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ORIGINAL ARTICLE

IN VITRO STUDIES ON ABIOTIC STRESS INDUCED RESPONSE IN THE MEDICINAL PLANT *BACOPA MONIERI* (L.) PENNELL

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Abstract: *Bocopa monnieri (L.) Pennell (Brahmi) is a plant that has been used for centuries in traditional Ayurvedic medicine and it has a long history as a medicinal plant in human civilization with diverse therapeutic applications specially focused on and treatment of neurological disease. Micropropagation by plant tissue culture is the widely used practical application of plant biotechnology, where new plant tissues are grown by transferring them into an in vitro condition later transferred to ex vitro environment. In the present study the cultured plants induced to grow under abiotic stress supplemented with salt (NaCl) stress (0.1 N) to observe the morphological changes within culture and also compare the different biochemical and molecular expression in stress induced and control plants.*
Keywords: *Bacopa*, *in Vitro*, NaCl, abiotic stress, root, shoot and molecular assay, biochemical assay.

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1. INTRODUCTION

Bacopa monnieri (L.) Pennell, commonly familiar in the name 'Brahmi' or water hyssop, is a small, creeping, perennial herbaceous plant, belonging to the family Scrophulariaceae. *B. monnieri* is an important medicinal plant in Indian traditional Ayurvedic medicines. Leaves and green shoots of this plant has been applied in Ayurveda treatment as a nerve tonic and treatment of neurological disease for millennia. In the 100-200 BC, *Bacopa* was recommended in Ayurvedic text (Charaka Samhita) for the treatment of wide range of mental illness [1, 2].

There were a number of stress induced studies, undertaken to assess the enzymatic changes occurring in plants [3]. Presently, *in vitro* stress induction on *Bacopa monnieri*, with 0.1 M concentration of NaCl solution was evaluated for biochemical expression and *ex vitro* transplantation studies. The outcomes of the studies have the potential to be utilized for large scale propagation of plants in soil having abiotic stress condition. The increased demand of this medicinal plant as a natural resource leads to the depletion of its natural habitat, making *Bacopa monnieri* a critically endangered species [4].

The aim of the present study is to observe the changes within *in vitro* growth, morphogenetic potential, level of

protein and different enzymes and expression of genes using the cDNA on abiotic stress induced plants and make a comparison with untreated plants of *Bacopa monnieri.*

2. MATERIALS AND METHODS

The internodal explants of *Bacopa monnieri (L.),* collected from pre cultured plants under *in vitro* condition. The explants were cut into pieces and placed in the Murashige and Skoog (MS) basal media [5] and MS media with 0.1 N salt (NaCl) stress (pH 5.7).

After the successful development of plantlets, they were allowed to undergo a period of acclimatization to adapt to the outside (*ex-vitro)* environmental conditions with different combination of composition i.e. soil, soil and sand (1:1) and soil, sand, soil rite (1:1:1) and Bavistin (0.1% w/v) was added in each set of combination. Various *in vitro* biochemical and molecular assay were conducted for to study the profile of *in vitro* behaviour.

Biochemical assay

The free amino acid contents were estimated according to the ninhydrin method of Lee and Takahashi [6]. The blue colour intensity was measured at 570 nm in spectrophotometer (Hitachi UH5300). The results were expressed in terms of µg amino acid g^{-1} FW.

The level of soluble protein is then estimated through the method of Lowry *et al.* [7].

Catalase assay (CAT)

Activities of catalase (CAT) was measured using the method of Kar and Mishra [8] with some modification. The CAT reaction solution (3 ml) contained 50 mM phosphate buffer (pH 7.0), 15 mM H_20_2, and 0.1 ml enzyme extract. Reaction was initiated by adding enzyme extract. Changes in absorbance of the reaction solution at 240 nm were read every 20 s. One-unit CAT activity was defined as an absorbance change of 0.01- unit min^{-1}. Measured leaves were crushed on ice bath by adding extraction buffer and β- Mercaptoethanol. The extract was centrifuged at 15000 rpm at 4°C for 20 minutes. The clear supernatant was carefully collected which contained the enzyme.

