common response to salinity (*e.g.* Binzel *et al*. 1987, Fedina *et al*. 2002, Yancey *et al*. 1982).

In conclusion we observed some similarities and dissimilarities in seedlings and leaf explants responses to NaCl *in vitro*. Understanding the physiological and biochemical mechanisms of salt resistance in *in vitro* cultures will lead to effective means for the development of plants with increased resistance to salt stress.

References

Bates, L.S., Waldren, R.P., Teare, I.D.: Rapid determination of free proline for water-stress studies. - Plant Soil **39**: 205-207, 1973.

Binzel, M., Hasegawa, P.M., Rhodes, D., Handa, S., Handa, A.K., Bressan, R.A.: Solute accumulation in tobacco cells adapted to NaCl. - Plant Phsiol. **84**: 1408-1415, 1987.

Cherian, S., Reddy, M.P.: Evaluating of NaCl tolerance in the callus cultures of *Suaeda nudiflora* Moq. - Biol. Plant. **46**: 193-198, 2003.

Dubey, R.S.: Effect of sodium chloride salinity on enzyme activity and biochemical constitutents in germinating salt tolerant rice seed. - Oryza **21**: 213-217, 1984.

Dubois, M., Gilles K.A., Hamilton, J.K., Rebers, P.A., Smith, F.: Colorimetric method for determination of sugars and related substances. - Anal. Chem. **28**: 350-356, 1956.

Elavumoottil, O.C., Martin, J.P., Moreno, M.L.: Changes in sugars, sucrose synthase activity and proteins in salinity tolerant callus and cell suspension cultures of *Brassica oleracea* L. - Biol. Plant. **46**: 7-12, 2003.

Fedina, I.S., Georgieva, K., Grigorova, I.: Light-dark changes in proline content of barley leaves under salt stress. - Biol. Plant. **45**: 59-63, 2002.

Hasegawa, P.M., Bressan, R.A., Zhu, J.-K., Bohnert, H.J.: Plant cellular and molecular responses to high salinity. - Annu. Rev. Plant Physiol. Plant mol. Biol. **51**: 463-499, 2000.

Jones, H.G., Jones, M.B.: Introduction: Some terminology and common mechanisms. - In: Jones, H.G., Flowers, T.J., Jones, M.B. (ed.): Plant Under Stress: Biochemistry, Physiology and Ecology and Their Application to Plant Improvement. Pp. 1-10. Cambridge University Press, Cambridge - New York 1989.

Lowry, O.H., Rosebrough, N.J., Farr, A.L., Randall, R.J.: Protein measurement with the Folin-phenol reagent. - J. biol. Chem. **193**: 265-275, 1951.

Mayer, A.M., Poljakoff-Mayber, A.: The Germination of Seeds. - Pergamon Press, Oxford - London - New York - Paris 1963.

Murashige, T., Skoog, F.: A revised medium for rapid growth and bioassay with tobacco tissue cultures. - Physiol. Plant. **15**: 473-497, 1962.

Nelson, N.: A photometric adaptation of the Somogyi method for the determination of glucose. - J. biol. Chem. **153**: 375-380, 1944.

Seyyednejad, M., Ebrahimzadeh, H., Talaie, A.: Carbohydrate content in olive Zard cv. and alternate bearing pattern. - Int. Sugar J. **103**: 84-87, 2001.

Shannon, M.C.: Adaptation of plants to salinity. - Adv. Agron. **60**: 75-119, 1998.

Shonjani, S.: Salt sensitivity of rice, maize, sugar beet, and cotton during germination and early vegetative growth. - Ph.D. Thesis. Institute of Plant Nutrition, Justus Liebig University, Giessen 2002.

Yancey, P.H., Clark, M.E., Hand, S.C., Bowlus, R.D.: Living with water stress: evolution of osmolyte systems. - Science **217**: 1214-1222, 1982.