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SHORT COMMUNICATION

Aluminium-Induced Morphogenic and Biochemical Variations of *Bacopa monniera*

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Shoots and roots of *Bacopa monniera* (L.) Wettst. have been regenerated from nodal segments on MS medium containing combinations of NAA and BAP. The cultures showed 100% regeneration on MS (sucrose 2%) medium added with NAA (0.2 mg L^{-1}), BAP (0.5 mg L^{-1}) and glutamine (50 mg L^{-1}). Supplemented with aluminium chloride (up to 400 μM), this medium could ensure successful survival of regenerants. All the regenerants, maintained on $AlCl_3$-supplemented medium for the last three years, failed to grow when transferred to $AlCl_3$-free media. Aluminium stress also induced synthesis of proline and proteins. The rate of photosynthesis decreased at increased aluminium concentrations.

Keywords: *Bacopa monniera*, aluminium, morphogenesis, proline, regenerants

Metals, which constitute a significant group of pollutants, become toxic to plant growth at higher concentrations (Chakravarty and Srivastava, 1992). One such metal, aluminium (Al), is found abundantly in the earth crust that comprises about 7% of the earth mass. Since many plant species are sensitive to micromolar concentrations of Al, its potential for soil toxicity is considerable (Taylor, 1988). Aluminium, a major constituent of soil, has recently been suggested as an important factor in forest decline as it is released from the soil under conditions of increased acidity (Ulrich *et al.*, 1980; Goldbold *et al.*, 1988; Hauhs *et al.*, 1989; Schulze, 1989). Aluminium proves toxic to a number of tree species (Goldbold *et al.*, 1988) and impairs growth of crop plants at high concentratoins (Taylor, 1988). The toxic effects of Al occur primarily in roots and then in shoots (Ryan *et al.*, 1993). Root stunting and thickening are typical symptoms of Al toxicity (Clarkson, 1969). Aluminium causes decrease in the hydraulic conductivity (Lpc) of root cells (Zhao *et al.*, 1987) and in the whole water conductivity (Lpr) (Kruger and Sucoff, 1989; Barcelo *et al.*, 1996; Gunse *et al.*, 1997).

Plant tissue culture is an excellent tool for study of stress tolerance. Tolerant plant lines can be produced by regenerating plants in cultures adapted to grow under the given stress (Nabors *et al.*, 1980; Kochba *et al.*, 1982; Ali *et al.*, 1997). Brahmi (*Bacopa monniera*) plant, used in medicine as memory vitalizer, is a small creeping herb of the family Scrophulariaceae. High frequency of regeneration makes it a model system for in vitro studies (Ali *et al.*, 1996).

The present study investigates the effect of Al on the morphogenic response of *Bacopa monniera* and determines the maximum concentration up to which the tolerant cultures could be raised. Variations in some stress-related parameters such as protein synthesis and proline contents (Pesci and Reggiani, 1992; Chakravarty and Srivastava, 1997) and photosynthetic rate (Robinson *et al.*, 1994) have been studied.

MATERIALS AND METHODS

Establishment of Cultures

Stem segments (20 mm) of *Bacopa monniera* (L.) Wettst. were procured from the Herbal Garden at Jamia Hamdard, New Delhi. The explants (500 pieces) were thoroughly washed with running tap water for 30 min and with 5% cetrimide (ICI, India) for 10 min. The washed segments were sterilized with freshly prepared 10% aqueous sodium hypochlorite for 10 min, and with 0.1% mercuric chloride for 5 min. They were then rinsed

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thoroughly with sterile distilled water, cut aseptically into 10 mm pieces and placed on Murashige and Skoog's (1962) medium (MS) gelled with 0.62% agar (Qualigen, India). MS (sucrose 3%) medium supplemented with NAA (0.1-0.2 mg L^{-1}), BAP (0.5-5.0 mg L^{-1}) and casein hydrolyzate (CH, 500 mg L^{-1}) was used for culture initiation.

Maintenance of the Cultures

The regenerants were maintained on MS (sucrose 2%) medium supplemented with NAA (0.2 mg L^{-1}), BAP (0.5 mg L^{-1}) and glutamine (50 mg L^{-1}). Growth and morphogenic response in terms of shoots per culture, height of regenerated shoots, increase in total fresh weight and the root formation were recorded in four-week-old cultures of 500 mg fresh weight with five shoots of 10-12 mm height grown on maintenance medium (MM) with various levels of $AlCl_3$ (50, 100, 150, 200, 250, 300 and 400 µM). All the cultures were maintained at 25±2°C in a culture room with 16 h photoperiod provided by fluorescent and incandescent light (total intensity 100 µmole m^{-2} s^{-1}).