Superoxide dismutase activity assay

Superoxide dismutase (SOD) activity was studied by using the nitro blue tetrazolium method. The reaction mixture contained 2.5 ml of 80 mM Tris-HCI buffer (pH 7.5) containing 0.12 mM EDTA and 10.8 mM TEMED, O.1 ml 0.0033% BSA, 0.1 ml 6 mM NBT, 0.1 ml of 0.6 MM riboflavin and 0.1 ml enzyme extract. The reaction was started by the addition of riboflavin, and the glass tube was shaken and placed under a fluorescent lamp (60 µmol $m^{-1}s^{-1}$). The reaction was allowed to proceed for 10 minutes and was stopped by switching off the light. The absorbance was measured at 560 nm. One unit of SOD was defined as the amount of enzyme that produced 50% inhibition of NBT reduction under assay condition. Enzyme activity was expressed as EU SOD mg protein min [9].

Estimation of free proline

Free Proline content was determined according to the procedure of Bates *et al*. [10]. Measured leaves were homogenized in 3% (w/v) aqueous sulfosalicylic acid and the absorbance of the red chromophore was measured spectrophotometer at 520 nm. The contents were expressed as µg fresh weight (FW).

Molecular assay

Plant materials (both treated and untreated) are washed and surface sterilized in 70% ethanol. Leaves weighed and

crushed in chilled autoclaved mortar and pestle with liquid nitrogen and Trizol for the extraction of RNA. Quantitative estimation of isolated RNA was done by gel electrophoresis method using 1 X TAE buffer at 70 V for 40 min. cDNA was isolated using this RNA through rt-PCR and this cDNA was used for semi quantitative assay with two gene specific primer (ACTIN i.e. housekeeping gene; forward primer 5'ACTGGTATTGTGCTTGACTCTG'3 reverse primer AGCTTCTCTTTAATGTCACGGAC'3) and NAC i.e. stress marker BrNAC2a forward primer CGGGATTTTCTCCTACGGCG reverse primer 5'GCTCTTCCTCAGCTACAGGC'3) in PCR followed by gel electrophoresis.

3. RESULTS AND DISCUSSION

In Vitro growth was observed in two sets i.e.; one as control, i.e., with MS basal media only (Figure 1 a-d) and another was MS nasal media, treated with NaCl (Figure 1 e-h) from the beginning to 21st day, at weekly interval.

