Ceylon Journal of Science 46(4) 2017: 97-104
DOI: http://doi.org/10.4038/cjs.v46i4.7472

RESEARCH ARTICLE

Influence of different media, medium strength and carbon sources on adventitious shoot cultures and production of bacoside A in *Bacopa monnieri* (L.)

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Received:23/07/2017; Accepted:18/09/2017

Abstract: *Bacopa monnieri* (L.) Wettst. (Plantaginaceae), commonly known as Brahmi, is used in Ayurveda as a medicinal herb, found throughout the Indian subcontinent in wet, damp and marshy areas. The herb yields pharmaceutically active compounds called bacosides. The current investigation was carried out to assess the potential for increasing biomass and the concentration of bacoside A from the *in vitro* regenerated shoots in different media (MS, B5, NN and N6), the strength of the MS medium (0.25, 0.50, 0.75, 1.0, 1.50 and 2.0X) and carbon source [sucrose, glucose, fructose, maltose, sucrose + glucose (1:1), sucrose + maltose (1:1), glucose + fructose (1:1), glucose + maltose (1:1), fructose + sucrose (1:1) and fructose + maltose (1:1)]. All the culture media were supplemented with 2 mg/L Kinetin (Kin) at pH 5.8. High performance liquid chromatography (HPLC) was used to determine the bacoside A content from the regenerated shoots. Optimum number of adventitious shoots (70.75 shoots per explant), fresh weight (2.344 g), dry weight (0.166 g) and bacoside A content (13.052 mg/g DW) were obtained from the cultures grown in the MS medium. Bacoside A production was highest in the shoots grown in the glucose + fructose combination (15.588 mg/g DW) among the different carbon sources tested. These findings suggest that *B. monnieri* culture requires full strength MS medium and a moderate level of sucrose. The present study is useful to obtain optimum production of biomass and bacoside A from the *in vitro* regenerated shoots of *B. monnieri.*

Keywords: Adventitious shoots; *Bacopa monnieri*; Bacoside A; Biomass; Carbon source.

INTRODUCTION

Bacopa monnieri (L.) Wettst. (Plantaginaceae), commonly known as Brahmi, is a medicinal herb, found throughout the Indian subcontinent in wet, damp and marshy areas. It is an important Ayurvedic medicine for the improvement of intelligence, memory and the revitalization of sensory organs (Sivarajan and Balachandran, 1994; Pravina *et al*., 2007). The Brahmi extract is known to possess anticancer and antioxidant properties (Elangovan *et al*., 1995; Tripathi *et al*., 1996). The nootropic activity of the extract has been attributed to the presence of two saponins, namely bacoside A and bacoside B, of which the former is more important (Singh *et al*., 1988; Dhawan and Singh, 1996; Singh *et al*., 1997). In addition to memory boosting activity, it is also claimed to be useful in the treatment of cardiac, respiratory and of neuropharmocological disorders like insomnia, insanity, depression, psychosis, epilepsy and stress (Russo and Borrelli, 2005). It was reported to possess anti-inflammatory, analgesic, antipyretic, sedative, free radical scavenging and also anti-lipidperoxidative activities (Russo and Borrelli, 2005). Increasing the bacoside A content in *B. monnieri* would have a number of benefits in pharmaceutical and industrial applications.

Plant cell and organ cultures are promising technologies to obtain plant-specific valuable metabolites (Verpoorte *et al*., 2002). Cell and organ cultures have a higher rate of metabolism than field grown plants because the initiation of cell and organ growth in culture leads to the fast proliferation of cells/organs and to a condensed biosynthetic cycle (Rao and Ravishankar, 2002). Further, plant cell/organ cultures are not limited by environmental, ecological and climatic