Biochemical and Physiological Estimations

Proline content of the regenerants was determined according to Bates *et al.* (1973). Samples of fresh material (300 mg) were homogenized in 10 ml of 3% aqueous sulphosalicylic acid. The homogenate was centrifuged at 9000×g for 15 min. Two ml aliquot of the supernatant was mixed in a test tube with 2 ml of freshly prepared acetic acid and acid ninhydrin to react for 1 h at 100°C. The reaction was terminated in an ice bath and the mixture was extracted with 4 ml of toluene. The extract was vigorously vortexed for 20 s. The chromatophore-containing fraction was then aspirated from the aqueous phase and its absorbance determined photometrically at 520 nm (Beckman 640 D, USA) using toluene for a blank. The total buffer-soluble proteins in the regenerants were estimated following the method of Bradford (1976). Fresh material (500 mg) was homogenized in 1 ml of phosphate buffer (pH 7.0). The crude homogenate was centrifuged at 5000×g for 10 min. A 0.5 ml aliquot of the supernatant was mixed with 0.5 ml of freshly prepared trichloroacetic acid (TCA) and centrifuged at 8000×g for 15 min. The pellet was dissolved in 1 ml of 0.1 N NaOH and 5 ml of Bradford reagent was added. Absorbance was recorded photometrically at 595 nm, using bovine serum albumin as standard. Li-6200 portable photosynthesis system (Li-Cor Inc., Lincoln, Nebraska, USA) was used for automatic measurement of the net rate of photosynthesis in the various samples.

RESULTS AND DISCUSSION

The regenerants were grown on MS medium supplemented with NAA (0.2 mg L^{-1}), BAP (0.5 mg L^{-1}), glutamine (50 mg L^{-1}) and with various concentrations of $AlCl_3$. The regenerants grown on maintenance medium without $AlCl_3$ served as control. They showed increase in number and height of shoots and the total fresh weight (Table 1). Eleven shoots per culture, with 3 cm height and 1.37 g increase in total fresh weight were recorded after 4 weeks. Rooting was 100%. On 50 µM $AlCl_3$-supplemented medium, the cultures showed an increase in number (17) and height (3.95 cm) of shoots and in the total fresh weight (1.68 g) of the regenerants after 4 weeks. Rooting was 100%. Even at 100 µM concentration of $AlCl_3$, the regenerants kept increasing in number (15) and height (3.85 cm) of shoots and the total fresh weight (1.61 g) as noted after 4 weeks. Roots differentiated in 100% cultures. Cultures grown directly on 150 µM of $AlCl_3$ showed an increase in number (113) and height (3.8 cm) of shoots per culture and the total fresh weight of regenerants (1.56 g) after 4 weeks. Rooting occurred in 100% cultures. An increase in number (12) and height of shoots (3.30 cm) and in the total fresh weight of regenerants (1.45 g) was apparent on 200 µM $AlCl_3$ after 4 weeks. Roots appeared in 95% cultures. The cultures grown on 250 µM $AlCl_3$ also increased in number (9) and height (3.0 cm) of shoots and the total fresh weight (1.35 g) of regenerants after 4 weeks. Root formation occurred only in 78% cultures.

Cultures grown on 300 µM of $AlCl_3$ exhibited an increase in number (7) and height (2.0 cm) of the regenerated shoots and the total fresh weight of regenerants (1.25 g) after 4 weeks. Rooting occurred in 60% cultures. A gradual increase in number of shoots per culture (7.0), height of shoots (1.5 cm) and the total fresh weight (1.18 g) of regenerants was recorded on 400 µM after 4 weeks. Roots developed in 60% cultures only. The cultures could not survive beyond 400 µM level of $AlCl_3$.

The cultures transferred on low concentrations (50-200 µM) of $AlCl_3$ exhibited stimulated growth of regenerants. However, a gradual decline in growth

occurred at higher (250-400 μM) concentrations of $AlCl_3$ (Table 1). Subsequently, the regenerants were able to grow in the presence of such higher levels of $AlCl_3$ (Data not presented). Moreover, the cultures growing on higher concentrations (250-400 μM) of $AlCl_3$ developed thinner roots. All the cultures are being maintained on Al-fortified medium for the last three years.

The cultures grown under Al stress (50-400 μM) showed an enhanced amount of proline in comparison to control. The proline content was correlative to the levels of $AlCl_3$. Similarly, the total protein content of the regenerants increased with increasing concentration of $AlCl_3$ in the medium (Table 1). The rate of photosynthesis in regenerants grown on $AlCl_3$ gradually decreased. This was highly correlative to the concentration of $AlCl_3$ in the medium (Table 1).