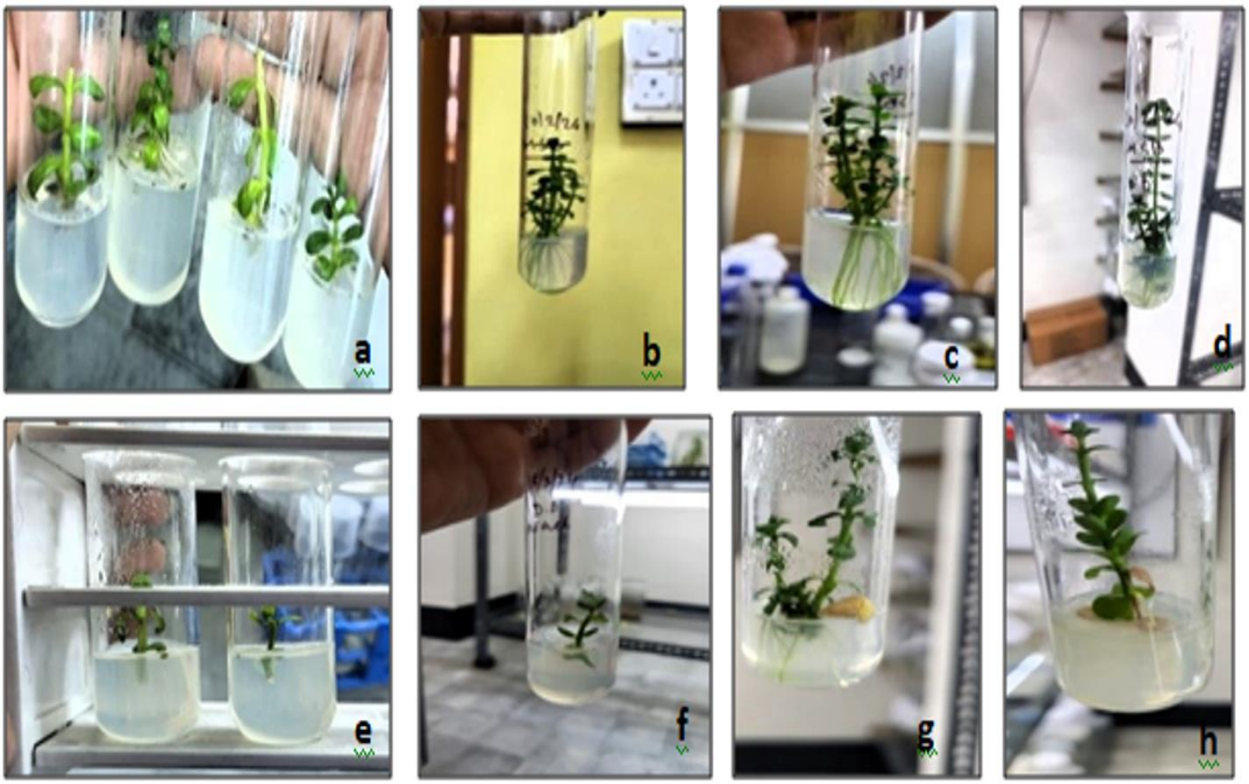

Figure 1. Development of *in vitro* generated plants under different parameters in MS basal media. a-d: In control condition (without NaCl) for 0 day (a), 7 days (b), 14 days (c) and 21 days (d). In MS media treated with NaCl for 0 day (e), 7 days (f), 14 days (g) and 21 days (h).

The untreated plantlets showed normal growth between 0 -21 days (Figure 2 A). However, reduced growth was observed in plants under stress was manifested by reduced elongation (Figure 2 B) of the plantlet and was visually observed within 0-7 days (Figure 1 e-f), which continued for subsequent observation of 14 and 21 days (Figure 1g-h) showing less rooting too.

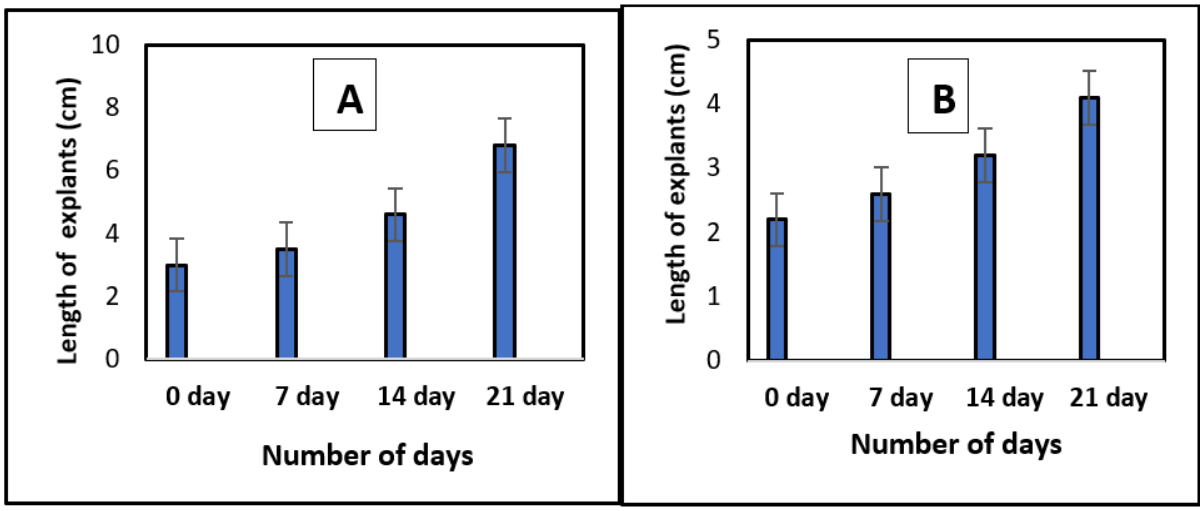

Figure 2. Increase in length or explants with time in control set (A) with MS basal media and NaCl treated MS media (B) on different time interval (0-21 days)