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conditions, and cells/organs can thus proliferate at higher growth rates than whole plant in cultivation. Several biotechnological advances have been developed in tissue culture that improve secondary metabolite production such as optimization of cultural conditions, selection of high-producing strains of lines, precursor feeding, elicitation, metabolic engineering, transformed root cultures, micropropagation, and bioreactor cultures, among others (Sarin, 2005; Murthy *et al*., 2014). Although carbohydrates are of prime importance for *in vitro* organogenesis, carbon metabolism *in vitro* is still not clearly understood (Kozai, 1991). It is well established that carbohydrate requirements depend upon the stage of culture and may show differences according to the species (Thompson and Thorpe, 1987). *In vitro* shoot regeneration methods have been established in Brahmi (Praveen *et al*., 2009; Naik *et al*., 2010, 2011, 2014). However effects of different media, medium strength and carbon sources on biomass accumulation and production of secondary metabolite in regenerated shoots have not been worked out. The present work reports the effects of different media, medium strength and carbon source on shoot regeneration, biomass accumulation from leaf explants of *B. monnieri* and production of bacoside A in regenerated shoots.

MATERIALS AND METHODS

Plant material and establishment of culture

Actively growing adventitious shoots of *B. monnieri* (L.) had been cultured for 2 months on Murashige and Skoog (MS, 1962) medium supplemented with 0.8% agar, 2% sucrose (w/v) and 2 mg/L Kin at pH 5.8 in the plant tissue culture laboratory, Department of Botany, Karnatak University, Dharwad, India (Praveen *et al*., 2009). From the *in vitro* adventitious shoots, leaf sections (5 × 5 mm) were cultured (abaxial surface down) into magenta boxes (Himedia, India) each containing 50 mL of Murashige and Skoog (MS) agar (0.8%) medium supplemented with 2 mg/L Kin. Different media such as MS, Gamborg's (B5) 1968, Nitsch and Nitsch (NN) 1969 and Chu's (N6) 1978, as well as various strengths of MS medium (0.25, 0.50, 0.75, 1.0, 1.50 and 2.0X) were tested depending on the objective of the experiment. In another set of experiments the effect of different carbon sources like sucrose, glucose, fructose, maltose, sucrose + glucose (1:1), sucrose + maltose (1:1), glucose + fructose (1:1), glucose + maltose (1:1), fructose + sucrose (1:1) and fructose + maltose (1:1) were studied for the shoot regeneration, biomass accumulation and bacoside A production. 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 to the same media concentration from where they have come from. After two months of culture, the explants were examined and number of adventitious shoots per explant, fresh weight of shoot clusters along with explant was recorded. Shoot clusters along with original explant were collected and oven dried at 60°C for one day and dry weight was recorded.

Extraction and HPLC analysis

Extraction and HPLC analysis of bacoside A were carried out by following the method of Murthy *et al*. (2006) 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 fractions were analysed using Shimadzu HPLC system equipped with Phenomenex C18, 5 µm (4.6 x 250 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 and column temperature was maintained at 30°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 *B. monnieri* extracts.

Statistical analysis

All the experiments were conducted with a minimum of 12 replicates and the experiment was repeated three times. The data were subjected to analysis of variance (ANOVA), and comparisons between the mean values of treatments were made by the least significant difference (Duncan post hoc) test ($P \leq 0.05$). Statistical analyses were performed using the

SPSS statistical package (SPSS ver. 17, SPSS Inc. Chicago, USA).

RESULTS

Influence of different media

In the present study, highest number of shoots (70.75 shoots per explant), fresh weight (2.344 g), dry weight (0.166 g) and bacoside A content (13.052 mg/g DW) were obtained in the MS medium, whereas B5, NN and N6 medium showed lesser quantities of the above parameters (Table 1) (Figure 1). Optimum number of shoots (38.75, 45.25 and 44.50 shoots per explant), fresh weight (1.423, 1.214 and 1.172 g), dry weight (0.096, 0.098 and 0.083 g) and bacoside A content (12.205, 9.346 and 12.162 mg/g DW) were obtained in B5, NN and N6 medium respectively.

