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The Effect of Heavy Metals on *In Vitro* Adventitious Shoot Production and Bacoside A Content in *Bacopa Monnieri* (L)

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Abstract

In the present study the effect of heavy metals ($MnSO_4$, $ZnSO_4$ and $CuSO_4$) on adventitious shoot regeneration, biomass and bacoside A accumulation in *Bacopa monnieri* was carried out. The leaf explants were cultured on Murashige and Skoog (MS) medium supplemented with 2.0 mg l^{-1} kinetin (Kin) with varying concentration of heavy metals (Control: Mn-0.10 mM, Zn-0.03 mM and Cu-0.0001mM; Mn: 0.20, 0.40, 0.80 and 1.60 mM; Zn: 0.06, 0.12, 0.24 and 0.48 mM; Cu: 0.02, 0.05, 0.10 and 0.20 mM). Optimum number of adventitious shoots (123.50 shoot/explants), fresh weight (3.826 g) and dry weight (0.226 g) of *Bacopa monnieri* were obtained in the medium with 0.12 mM Zn concentration. The highest production

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of bacoside A content (28.090 mg g^{-1} DW) was recorded in *Bacopa monnieri* leaf explant cultured in the medium with 0.20 mM Cu concentration.

Keywords: *Bacopa monnieri*, Bacoside A, Biomass, Heavy metals

Introduction

Plant, cell, tissue and organ culture techniques have emerged as an escapable tool with the possibilities of complimenting and supplementing the conventional method in plant breeding, plant improvement, biosynthetic pathways, etc. Plant tissue culture plays a major role in conservation of germplasm, rapid clonal propagation, regeneration of genetically manipulated superior clones, production of secondary metabolites and ex vitro conservation of valuable phytodiversity [1]. This technique has several potential applications in crop improvement, and efficient regeneration is a prerequisite in such improvement programmes.

Bacopa monnieri (L.) Wettst (Plantaginaceae), also referred as Brahmi or Jal brahmi is an important Ayurvedic system of medicinal herb, found throughout the Indian subcontinent in wet, damp and marshy areas [2]. It is used for centuries as a brain tonic, memory enhancer, revitaliser of sensory organs, anti-anxiety, cardio-tonic, diuretic, antidepressant and anticonvulsant agent [3]. The pharmacological properties of Brahmi are mainly due to the presence of major bioactive saponins called 'bacosides' which are complex mixtures of structurally closely related compounds, glycosides of either jujubogenin or pseudojujubogenin. Bacoside A is a major chemical entity shown to be responsible for memory-facilitating action of Brahmi [4]. The composition of bacoside A has been established very recently as a mixture of triglycosidic saponins, bacoside A_3, bacopaside II, jujubogenin and bacopasaponin C [5].

Plant tissue culture techniques have previously been found useful in the selection of metal tolerant plants [6, 7, 8]. Metal ions such as Cu^{2+}, Mn^{2+}, Zn^{2+} and Fe^{2-} are essential trace nutrients taking part in redox reactions, structural configuration of several enzymes and nucleic acid metabolism [9]. At higher concentration, however, they become strongly poisonous, causing inhibition of growth and

metabolism and even death of the organism [9, 10, 11]. Although, these heavy metals are widely used as microelements, their effective roles on morphogenesis in medicinal herbs need extensive elaboration. An optimized protocol for the *In vitro* shoot regeneration of Brahmi has been established in our previous studies [12, 13, 14, 15]. Earlier researchers explained the effect of Cd, Cu and Zn on morphogenic and proline content of *Bacopa monnieri* regenerants from nodal explants in three different experiments [16, 17, 18]. Since stress has been implicated in secondary metabolite production, So in the light of earlier works, the present work was undertaken to assess the role of metal stress on shoot regeneration, biomass accumulation and production of bacoside A from regenerated shoots of *Bacopa monnieri* leaf explants.

