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Proline accumulation, protein pattern and photosynthesis in *Bacopa monniera* regenerants grown under NaCl stress

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Abstract

Shoots of *Bacopa monniera* exhibited 100 % regeneration on Murashige and Skoog medium with 2 % sucrose, 0.2 mg dm^{-3} 1-naphthaleneacetic acid, 0.5 mg dm^{-3} 6-benzylaminopurine and 50 mg dm^{-3} glutamine. When the medium was supplied with various concentrations (5 - 15 g dm^{-3}) of sodium chloride, proline content in regenerants was six times higher than in the control. With increasing NaCl concentration photosynthetic rate decreased and fresh mass and root length of regenerants declined. NaCl also induced formation of new proteins.

Additional key words: CO_2 uptake, *in vitro* culture, salinity.

Introduction

Numerous cell-suspension culture lines able to grow in the presence of salt-induced osmotic stress have been established for the purpose of studying cellular adaptation to salinity stress (Kavi Kishore 1988, 1989, Plaut *et al.* 1991, Locy *et al.* 1996, Ali *et al.* 1997, Purohit *et al.* 1998). Salinity affects dry matter allocation, ion relations, water status, and many other physiological processes, biochemical reactions, *etc.* (Greenway and Munns 1980). Many plant species respond rapidly to stress by increasing the concentration of compatible solutes involved in osmoregulation. Proline accumulation has often been observed to occur in plants subjected to environmental stresses; it acts as a cytoplasmic osmoticum, counteracting the effect of salt accumulated in the vacuole (Steward and Lee 1974, Voetberg and Sharp 1991), as a protective agent for cytoplasmic enzymes (Paleg *et al.* 1984), as a

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Abbreviations: BAP - 6-benzylaminopurine; EDTA - ethylenediaminetetraacetic acid; MS medium - Murashige and Skoog's medium; NAA - 1-naphthaleneacetic acid; SDS - sodium dodecyl sulphate.

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reservoir of nitrogen and carbon source for post-stress growth (Fukutaku and Yamada 1984), or even as a stabilizer of machinery for protein synthesis (Kardpal and Rao 1985). In higher plants, proline biosynthesis route is generally thought to follow the glutamate pathway. The role of proline in imparting resistance to salt stress, however, continues to be controversial: some workers consider enhanced proline content as simply a stress effect rather than a cause of stress tolerance (Mofiah and Michel 1987). Proline accumulation induced by NaCl has been shown to correlate with growth inhibition (Lin and Kao 1996).

It has been postulated that the specific proteins whose synthesis is induced under stress are critical to the plant survival. Saline environment is generally correlated to the synthesis of new proteins (Ericson and Alfinito 1984, Ramagopal 1986, Singh *et al.* 1987, Chretien *et al.* 1992). Changes in gene expression, transcription, and translation often occur during acclimation and are thus thought to be involved in the induction of tolerance (Sinha and Häder 1996). Investigations dealing with the effects of environmental stresses on specific protein synthesis have been performed also on cell cultures (Liu and Li 1991).

High frequency of *in vitro* regeneration in *Bacopa monniera* (Ali *et al.* 1996) prompted us to test tolerance potential of the regenerants against salt stress. Successful maintenance of cultures on salt-supplemented medium for the last three years, establishes adaptation of the cultures (Ali *et al.* 1997). This study aims to enhance the understanding of the role that proline accumulation and new protein synthesis play in *B. monniera* under salt stress. The study also addresses the effect of salt stress on photosynthetic performance in the regenerants of *Bacopa monniera*.

Material and methods

Establishment of cultures: Stem segments (20 mm) of *Bacopa monniera* (L.) Wettst. were procured from the Herbal Garden at Jamia Hamdard. The explants (500 pieces) were thoroughly washed under running tap water for 30 min and with 5 % *Cetrimide* (*ICI*, Bombay, India) for 10 min. The washed stem segments, sterilized with 10 % sodium hypochlorite for 10 min and with 0.1 % mercuric chloride for 5 min, were rinsed thoroughly with sterile distilled water before implantation. For culture initiation, explants were cut aseptically into 10 mm pieces and placed on MS (Murashige and Skoog 1962) medium supplemented with 3 % sucrose, NAA (0.1 - 0.5 mg dm^{-3}), BAP (0.5 - 5.0 mg dm^{-3}) and casein hydrolysate (500 mg dm^{-3}), gelled with 0.62 % agar (*Qualigen*, Bombay, India) and pH adjusted to 5.8 before autoclaving (for 15 min at 121 °C and 15 kg cm^{-2}). The cultures were maintained on the maintenance medium (MM), *i.e.* MS with 2 % sucrose, 0.2 mg dm^{-3} NAA, 0.5 mg dm^{-3} BAP, and 50 mg dm^{-3} glutamine. This medium was also supplied with NaCl (5, 10 and 15 g dm^{-3}). Fresh mass and root length in regenerated shoots were monitored at regular intervals. All the cultures were maintained at temperature 25 ± 2 °C, relative humidity in a culture room 55 ± 5 %, 14-h photoperiod, and irradiance of 100 μmol(photon) m^{-2} s^{-1}.