Influence of MS medium strength

We have tested the effect of various MS medium strength on adventitious shoot regeneration, biomass and bacoside A accumulation from leaf explants of *B. monnieri*. Our study showed that highest number of shoots (71.00 shoots per explant), fresh weight (2.390 g), dry weight (0.164 g) and bacoside A content (13.092 mg/g DW) were obtained in full-strength MS medium (Table 2) (Figure 2). Interestingly, explants grown in 1.5X strength MS medium though produced lowest number of shoots (8.50 shoots per explant), fresh weight (0.755 g) and dry weight (0.055 g) it also accumulated higher bacoside A content (12.97 mg/g DW). Double strength (2.0X) MS medium also showed higher concentrations of bacoside A (12.582 mg/g DW) without producing much of the shoot (17.25 shoots per explant). Remaining media strength i.e. 0.25, 0.5 and 0.75X produced 25.75, 58.50 and 52.25 shoots per explant respectively and accumulated bacoside A at the intermediate levels (7.403, 9.781 and 11.448 mg/g DW respectively).

Table 1: The effect of different media on adventitious shoot regeneration and biomass accumulation from leaf explants of Bacopa monnierix xData were collected over two months of culture. Values represent the mean ± SE. Mean values following the same letter within columns are not significantly different, according to Duncan's multiple range ($p \leq 0.05$) test.

Media	Mean no. shoot per explants	Mean fresh weight (g)	Mean dry weight (g)
MS	70.75 ± 1.376a	2.344 ± 0.038a	0.166 ± 0.001a
B5	38.75 ± 0.629c	1.423 ± 0.005b	0.096 ± 0.003b
NN	45.25 ± 0.629b	1.214 ± 0.015c	0.098 ± 0.001b
N6	44.50 ± 1.892b	1.172 ± 0.002c	0.083 ± 0.001c

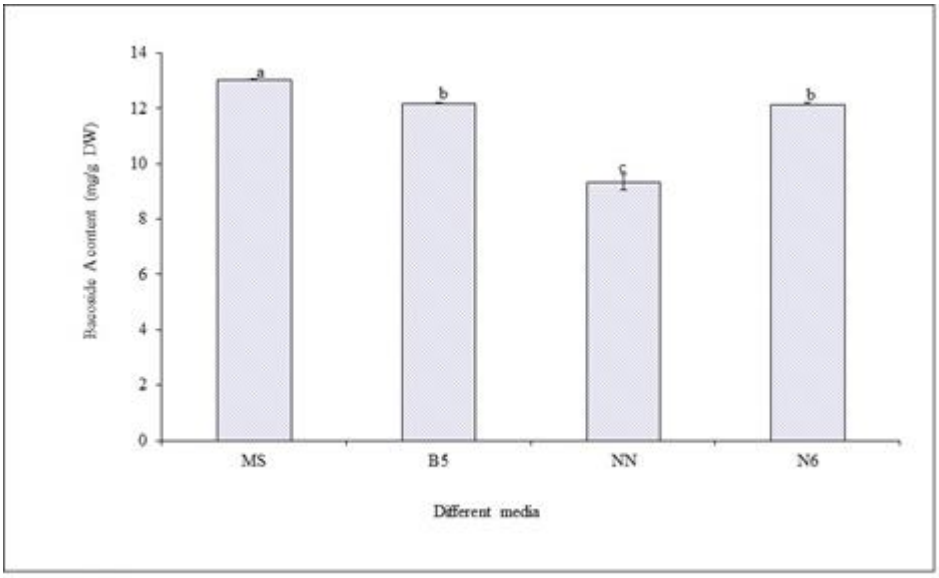

Figure 1: Effect of different media on bacoside A accumulation from leaf explants of *B. monnieri* cultured for two months, supplemented with 2 mg/L Kin and 2% sucrose at pH 5.8. Data were collected over two months of culture. Bars represent the standard errors, mean values following the same letter are not significantly different, according to Duncan's multiple range ($p \leq 0.05$) test .

