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Full Length Research Paper

Responses of *Bacopa monnieri* to salinity and drought stress *in vitro*

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***Bacopa monnieri* has been chosen as a model medicinal plant to study the abiotic stress (salinity and drought) under *in vitro* system. Distinct morphological changes on organogenesis and callogenesis were observed on stress application. The proline and protein content was also investigated and it was found that the proline increases whereas protein content decreases on stress in the plants under *in vitro* conditions.**

Key words: *Bacopa monnieri*, morphogenesis, drought stress, proline, protein, salinity stress.

INTRODUCTION

Bacopa monnieri L. *(*Family Scrophulariaceae) also refer-red to as *B. monniera*, *Herpestis monniera*, water hyssop, and "*Brahmi*," has been used in the Ayurvedic system of medicine for centuries (Debnath et al., 2006). Tradi-tionally, it was used as a brain tonic to enhance memory development, learning, concentration and to provide relief to patients with anxiety or epileptic disorders. The plant has also been used in India and Pakistan as a cardiac tonic, digestive aid, and to improve respiratory function in cases of bronchoconstriction. *Bacopa* 's antioxidant pro-perties may offer protection from free radical damage in cardiovascular disease and certain types of cancer (Mukherjee and Dey, 1966).

Environmental stress severely restricts the distribution and productivity of plants. In particular, salinity and drought are two major abiotic factors that limit crop productivity (Abdelbe et al., 2003; Misra et al., 1990, 2002). Tissue culture is also an efficient means to study the effect of abiotic stress on the cell metabolism (Das et al., 1990, 1992; Misra et al., 1990, 2002). Cellular accli-mation to unfavorable environments can facilitate plants for continued survival and growth. One such mechanism, that is ubiquitous in plants, is the accumulation of certain organic metabolites of low molecular weight that are known collectively as compatible solutes. Metabolites that serve as "compatible solutes" differ among plant species and include polyhydroxylated sugar alcohols (e.g. manni-tol, sorbitol, trehalose etc.), free amino acids (notably pro-line), quaternary ammonium compounds (e.g. glycinebe-taine, prolinebetaine, -alaninebetaine, and choline-*O*-sulfate), and the tertiary sulfonium compound 3-dimethyl-sulfoniopropionate (DMSP) (Bohnert et al., 1995; Misra et al., 1990, 2002). These compounds are reported to play a pivotal role in cellular osmotic adjustment in response to osmotic and salt stresses (Bohnert et al., 1995; Misra et al., 1990; 2002). The objectives of the present study was to study the changes in morphogenetic potential and proline and protein content of the *in vitro* grown shoots of medicinally useful plant *Bacopa monnieri* under salinity and drought stress.

MATERIALS AND METHODS

In vitro studies

B. monnieri L. plant was obtained from a nursery from Kolkatta, India. Shoot apex and nodal explants of 1 - 1.2 cm were collected from a young plants grown in a greenhouse (temperature of 28 ± 5°C and partial shade conditions). They were watered twice a day. Explants were washed with running tap water for 20 min, immersed in Tween 20 solution for 10 min, washed thrice with double-distilled water, sterilized using 0.1% (w/v) $HgC1_2$ for 5 min and then rinsed three times with sterile distilled water. *Bacopa* explants were inoculated on MS medium (Murashige and Skoog, 1962), supplemented with 5 mg/l BAP (Debnath et al., 2006). Agar (0.8%, Himedia, Mumbai) was added and the pH of MS medium was adjusted to 5.8 prior to autoclaving. The cultures were maintained at a temperature of 25 ± 2°C and 16 h photoperiod. The shoot and nodal explants after 15 - 20 days showed callogenesis as well as morphogenesis (Figure 1A). The proliferating shoots were separated and re- inoculated on MS medium supplemented with 5 mg/L BAP (Figure 1B, C).

Stress treatments

In vitro stress treatment studies on the proliferating shoots in MS + 5 mg/L BAP was done by transferring the shoots to the same medium supplemented with NaCl (0 - 1%, for salinity stress) and mannitol (0 - 10%, for drought stress). These growing explants (callus + shoot + root) were retrieved from the modified MS medium at 5, 15 and 30 days interval and extraction and biochemical quantification for proline and protein was carried out.