Materials and Methods

Preparation of explant material

Actively growing adventitious shoots of *Bacopa monnieri* (L.) had been cultured for 2 months on 0.8% agar and 2% sucrose (w/v) Murashige and Skoog (MS) medium supplemented with 2 mg l^{-1} Kin at pH 5.8 were maintained in the Plant Biotechnology Laboratory, Department of Botany, Karnatak University, Dharwad, India. From the *in vitro* adventitious shoots, leaves were aseptically separated, these leaves were used as the explants source for heavy metal (Mn, Zn, and Cu) supplement experiments.

Heavy metal treatment

Leaf sections (5 x 5 mm) were cultured (abaxial surface down) into culture bottles each containing 50 ml of MS agar (0.8%) medium supplemented with 2.0 mg l^{-1} Kin. Effect of heavy metals was examined by varying the concentrations of manganese ($MnSO_4.H_2O$, 0.1 mM normal content in MS medium served as a control, 0.2, 0.4, 0.6, 0.8 and 1.6 mM) zinc ($ZnSO_4.7\ H_2O$, 0.03 mM normal content in MS medium as control, 0.06, 0.12, 0.24, and 0.48 mM) and copper ($CuSO_4.5\ H_2O$, 0.0001 mM normal copper content in MS medium as control, 0.02, 0.05, 0.1 and 0.2 mM). All cultures were incubated in the growth chambers at 25±1°C, with a 16 h photoperiod (40 µmol m^{-2} s^{-1}) provided by 40-W fluorescent lamps

(Philips, Kolkata, India). After one month, the explants were subcultured with the same media concentration where they have come from. After two months of culture, the explants were examined, and the number of adventitious shoots per explant, fresh weight of the shoots cluster along with explant was recorded. Shoot clusters along with original explant were collected and oven dried at 60^{o}C for one day and dry weight was recorded.

Extraction and quantification of bacoside A

Extraction and High-performance liquid chromatography (HPLC) analysis of bacoside A were carried out by following the method of Murthy et al. [19] with some modifications. Thirty milligram of powdered plant material was extracted in 25 ml of 70% methanol by heat-refluxing for 45 minutes and filtered through 0.45 µm membrane filters. The bacoside contents were analysed using a Shimadzu HPLC system equipped with Phenomenex C18, 5 µm (4.6x250 mm) column, LC10AT VP lamps, SCL-10AVP system controller, SIL-10 AD VP auto-injector, SPD-M10 AVP photodiode array detector. The mobile phase was a mixture of acetonitrile and water (60:40, v/v) at a flow rate of 1 ml min^{-1} and column temperature was maintained at 30^{o}C. The detection wavelength was set at 205 nm. The injection volume was 20 µl. The chromatography system was equilibrated by the mobile phase. The standard bacoside A was purchased from Chromadex (Laguna Hill, CA, USA). The standard bacoside A chromatogram was used to quantify the concentrations of bacoside A in *Bacopa monnieri* extracts.

Statistical analysis

All the experiments were conducted with a minimum of 12 replicates, and the experiment was repeated thrice. The data were analysed by using SPSS ver. 17.0 (SPSS Inc. Chicago, USA) statistical software. The significance of differences among means was carried out using Duncan's multiple range test (DMRT) at $P \leq 0.05$. The results are expressed as the means ± SE of three experiments.

Results and Discussion

The effects of different heavy metals and varying concentrations play a major role in the biomass accumulation and secondary metabolite production [16, 18, 20]. After one month of culture on MS medium supplemented with 2 mg l^{-1} Kin adventitious shoot initiations were observed from leaf explants (Figure 1A). In the control media after two months 70.75 shoots/explant were observed (Table 1; Figure 1B). Table 1 shows how shoot growth and biomass of *Bacopa monnieri* was affected by the various concentrations of heavy metals and figure 2 depicts the accumulation of bacoside A content. In the present study, highest number of shoots (123.50 shoots/explant; Figure 1C), fresh weight (3.826 g) and dry weight (0.226 g) were obtained in the medium with 0.12 mM $ZnSO_4$. Optimum bacoside A content (28.090 $mg.g^{-1}$ DW) was observed in the medium with 0.20 mM $CuSO_4$. Heavy metals are an essential micronutrient of the medium which is required for normal growth, development and interferes with numerous physiological functions. It constitutes of protein component of several enzymes, catalysing redox reactions in mitochondrial chloroplast, cell wall and cytoplasm of plant cells [21]. Also, it plays a major role in protein synthesis, phytohormonal activity, enzyme activation, membrane integrity and detoxification of superoxide radicals [22].