Table 2: The effect of various medium strength on adventitious shoot regeneration and biomass accumulation from leaf explants of *Bacopa monnieri* [x]

[x]Data were collected over two months of culture. Values represent the mean ± SE. Mean values following the same letter within columns are not significantly different, according to Duncan's multiple range ($p \leq 0.05$) test.

Media Strength (X)	Mean no. shoot per explants	Mean fresh weight (g)	Mean dry weight (g)
0.25	25.75 ± 0.381d	1.516 ± 0.021d	0.116 ± 0.003c
0.5	58.50 ± 0.288b	2.226 ± 0.070b	0.160 ± 0.001ab
0.75	52.25 ± 0.629c	2.088 ± 0.049c	0.156 ± 0.001b
1.0	71.00 ± 1.527a	2.390 ± 0.034a	0.164 ± 0.002a
1.5	8.50 ± 0.288f	0.755 ± 0.013f	0.055 ± 0.003e
2.0	17.25 ± 0.946e	1.250 ± 0.004e	0.105 ± 0.002d

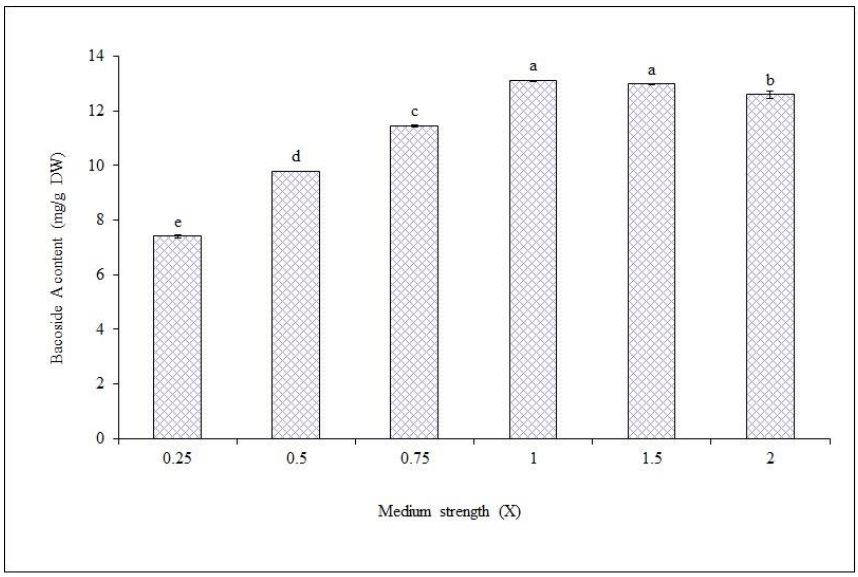

Figure 2: Effect of various medium strength on bacoside A accumulation from leaf explants of *B. monnieri* cultured for two months on MS medium supplemented with 2 mg/L Kin and 2% sucrose at pH 5.8. Data were collected over two months of culture. Bars represent the standard errors, mean values following the same letter are not significantly different, according to Duncan's multiple range ($p \leq 0.05$) test.

Influence of different carbon sources

In the present study, the effect of different carbon sources like sucrose, glucose, fructose, maltose, sucrose + glucose (1:1), sucrose + maltose (1:1), glucose + fructose (1:1), glucose + maltose (1:1), fructose + sucrose (1:1) and fructose + maltose (1:1) were studied for the adventitious shoot regeneration, biomass accumulation and bacoside A production. Sucrose induced an optimum number of adventitious shoots (70.75) from the explants followed by sucrose + maltose, fructose + maltose and glucose + fructose (64.00, 63.50 and 61.75 shoots per explant respectively) remaining carbohydrates treated leaf explants produced a lesser number of shoots (Table 3). Sucrose also induced maximum biomass (fresh weight and dry weight). Bacoside A production was highest in the glucose + fructose combination (15.588 mg/g DW; Figure 3) followed by sucrose + maltose (14.468 mg/g DW) and fructose alone supplemented medium (14.186 mg/g DW). In glucose + maltose combination, both adventitious shoot production and bacoside A accumulation was the lowest among all the tested combinations (19.00 shoots per explant; 10.940 mg/g DW).