The proline content was determined by the method of Bates *et al.* (1973). *Li-6200* portable photosynthesis system (*Li-Cor*, Lincoln, USA) was used for automatic measurement of the net photosynthetic rate in various samples. For protein analysis (SDS polyacrylamide gel electrophoresis), the sample extract (1000 mg each) was homogenized with 0.03 cm^3 extraction buffer, consisting of 0.2 M phosphate buffer with 5 % β-mercaptoethanol, 1 % SDS, 10 % sucrose, 0.15 % $MgCl_2$ and 0.14 % EDTA. The crude homogenates were then centrifuged at 12 550 *g* for 30 min at 4 °C to remove cellular debris. The supernatant was denatured by heating and then mixed with bromophenol blue. Such samples (0.075 cm^3 supernatant) were loaded in the well along with 0.01 cm^3 of protein in one well. SDS-PAGE was carried out following the method of Laemmli (1970). After completion of run, the gel was stained overnight in a staining solution (Coommassie brilliant blue) and finally destained by ethanolic destainer to analyse the banding pattern.

Results

Growth response: NaCl (5, 10 and 15 g dm^{-3}) showed adverse effects on the growth of regenerants, as evidenced through necrosis at the proximal end after 4 week growth. These regenerants regained appreciable growth after 16 weeks (Table 1). However, the growth of regenerants was less affected at low concentration of NaCl (5 g dm^{-3}) than at higher concentrations (10 - 15 g dm^{-3}). NaCl reduced shoot and root length, fresh mass and leaf size (Table 1).

Table 1. Total fresh mass [g vessel $^{-1}$] and root length [cm] of *Bacopa monniera* grown *in vitro* under different concentration of NaCl [g dm^{-3}] for 4 to 20 weeks (500 mg of fresh mass was transferred every fourth week in each culture vial). Values represent means ± SE based on 24 replicates; the experiment was repeated twice.

	NaCl	4 weeks	8 weeks	12 weeks	16 weeks	20 weeks
Fresh mass	0	1.37 ± 0.05	1.34 ± 0.03	1.38 ± 0.06	1.36 ± 0.04	1.35 ± 0.05
	5	1.22 ± 0.08	1.11 ± 0.04	1.19 ± 0.03	1.24 ± 0.07	1.32 ± 0.05
	10	0.95 ± 0.03	0.88 ± 0.07	0.92 ± 0.04	1.11 ± 0.02	1.16 ± 0.06
	15	0.78 ± 0.05	0.63 ± 0.07	0.55 ± 0.03	0.68 ± 0.04	0.68 ± 0.02
Root length	0	2.50 ± 0.06	2.52 ± 0.09	2.55 ± 0.05	2.57 ± 0.03	2.57 ± 0.08
	5	2.25 ± 0.03	2.15 ± 0.05	2.32 ± 0.08	2.39 ± 0.09	2.45 ± 0.03
	10	1.79 ± 0.07	1.62 ± 0.08	1.83 ± 0.07	1.95 ± 0.03	2.12 ± 0.08
	15	0.68 ± 0.09	0.61 ± 0.04	0.69 ± 0.04	0.72 ± 0.09	0.95 ± 0.04

Proline accumulation: Proline content was six times higher at 15 g dm^{-3} NaCl concentration in the medium than in the control. It was dependent on NaCl concentration and exposure time (Table 2).

Table 2. Proline content in regenerants of *Bacopa monniera* grown under NaCl stress for 4, 12 and 20 weeks. Values represent mean ± SE based on three replicates; the experiment was repeated twice

NaCl [g dm^{-3}]	Proline [µg g^{-1}(f.m.)] 4 weeks	12 weeks	20 weeks
0	21.7 ± 0.16	21.2 ± 0.26	21.8 ± 0.23
5	98.0 ± 0.21	103.0 ± 0.18	110.0 ± 0.27
10	111.0 ± 0.11	116.0 ± 0.15	125.0 ± 0.13
15	124.0 ± 0.14	129.0 ± 0.22	135.0 ± 0.29

Rate of photosynthesis: Photosynthetic rate was higher in the control and declined sharply in the salt-supplemented cultures. The rate was 13.1, 12.1, and 11.0 µmol(CO_2) m^{-2} s^{-1} in the cultures grown on 5, 10 and 15 g dm^{-3} NaCl, respectively, whereas, it was 15.3 µmol(CO_2) m^{-2} s^{-1} in the control after 4 week growth (Fig. 1).