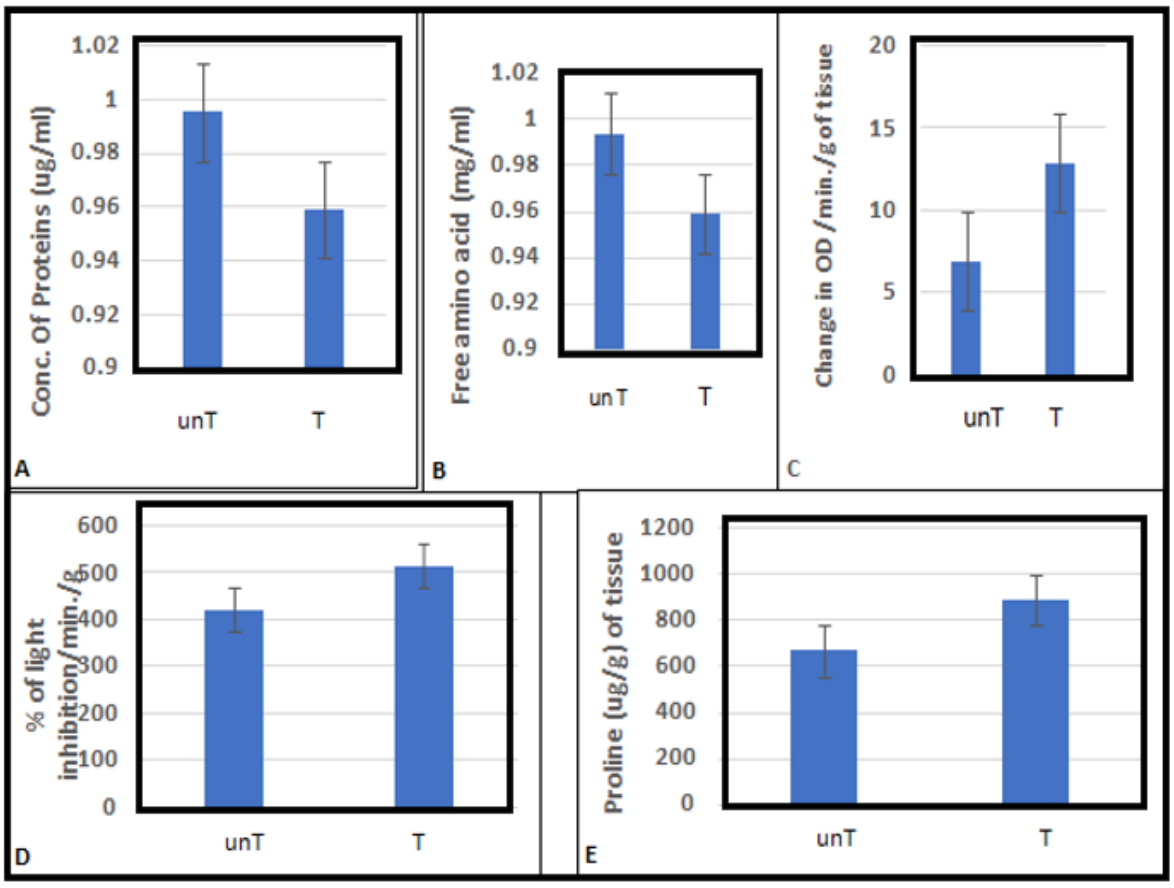

Figure 3. Various parameters in *in vitro* generated plants grown in both untreated (unT, control, MS basal media) and treated (T, MS media threated with NaCl). A: Protein concentration, B: Free amino acid content, C: Catalase activity, D: Superoxide dismutase and E: Proline content. Bars indicate standard deviation.