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Table 3: The effect of different carbon sources on adventitious shoot regeneration and biomass accumulation from leaf explants of *Bacopa monnieri* [x]

[x]Data were collected over two months of culture. Values represent the mean ± SE. Mean values following the same letter within columns are not significantly different, according to Duncan's multiple range ($p \leq 0.05$) test.

Carbon sources (2%)	Mean no. shoot per explants	Mean fresh weight (g)	Mean dry weight (g)
Sucrose	70.75 ± 1.376a	2.272 ± 0.008a	0.165 ± 0.002a
Glucose	39.25 ± 1.842e	1.178 ± 0.010e	0.091 ± 0.001d
Fructose	52.75 ± 1.181d	1.170 ± 0.003e	0.114 ± 0.001c
Maltose	28.25 ± 1.181f	0.510 ± 0.008g	0.085 ± 0.003d
Sucrose + Glucose	36.75 ± 1.520e	1.342 ± 0.013d	0.093 ± 0.002d
Sucrose + Maltose	64.00 ± 1.527b	1.860 ± 0.010c	0.113 ± 0.003c
Glucose + Fructose	61.75 ± 1.145bc	2.048 ± 0.028b	0.125 ± 0.003b
Glucose + Maltose	19.00 ± 0.577g	0.894 ± 0.012f	0.066 ± 0.003e
Fructose+ Sucrose	58.25 ± 1.842c	1.185 ± 0.009e	0.091 ± 0.002d
Fructose+ Maltose	63.50 ± 1.322b	2.031 ± 0.024b	0.131 ± 0.003b

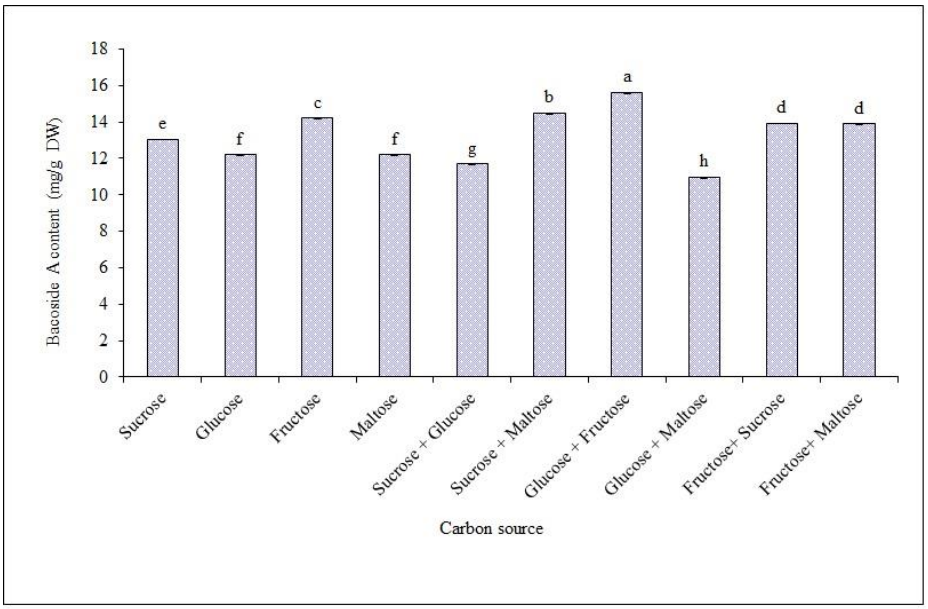

Figure 3: Effect of different carbon sources on bacoside A accumulation from leaf explants of *B. monnieri* cultured for two months on MS medium supplemented with 2 mg/L Kin at pH 5.8. Data were collected over two months of culture. Bars represent the standard errors, mean values following the same letter are not significantly different, according to Duncan's multiple range ($p \leq 0.05$) test.