Proline estimation

Proline was measured as described by Bates et al. (1973). 100 mg of frozen plant (callus + shoot + root) material was homogenized in 1.5 ml of 3% sulphosalicylic acid and the residue was removed by centrifugation. 100 µl of the extract was thoroughly treated with 2 ml glacial acetic acid and 2 ml acid ninhydrin (1.25 g ninhydrin warmed in 30 ml glacial acetic acid and 20 ml 6 M phosphoric acid until dissolved) at 100°C in a boiling water bath for 1 h. The reaction was then terminated instantly by dipping in an ice bath. The reaction mixture was extracted with 1 ml toluene. The chromophore containing toluene was warmed to room temperature and its optical density was measured at 520 nm. The amount of proline was determined from a standard curve in the range of 20 – 100 µg.

Protein estimation

Protein extraction was performed as described by Hurkman and Tanaka (1986). 1 g fresh tissue was grinded in a chilled mortar and pestle with 2.5 mL of Tris (pH 8.8) buffered phenol and 2.5 mL of extraction media (0.1 M Tris-HCl pH 8.8, 10 mM EDTA, 0.4% 2-mercaptoethanol, 0.9 M sucrose) . The homogenate was centrifuged for 10 min at 5000 g for 4°C. The aqueous phase at the bottom was mixed with 2.5 mL of extraction media and phenol by vortexing. The phenol phase at the top of the extract was separated twice by this method and pooled together. Proteins was precipitated from the phenol extract by adding 5 volumes of 0.1 M ammonium acetate in 100% methanol (stored at -20°C) and centrifuging at 20,000 g, 4°C for 20 min. The precipitate protein was incubated overnight at - 20°C .

Protein quantification was performed as described by Lowry et al. (1951). To 1 ml of above solution, 5 ml of freshly prepared alkaline $CuSO_4$ reagent [1 ml of Alkaline Na_2CO_3 reagent to 50 ml of Copper Sulphate reagent] was added, mixed properly and after 10 min Folin's reagent (0.5 ml) was added. The solution was mixed instantaneously and allowed to develop the colour. Absorbance was recorded at 750 nm.

RESULTS

In the present study, the explants responded to the MS medium supplemented with 5 mg/l BAP. It was found that the nodal explants formed 25 - 30 multiple shoot proliferation along with some callus formation within 15 - 20 days (Figure 1A). After subculturing on the same media, these shoots started to elongate after 20 - 25 days (Figure 1B, C). These explants from the *in vitro* cultures as well as fresh cultures were used as inoculum and subjected them to salinity (NaCl) and drought (mannitol) stress. Growth rate of the plantlets (from both the *in vitro* and fresh cultures) in NaCl or mannitol containing media was slow compared to the growth in the control medium. Among all the plantlets under *in vitro* abiotic stress no significant morphogenic growth was observed within 5 days. In the control medium there was an evidence of multiple shoot bud initiation after 5 days. About 25% of the shoot explants in NaCl containing medium showed green and compact callus at the cut ends after 10 days of incubation, which then differentiated to shoot buds in 10 days of subculture on media with 0.2% (w/v) NaCl (Figure 1D). About 70 - 90% nodal explants showed shoot organogenesis in media with lower concentrations (0 - 0.8% w/v) of NaCl after 15 days (Figure 1E). No shoots development was observed at 1% NaCl concentration. Yet the plantlets were viable and tolerant to 1% (w/v) NaCl concentration. The maximum number of shoots per culture was 20 at 0.2% (w/v) NaCl. After 45 days of culture in 1% (w/v) NaCl yellowing and necrosis of plantlets were observed. No root formation was found under salt stress.

The cultures maintained in MS with 5 mg/l BAP and varying concentration of mannitol also showed distinct morphogenic responses such as multiple shoot proliferation with some callusing (Figure 1F - H). Morphological differentiation shifted from shoot organogenesis to root formation with an increase in mannitol concentration. In the medium without mannitol 20 - 25 visible shoots differentiated from the green callus, where as at 2% (w/v) mannitol ---- shoots with 2 elongated thin roots (1 cm) (Figure 1F), at 4% (w/v) mannitol ---- shoots with 2 - 3 stouter roots, at 6% (w/v) mannitol ---- shoots with 3 - 4 (0 - 1.5 cm) roots; at 8% (w/v) mannitol 4 - 5 shoot buds with 3 roots of length 3 - 3.5 cm (Figure 1G); and 10% (w/v) mannitol shoot with 4 - 5 roots of length 4 cm (Figure 1H) but no significant increase in shoot growth even after 30 days. Among the plants under water stress all the plants were viable till 45 days indicating that *Bacopa* can tolerate both NaCl and water stress successfully.