Soluble protein content (Figure 3A), in NaCl treated stressed plant (T) was found to be lower, compared to the untreated control plants. Similarly, free amino acids content (Figure 3B) in the tissue was more in control plants than in NaCl stressed plants. This is perhaps due to less amount of protein synthesis due to osmotic adjustment taking place in the regenerated plants under stress [11].

However, on the contrary, the enzyme activity was observed to be increased in the regenerated plants under stress. Catalase activity was approximately double (Figure 3C) with respect to untreated plants. Catalase has proven role of nullifying oxidative damage due to stress, which is evident here as well [12].

Another enzyme SOD (Super Oxide Dismutase) Showed 20% increased activity in stressed plant (Figure 3 D). Activity of this enzyme appears to be a stress marker, which tries to stabilize the plant metabolism drastically [13]. The Proline content indicates the rise at the level of 15% (Figure 3E) in salt stressed regenerated plant. Plants exposed to NaCl molecules show physiological, anatomical and biochemical response to overcome adverse effect of salinity. Proline, Catalase Superoxide dismutase and free amino acids are known to help to overcome the stress [8]. The interrelationships of these molecules may play crucial roles in maintaining cellular integrity against stress of salinity y and act outer environment.

Proline is the amino acid, is reported to be accumulated in the cells, in response to different stress conditions like drought and salinity. It acts as osmo-protectant by helping to scavenge free radicals and help in the resilience by stabilizing enzymatic reactions and protecting the cell structure through appropriate turgor pressure [14].

Superoxide dismutase (SOD) is reported to help in the mitigation of the oxidative damage in tissue by neutralization of superoxide radicals which are a kind of harmful reactive oxygen species (ROS) [15].

The activity of catalase reduces H_2O_2 concentration in cellular environment by breaking it into water and oxygen. In this way, catalase detoxifies and prevents overall cellular damage. Through these overall observations, the present study depicts that regenerated *Bacopa monnieri* plant is showing adaptive strategies to cope and survive in saline environment [16].

Ex-vitro survival rate was comparatively high in soil-sand-soil rite (1:1:1) mixture (Figure 4 a-c)

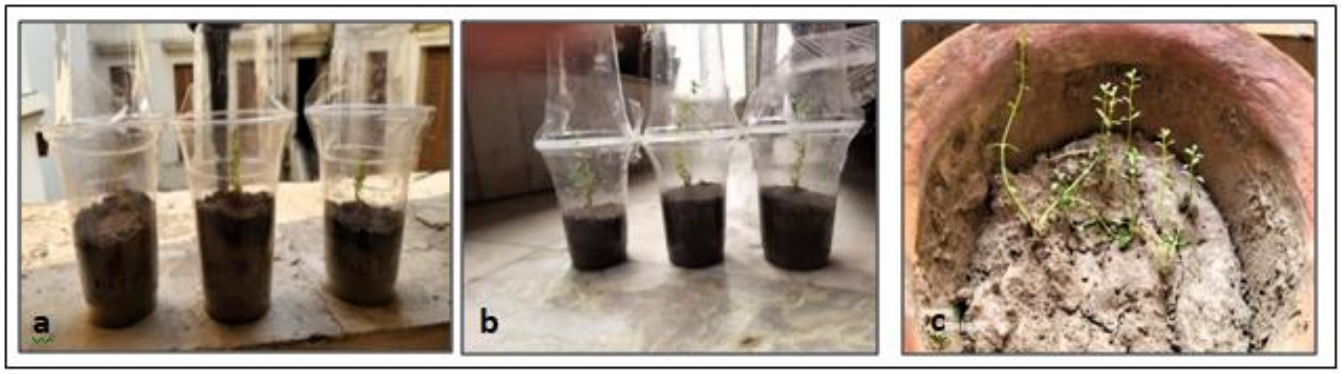

Figure 4. Transplanted plants being acclimatized *ex vitro* under natural conditions in different soil compositions. a. Soil, sand and soil rite (1:1:1) b. Soil and sand (1:1) c. Soil