DISCUSSION

The degree of growth, differentiation and metabolite accumulation varies considerably with the medium constitution (Bhaskaran and Jayabalan, 2005). Our results are corroborated with the observations made by Bhaskaran and Jayabalan, (2005) in *Eclipta alba* where the MS medium was superior over B5 and SH media for a number of adventitious shoot production. MS medium was shown to be more effective than other media by various investigators (Tefera and Wannakrairoj, 2004; Gao *et al*., 1999; Komalavalli and Rao, 2000; Ndoye *et al*., 2003). As nitrogen is to be a constituent of plant cell components, its deficiency inhibits plant growth. Total nitrogen content and nitrate:ammonium is very critical in nitrogen nutrition (Ramage and Williams, 2002). The nitrate:ammonium ratio strongly influences the pH of the medium, which in turn determines the absorption of other nutrients (Tefera and Wannakrairoj, 2004). Since the MS medium possesses relatively higher

nitrate:nitrogen, it could have exerted the profound effect on shoot growth of this plant species.

The optimum nutrient concentration is a critical determinant in controlling the growth of the cells/organs and the accumulation of secondary metabolites (Murthy et al., 2008). In contrast to our results, Wu *et al*., (2006) reported that half-strength MS medium was effective to enhance biomass accumulation and phenolic synthesis in adventitious roots culture of *Echinacea angustifolia* among varied strength MS (0.25–2.0X) media tested.

Different types of sugars (sucrose, fructose, maltose, sorbitol or glucose) used in the medium may affect biomass and secondary metabolite accumulation, and it is essential to work out suitable sugar combinations in the culture medium to obtain higher levels of secondary metabolites (Rao and Ravishankar, 2002). Contrary to our results, in *Stevia rebaudiana,* fructose performed well followed by sucrose, maltose and glucose in keeping shoot number constant (Preethi *et al*., 2011). Similar to our findings in *Gymnema sylvestrae,* sucrose was proved to be the best among various carbohydrate sources tested for inducing maximum number of shoots and gymnemic acid production (Praveen *et al.,* 2011). Sucrose proved to be better for shoot proliferation than other carbon sources in micropropagation of several plant species such as *Pogostemon cablin* (Kumara et al., 2010) and *Centella asiatica* (Anwar *et al*., 2005).

Uptake and utilization of different sugars might vary with different plant cells as reported by Srinivasan *et al*. (1995) and might be due to enzyme systems present in them for preferential utilization. In *B. monnieri* shoot cultures 2% sucrose favoured biomass accumulation, whereas sucrose-free medium accumulated maximum amount of bacoside A content (Naik *et al*., 2010).

CONCLUSIONS

The present study of adventitious shoot cultures of *B. Monnieri* has demonstrated the influence of different media, media strength and type of carbohydrate used in biomass and secondary metabolite accumulation. The highest number of adventitious shoots, fresh weight, dry weight and the production of bacoside A content were recorded in the MS medium among the different media and media strengths tested. Of the various carbon sources used, the glucose + fructose combination influenced maximally for the bacoside A production at optimum levels. These findings suggest that *B. monnieri* culture requires full strength MS medium and a moderate level of sucrose. Our results are promising and can be extrapolated for the large scale production of bacoside A via tissue culture.

ACKNOWLEDGMENTS

This work was partially financed by Council of Scientific and Industrial Research [No. 38(1098)/04/EMR-II] and University Grants Commission under the Special Assistance Programme, New Delhi, India.

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