The proline and protein content were estimated after a period of 5, 15 and 30 days of stress *in vitro*. Proline content increased with an increase in both mannitol induced osmotic stress and NaCl induced salinity stress, as well with culture age (Figure 2A, B). Salinity or osmotic stress induced a gradual decrease in the plantlets with age and increase in the mannitol and NaCl concentrations (Figure 2C, D). The effect of osmotic stress on protein content depended on the concentration of NaCl. At lower concentration of NaCl and mannitol, there was an increase in protein content also, but at higher concentration caused it to decline. There was a similar response on mannitol treatment also.

DISCUSSION

Reduced growth of tissues in stressful media is a usual phenomenon (Das et al., 1990, 1992; Misra et al., 1996; 1997a, b) and it has been interpreted that a metabolism is channeled to resist the stress. Growth response of plantlets in NaCl or mannitol was different (Figure 1).

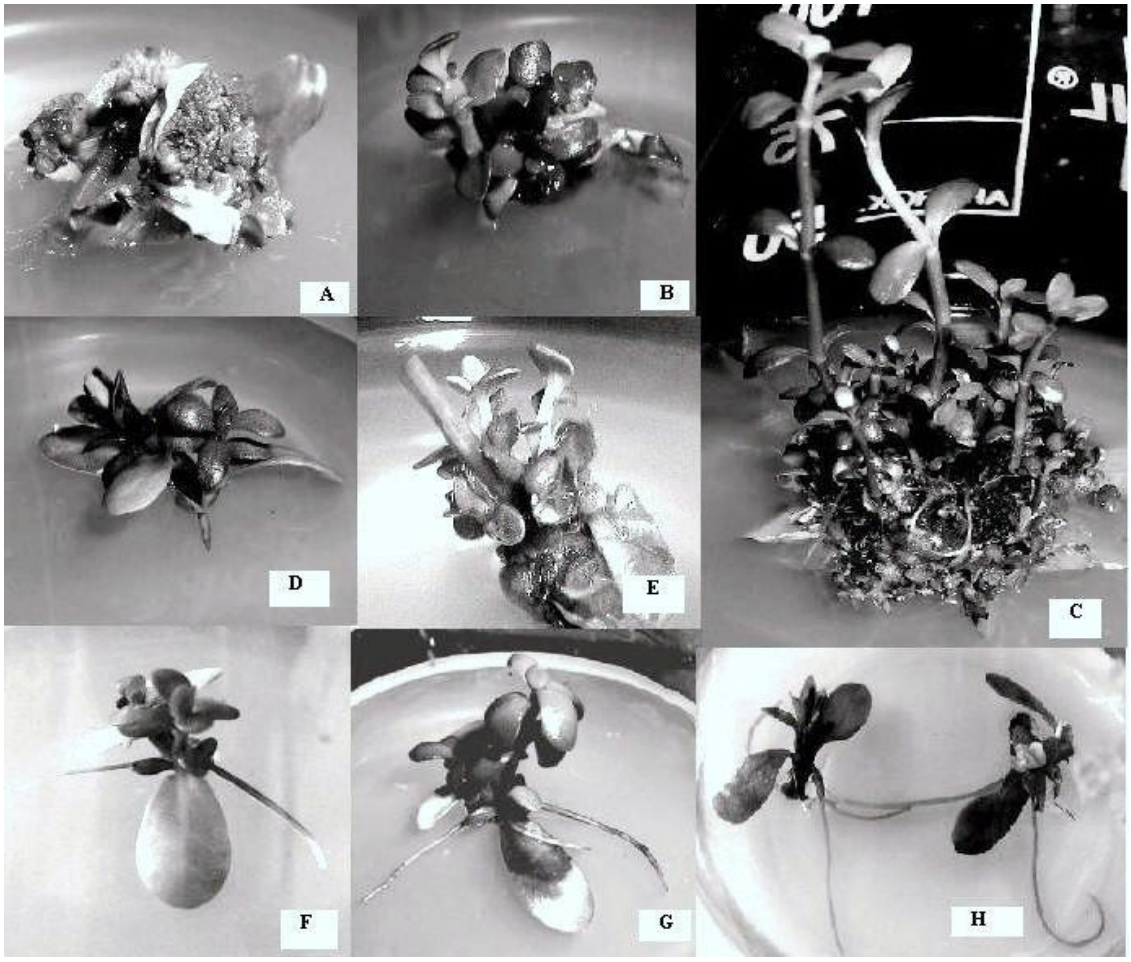

Figure 1. Effect of NaCl and mannitol on *Bacopa monnieri*
(A) 25 - 30 multiple shoot proliferation along with some callus formation within 15 - 20 days MS medium supplemented with 5 mg/l BAP from the nodal explants of *Bacopa monnieri* formed. (B) and (C) After subculturing on the same media these shoots elongated after 20 days and after 25 days. (D) Differentiated of shoot buds after 15 days of subculture on modified MS supplemented with 0.2% NaCl. (E) Shoot proliferation on modified MS supplemented after 15 days of subculture with 0.4% NaCl. (F) On modified MS supplemented with 2% mannitol, after 15 days of subculture 2 elongated thin roots (1 cm) from nodal parts appeared. (G) On modified MS supplemented with 8% mannitol concentration after 15 days of subculture 3 roots appeared. (H) On modified MS supplemented with at concentration 10% mannitol after 15 days of subculture, 4 - 5 roots of length appeared.