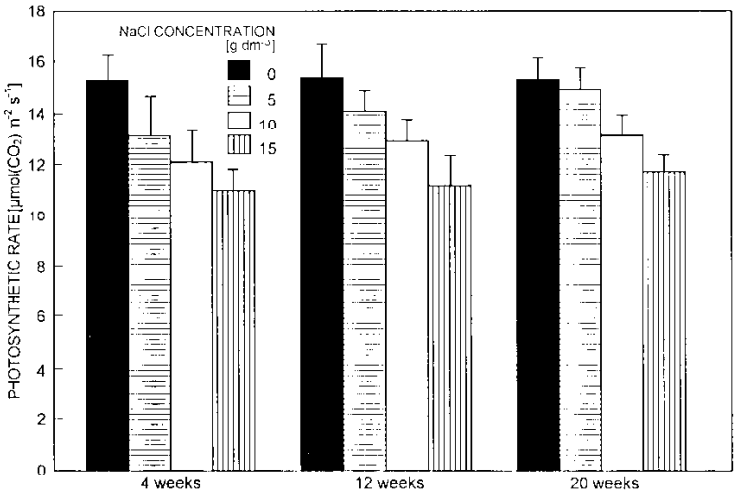

Fig. 1. Net photosynthetic rate of *Bacopa monniera* regenerants are affected by NaCl concentration and treatment duration

Protein pattern: The SDS-PAGE protein profile of the regenerants grown on NaCl (5, 10 and 15 g dm^{-3}) exhibited extra bands of ~58-60, ~62-64 and ~66-68 kD as compared to control. Three more bands ~88-90, ~92-94 and ~104-106 kD, which were absent at 5 g dm^{-3}, were also seen at higher NaCl concentration. The intensity of all the bands was more pronounced in the NaCl-treated cultures.

Discussion

A good correlation does not always exist between the salt tolerance of cell suspension or callus and that of whole plants (Tal 1984). The shoot-tissue regenerants are less prone to somaclonal variation and appear to offer a better system for testing and selecting the salt tolerance. *Bacopa* shoots easily propagated *in vitro* (Ali *et al.* 1996), and withstood salt stress for varying periods. The fresh mass and root length in control plantlets grew faster and was hampered by NaCl supply. Negative effect of NaCl on fresh mass of regenerants was directly related to salt concentration (Kavi Kishore 1988, 1989). Root length inhibition in *Bacopa* may be an indicator of susceptibility to NaCl. We have also noted that inhibition in fresh mass and root formation was not only concentration but also treatment duration dependent. Regenerants on low NaCl concentration could resume normal growth when they were grown for a longer period (Table 1). Also the stepwise transfer from lower to higher concentrations is more promising than the direct treatment (Ali *et al.* 1997).

Plant cells exposed to NaCl undergo osmotic adjustment through a double mechanism: accumulation of NaCl in the vacuole and accumulation of organic solutes (proline, betaine, polyoles) in the cytoplasm (*e.g.* Yancey *et al.* 1982). The stress signal should induce a loss of feedback inhibition of the key enzyme of proline biosynthesis, Δ-pyrroline-5-carboxylate synthetase (Delauney and Verma 1990, 1993), which results in the proline accumulation. Many recent studies support the hypothesis of a positive correlation between the ability of plant for proline accumulation and the degree of tolerance (Delauney and Verma 1993, Perez Alfocea 1994, Lin and Kao 1996, Martinez *et al.* 1996, Gzink 1996, Ali *et al.* 1998). Proline accumulation in *Bacopa* under salt stress may be preventive to damage from cellular dehydration by balancing the osmotic strength of cytoplasm as suggested by Gzink (1996) in the case of suger beet.

In the regenerants of *B. monniera*, photosynthetic rate declines with increased NaCl concentration. This may be associated with decreased pigment concentration and stomatal index (*e.g.* Choudhury and Choe 1996, Muthuchelian *et al.* 1996).

Earlier studies confirm the differences in protein composition between salt-adapted cultured cells and unadapted cells (Ericson and Alfinito 1984, Ramagopal 1986). The newly synthesized proteins were considered to be salt-adaptation proteins (Ramagopal 1986). 26 kD protein, synthesized in cultured tobacco cells exposed to ABA (Singh *et al.* 1987), may be responsible for accelerating adaptation of cells to NaCl stress (La Rosa *et al.* 1985). The *Bacopa* cultures grown under NaCl stress suggest that the NaCl induces new protein synthesis. These results are corroborated by the earlier findings on *Lycopersicon* (Liu and Li 1991) and *Anabaena* sp. (Sinha and Häder 1996). It is hard to demonstrate unambiguously that observed change in protein gene expression contributes to the survival, probably only some of these proteins are involved in stress tolerance. It is possible that in some cases the synthesis of proteins indicates sensitivity to a stressor rather than being a part of a tolerance mechanism. According to Chretien *et al.* (1992), changes in the electrophoretic pattern of proteins in salt-stress cultures could be suggested as an adaptive response. However, further investigation of properties of the newly-formed proteins in the

cultures under salt stress will help in understanding the mechanism of tolerance and facilitate selection of salt-tolerant plants of *B. monniera via* tissue culture technique.

The results indicate that proline accumulation and formation of new proteins are useful stress-indicators in *B. monniera*.

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