Increasing concentration of Mn in culture media caused a significant decrease in shoots/explants when compared to control (Table 1). Highest 123.50 shoots/explants were observed at medium containing 0.12 mM Zn concentration, beyond 0.12 mM concentration shoots/explants were decreased. In medium containing 0.02 mM Cu concentration optimum of 122.00 shoots/explants was observed. The higher the concentration of Cu in the medium, shoots/explants were decreased and found 17.25 shoots/explants at 0.20 mM concentration (Figure 1D). Our results are by the earlier report obtained in *Lepidium sativum, Ailanthus altissimia, Withania somnifera* [8, 10, 20]. Increase in Cu concentration above an optimum level (0.10 mm) causes deleterious effects on shoot regeneration. At higher Cu level, it becomes toxic and induces the deficiency of their essential elements, inhibits the growth and alteration in plasma membrane permeability [23].

Increasing concentration of Mn in a media cause decrease in biomass (fresh weight and dry weight) when compared to control. There was a significant increase in biomass (fresh weight 3.826 g and dry weight 0.226 g) of the regenerated shoots in *Bacopa monnieri* at 0.12 mM Zn concentration in media and found lowest biomass (fresh weight 0.145 g and dry weight 0.012 g) at 0.20 mM Cu concentration in the media. The results were corroborated with the earlier reports [10, 20].

After two months of culture on MS media, the secondary metabolite bacoside A content of control shoots were found to be 12.515 mg g^{-1} DW. The significant increase in bacoside A content was observed in leaf explants exposed to different concentration of Mn, Zn and Cu in MS media. The maximum bacoside A content 28.090 mg g^{-1} DW of *Bacopa monnieri* regenerated shoots were observed at 0.20 mM Cu concentration cultured on MS medium (Figure 2). In the similar way, lepidine content was enhanced at higher concentration of Zn and Cu [10]. An increase in the proline content was correlated to enhanced levels of Zn and Cu supplemented medium. The present results corroborating with the earlier findings in *Bacopa monnieri* in which higher concentration of Cu enhances the proline content [18]. Bacoside A content in stressed regenerants was more than in those grown on control.

Conclusion

The cultures of *Bacopa monnieri* exhibit varying responses to various concentrations of $MnSO_4$, $ZnSO_4$ and $CuSO_4$. Incorporation of $ZnSO_4$ and $CuSO_4$ in the medium stimulates the growth and bacoside A content production. The altered levels of heavy metals proved that every plant species have a particular level of heavy metals requirements. Therefore, catering the need of a particular plant further requires the extensive evaluation of tissue culture medium to optimize the regeneration potential. The protocol could improve the understanding of growth and differentiation in plants at physiological and biochemical levels. The present investigation elucidated that the cultures of *Bacopa monnieri* can serve as a potential source of bacoside A content under suitable conditions. Zn and Cu proved to be more effective in enhancing the biomass and bacoside A content.

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Table 1. Effect of different concentrations of heavy metals on adventitious shoot regeneration and biomass accumulation from leaf explants of *Bacopa monnieri* cultured for two months on MS medium supplemented with 2 mg l^{-1} Kin and 2% sucrose at pH 5.8.