Mannitol is a non-ionic osmoticum whereas NaCl is ionic in nature. The results revealed that tissue growth was more impaired due to the effect of an ionic osmoticum than a non ionic one. One of the mechanisms of salt and drought stress in plants is the increase of root length and root branches (Ehsanpour and Amini, 2003; Misra et al., 1990, 2002). In the present study also there was increase in root length and root branches but only in increasing concentrations of mannitol of *Bacopa* suggesting that this plant can tolerate both drought and salt stress.

Proline continues to be the most studied molecule in the decade (Das et al., 1990; Misra et al., 1996). Higher plants accumulate free proline in response to external salt and drought stress (Misra et al., 1990; 2002). Proline acts as an osmoticum, a protective agent of enzyme and cellular structure and a storage compound of reducing nitrogen for rapid re-growth after stress are relieved. In this study also an increase in free proline content has been observed in all *in vitro* plants with increasing NaCl and water stress in the medium. The results of present study are in agreement with the earlier reports on the free proline accumulation under stress in seedlings, plants as well in callus cultures (Das et al., 1990; Misra et al., 1990, 1996, 2002) . *In vitro* culture of plant tissues mimic the response of field grown when subjected to stress treatment (Das et al., 1990, 1992; Misra et al., 1990, 1996, 2002). The increase in free proline content in stressed tissue *in vitro* or seedlings or plants grown in natural conditions showed a simultaneous decrease in the protein content (Das et al., 1990, 1992; Misra et al., 1990; 1995a,