Aluminium concentrations in mineral soil solutions are usually much below 1 mg L^{-1} (~37 μM) at pH values higher than 5.5, but rise sharply at lower pH. Ability of some plant species (accumulators) to tolerate high aluminium contents in the tissue, and the toxic effects of high aluminium levels in soil or nutrient solutions on plant growth were well studied (Marschner, 1995). There is no convincing evidence that aluminium is an essential mineral element even for accumulator species. However, beneficial effects (growth stimulation) of low aluminium concentrations have been noted on sugar beet and maize (Bollard, 1983). In the tea plant, an aluminium-tolerant crop species, growth stimulation occurs at as high concentrations of Al as 1000 μM (Matsumoto *et al.*, 1976) or even 6400 μM (Konishi *et al.*, 1985). The rate of root elongation in *Picea abies* seedlings was inhibited by 63-73% within 24 hours on exposure to Al at 800-1200 μM (Goldbold *et al.*, 1988). Meredith (1978) noted that callus from two tomato cultivars with different level of tolerance to aluminium at the whole plant level, responded to aluminium differently, thus suggesting that cultures can be used for detecting varietal differences in metal tolerance. Conner and Meredith (1985) regenerated fertile plants from aluminium-resistant callus cultures of *Nicotiana plumbaginifolia* after recurrent selection.

In the present investigation, the regenerants of *Bacopa monniera* could survive up to 400 μM of $AlCl_3$. The cultures grown on higher concentrations (250-400 μM) of $AlCl_3$ showed retarted growth rate of the regenerants up to 12 weeks, compared to control and lower Al levels; subsequently recovery took place by the 16th week (Data not presented). Beyond 400 μM level, the cultures could not grow. In fact, regenerants grown on lower concentrations (50-200 μM) of $AlCl_3$ showed better growth as compared to control (without $AlCl_3$), substantiating the observations of Bollard (1983) in maize and suger beet plants. Successful maintenance of regenerants on $AlCl_3$ for the last three years is indicative of their adaptability towards Al (Fig 1A-D). When transferred on to aluminium-free media, these regenerants could not survive, thus indicating that they had become metallophilic. The root elongation or inhibition rate is widely used as a reliable indicator for varietal differences in Al tolerance (Hanson and Kamprath, 1979). The regions of root where pH effect is probably most critical are the root cap and root apical meristem. Bennet *et al.* (1987) found that aluminium-treated

Table 1. Effect of $AlCl_3$ on morphogenic response (increase in number of shoot per culture, height of shoot, root development and total fresh weight), proline and protein contents and rate of photosynthesis

$AlCl_3$ (μM)	Increase in shoots/ culture	Increase in shoot height (cm)	Increase in fresh weight/ culture (g)	Rooting (%)	Proline content* (μg/g fr.wt.)	Protein content* (mg/g fr.wt.)	Rate of* photosynthesis (μmole CO_2 $m^{-2}s^{-1}$)
00	11±2.60	3.0 ±0.98	1.37±0.95	100	21.87±2.23	6.26±0.52	15.30±1.16
50	17±2.53	3.95±0.86	1.68±0.98	100	44.40±2.94	7.21±0.73	14.20±1.39
100	15±2.60	3.85±0.84	1.61±0.89	100	47.30±2.99	7.51±0.62	14.30±1.38
150	13±2.85	3.8 ±0.88	1.56±0.84	100	56.25±2.91	8.22±0.52	14.40±1.33
200	12±2.45	3.3 ±0.79	1.45±0.88	95	58.87±2.91	9.12±0.56	14.50±1.45
250	9±2.11	3.0 ±0.59	1.35±0.78	78	93.90±2.86	9.31±0.77	14.75±1.51
300	7±2.15	2.0 ±0.55	1.25±0.83	60	69.60±2.65	9.43±0.72	14.11±1.38
400	7±2.18	1.5 ±0.48	1.18±0.86	60	72.57±2.60	9.84±0.82	14.00±1.36

Values represent mean ± S.D. based on 24 replicates and experiment was repeated twice.
*Values represent mean ± S.D. based on 3 replicates and experiment was repeated twice.
Five shoots of 1-1.5 cm height with 500 mg fresh weight were initially inoculated in each culture vial.