Metals	Concentration (mM)	Mean no. shoots/explants	Mean fresh Wt (g)	Mean dry Wt (g)
Control	Mn-0.10, Zn-0.03 and Cu-0.0001	70.75±1.10c	2.344±0.030d	0.166±0.002d
Mn	0.20	28.25±1.43f	0.916±0.022j	0.070±0.002i
	0.40	29.50±1.04f	1.072±0.058i	0.083±0.002h
	0.80	57.75±1.03e	2.222±0.046e	0.155±0.003e
	1.60	54.25±1.43e	2.084±0.029f	0.145±0.002e
Zn	0.06	76.00±1.95b	2.997±0.040c	0.184±0.002c
	0.12	123.50±3.30a	3.826±0.055a	0.226±0.003a
	0.24	63.75±1.31d	2.441±0.075d	0.154±0.002e
	0.48	56.25±1.75e	1.400±0.024h	0.106±0.006g
Cu	0.02	122.00±1.58a	3.170±0.028b	0.198±0.001b
	0.05	64.75±1.49d	1.157±0.027i	0.089±0.001h
	0.10	75.50±1.65b	1.729±0.035g	0.118±0.006f
	0.20	17.25±1.10c	0.145±0.005k	0.012±0.001j

Data were collected after two months of culture. Mean values in column followed by the different letters are significantly different according to the Duncan's multiple range ($p \leq 0.05$) test.

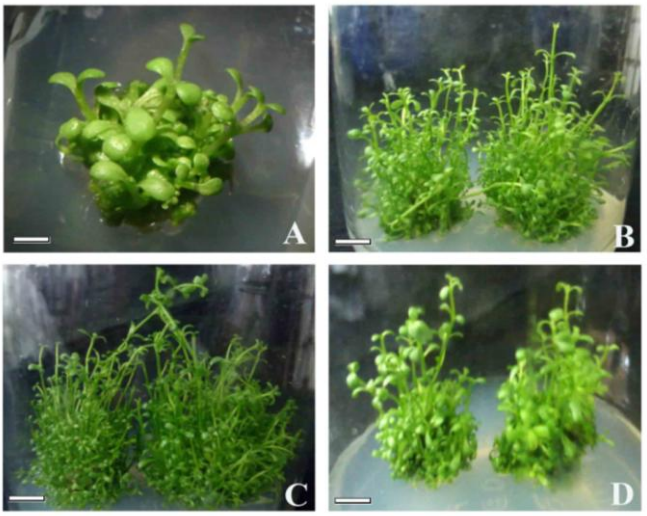

Fig 1. A. Shoot initiation from leaf explants of *Bacopa monnieri* on MS medium supplemented with 2 mg l^{-1} Kin and 2% sucrose at pH 5.8 after one month culture (Bar = 1.8 mm). B. Adventitious shoot regeneration after two months of culture (Bar = 9.0 mm). C. Adventitious shoot regeneration from leaf explants of *Bacopa monnieri* on MS medium along with Zn (0.12 mM), after two months of culture (Bar = 8.9 mm). D. Adventitious shoot regeneration from leaf explants of *Bacopa monnieri* on MS medium along with Cu (0.20 mM), after two months of culture (Bar = 7.3 mm).

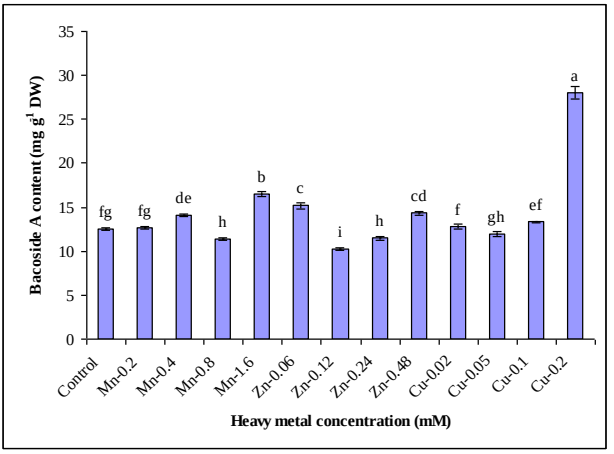

Fig 2. Effect of different concentrations of heavy metals on bacoside A accumulation from leaf explants of Bacopa monnieri cultured for two months on MS medium supplemented with 2 mg l-1 Kin and 2% sucrose at pH 5.8. Bars represent the standard error; mean values following the same letter are not significantly different, according to Duncan's multiple range ($p \leq 0.05$) test.