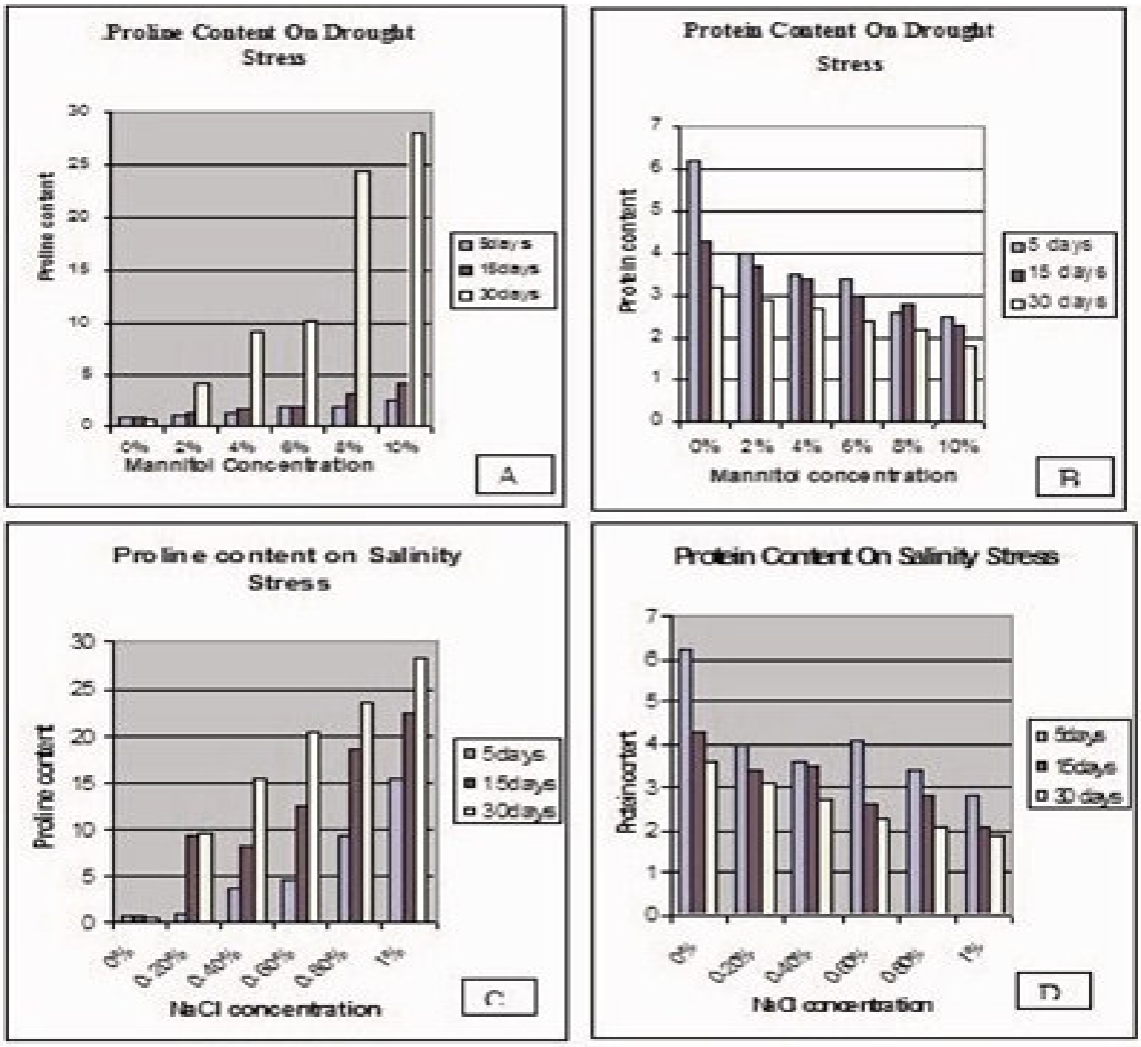

Figure 2. Changes in the proline and protein content of regenerated shoots of *Bacopa monnieri* grown in MS + 0.5 mg/L BAP supplemented with mannitol 0 - 10% w/v (A, B) and NaCl 0 - 1% w/v (C, D). (Each data is an average of 5 - 10 replicates).

b; 1996; 1997a, b; 2002). The present study on *Bacopa* tissue culture under mannitol or NaCl stress corroborates with these findings. These authors suggested that the increase in the proline content in stressed tissue was due to enhanced synthesis of proline and /or stress induced decreased incorporation of proline to other macro-molecule synthesis such as protein, which is degraded by the induction of rapid turn-over processes with an increase in proteolytic enzymes. The higher proline content could be due to enhanced activity of ornithine amino transferase (OAT) and Pyrroline 5-carboxylate reductase (P5CR), the enzyme involved in proline biosynthesis as well as due to the inhibition of proline oxidase, proline catabolising enzymes.

Conclusion

The growth of *Bacopa* for more than 45 days in tissue culture and the NaCl or mannitol stress induced increase in proline content more than 20 times that of the control is a clear indicator that this medicinally important plant can be grown in adverse soil conditions such as salt and water stress affected areas.

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