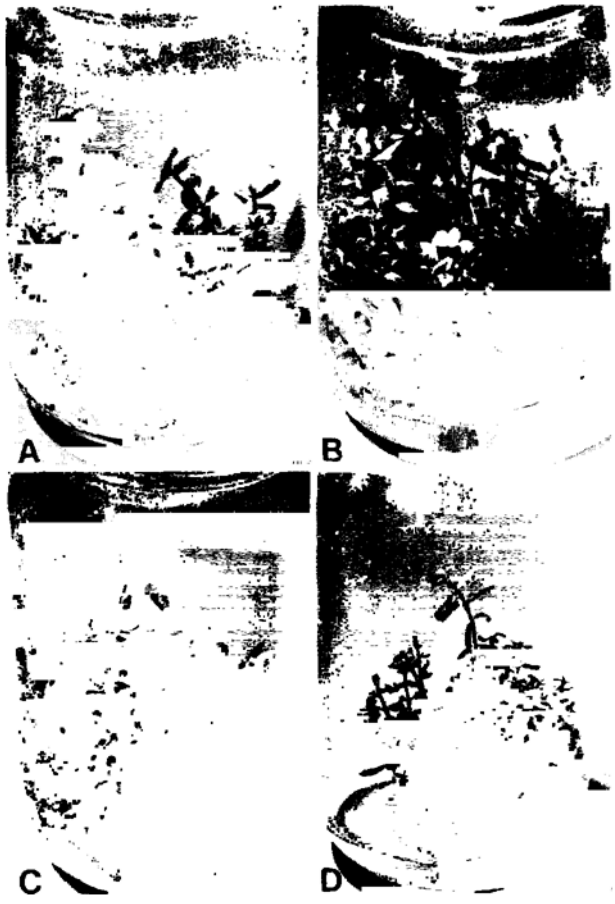

Fig. 1. *Bacopa monniera*. Three-year old cultures subcultured every 4 weeks on control and various concentrations of $AlCl_3$. A, Regenerants on control showing normal growth; B, Regenerants on 50 µM $AlCl_3$; C, Regenerants on 200 µM of $AlCl_3$, Note; Regenerants on 50 and 200 µM $AlCl_3$ showed luxuriant growth; D, Regenerants on 400 µM $AlCl_3$ resumed the growth.

corn roots showed increased H^+ efflux near the root apex and root cap, compared to the control roots which were slower in H^+ efflux. They hypothesized that the primary site of Al injury is in the root cap and that effects on cell division and differentiation in the root meristem are mediated via hormones produced in the root cap (Binnet *et al.*, 1987). Kinraide (1988) found a different pattern and showed that control wheat root did not acidify the apical regions, while Al-treated root often acidify the rhizosphere around the apex. Similar observations have been noted for the roots of *B. monniera* regenerants grown at high concentrations of aluminium. The appreciable growth of regenerants under aluminium stress signifies the role of Al as stimulator of growth in *Bacopa monniera* (Fig 1B-D). Fully-grown plantlets were successfully transferred to pots and maintained under field conditions.

Proline biosynthesis is said to be controlled by an enzyme pyrroline-5-carboxylate synthetase (P_5-Cs), which is regulated by proline via feedback inhibition (Delauney and Verma, 1993). A loss of this feed back regulation was observed under water stress. This could account for the high accumulation rates of proline under stress (Delauney and Verma, 1993). Differential tolerance to metal toxicity may also be due to differences in the structure and function of membranes or to the differential gene expression through different biochemical pathways (Foy *et al.*, 1978). Changes in the permeability of membrane lead to water-stress like conditions, causing increase in proline levels (Pesci and Reggiani, 1992). Changes in proline content due to Al, in the present study also suggest that permeability of membranes may be affected. Proline is also believed to protect plant tissues against stress by acting as N-storage compound, osmosolute and hydrophobic protectant for enzymes and cellular structures (Greenway and Munns, 1980). The increase in proline content of *Bacopa* plantlets indicates that they tend to protect themselves from the toxicity of $AlCl_3$.

Estimation of the total protein content in *Bacopa* regenerants has revealed a considerable increase in response to enhanced levels of aluminium. This substantiates many earlier findings on total proteins under stress conditions (Mehrotra and Bisht, 1990; Liu and Li, 1991; Chretien *et al.*, 1992; Chakravarty and Srivastava, 1997). Presence of Cd was thought by Hirt *et al.* (1989) to stimulate mRNA synthesis leading to increase in total protein.

Photosynthesis is inhibited by heavy metals due to decrease in chlorophyll biosynthesis and transpiration (Goldberg *et al.*, 1980; Bhardwaj and Mascarenhas, 1989). Miller *et al.* (1973) have demonstrated Cd-related inhibition in the mitochondrial electron transport in dark. We have noticed minor reduction in photosynthetic rate of *Bacopa monniera* in the presence of aluminium.

Thus, *Bacopa* exhibits various responses to different concentrations of $AlCl_3$. Higher concentrations bring the morphogenic potentiality down. The response is correlated to changes in biochemical parameters such as proline and protein contents which may be an indication of toxicity or tolerance. The regenerants of *B. monniera* can thus be grow even at higher levels of aluminium by keeping the regenerants on aluminium-supplemented medium for longer durations. These observations may assist in

generating aluminium-tolerant plants of *Bacopa monniera*.

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