RNA was isolated following the standard protocol [17]. In the NaCl treated plant, RNA (Figure 5a) was showing

trails in the gel. The comparison of expression of two RNA namely ACTIN and NAC (nitrogen assimilation control) stress marker revealed that the expression of ACTIN levels is relatively similar across these two conditions i.e., salt treated and untreated plants.

However, in stressed plant, the results were distinct, i.e. the expression of NAC was very high (Figure 5b) compared to untreated plants.

NAC genes are known as transcription factors perhaps initiate downstream stress responsive genes, regulating both biotic and abiotic stress response in plants [18]. Such transcriptome response related to NAC gene is somehow evident from this result in the regenerated plants of *Bacopa monnieri* (L.) Pennel.

This is observed that the over expression of NAC is due to stress and the gall inducing the protein is synthesized more to synthesize protein /metabolites to protect the *Bacopa monnieri* plant and NaCl stress. Over expression of NAC gene leads to more transcription factors which helps in stress response and gene expression.

All these changes perhaps are the complex physiological and biochemical responses that the plant exhibit to survive and adapt to challenging environmental conditions, which needs further detailed study.

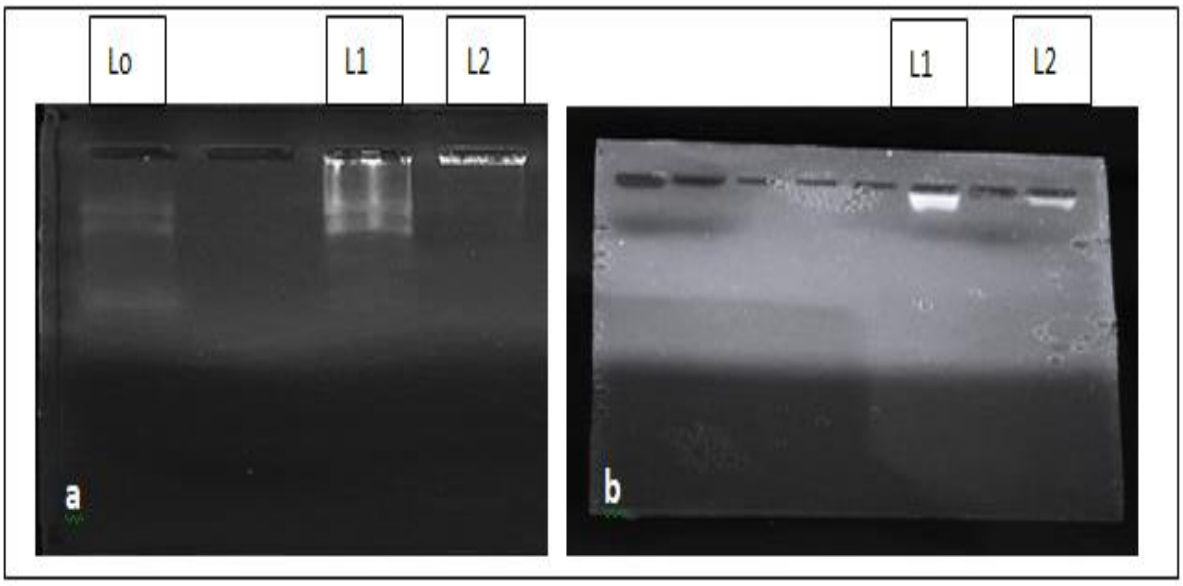

Figure 5. Gel electrophoresis (L1- Treated and L2- Untreated). a. RNA extract b. Post rtPCR amplification differential expression of ACTIN and NAC from cDNA)

4. ACKNOWLEDGEMENTS

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