Int J Pharm Bio Sci 2012 July; 3(3): (B) 848 - 855

Research Article **Biotechnology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

EFFECT OF MUTAGENS ON THE *IN VITRO* ADVENTITIOUS SHOOT GROWTH AND BACOSIDE A ACCUMULATION IN *BACOPA MONNIERI* (L.)

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ABSTRACT

Mutations were induced in tissue cultured raised *Bacopa monnieri* (L.) plantlets by treating *in vitro* derived leaves with gamma rays at (γ – rays) dose rate of 0, 10, 20, 40 and 80 gray (Gy) and 0.5% of ethyl methanesulphonate (EMS) at 0, 0.5, 1.0, 1.5, 2.0 and 2.5 hours (h). Xantha mutants were observed with γ - rays at 10, 20 and 40 Gy. All the leaf explants treated with 80 Gy were either necrotic or irregenerable. Both γ and EMS treatment exhibited a reduction in survival rate, number of adventitious shoots, fresh weight and dry weight (DW). The γ - rays doses at 10, 20 and 40 Gy induced mutation in *Bacopa monnieri* leaf explants and five lines were obtained with increased in the bacoside A content. The lines obtained are D15 (40 Gy) which produced maximum amount of bacoside A (22.567 mg g^{-1} DW) followed by B11 (10 Gy), C18 (20 Gy), D17 (40 Gy) and B6 (10 Gy) lines which accumulated 21.051, 19.320, 17.163 and 14.133 mg g^{-1} DW respectively compared with control (13.750 mg g^{-1} DW). The leaf explants treated with EMS did not show any significant increase with respect to the bacoside A content.

KEYWORDS : ***Bacopa monnieri,*** **Bacoside A, Ethyl methanesulphonate, γ – rays, and High performance liquid chromatography.**

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INTRODUCTION

Bacopa monnieri (L.) Pennell. (Scrophulariaceae) 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 revitalisation of sensory organs[1]. The brahmi extract is known to possess anticancer and antioxidant properties[2, 3]. 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[4, 5]. 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[1]. It was reported to possess anti inflammatory, analgesic, antipyretic, sedative, free radical scavenging and also anti-lipidperoxidative activities. Increasing the amount of bacoside A content in *Bacopa monnieri* would have a number of benefits in pharmaceutical and industrial applications.

Induction of mutations based on the use of ionizing radiations and chemical mutagens is one of the major breeding approaches for plant improvement. Combination of such techniques with a variety of *in vitro* culture methods can speed up breeding programmes, from generation of variability, through selection, to multiplication of the new genotypes[6]. The quality of secondary metabolites in plants can be improved by the use of ionizing radiation, such as X-rays, γ - rays, alpha and beta particles, proton and neutrons[7, 8]. In *Wasabia japonica* the concentration of allyl isothiocyanate accumulation was increased using the ionizing radiation[9] and in *Digitalis obscura* the production of cardenolide was increased using the γ – rays[10].

Similarly ethyl methanesulphonate (EMS) is a chemical mutagen of the alkylating group and has been commonly used in plant breeding because it can cause high frequency of gene mutations and low frequency of chromosome aberrations[11]. This mutagen has been used to treat seeds and recently to treat *in vitro* explants of many species[12]. *In vitro* shoot regeneration methods have been established in *Bacopa monnieri*[13, 14]. The objectives of the present studies were to induce mutations in the shoot cultures of *Bacopa monnieri* by treating with γ – rays and EMS and to examine their *in vitro* shoot growth and bacoside A content.

MATERIALS AND METHODS

(i) Induction of adventitious shoots

The shoot cultures were initiated by culturing *in vitro* grown adventitious shoots of *Bacopa monnieri* in MS[15] medium supplemented with 2% sucrose and 2 mg l^{-1} Kinetin (KIN) gelled with 0.8% agar[14]. From these *in vitro* grown adventitious shoots, leaves were aseptically separated, and these leaves were used as the explant source for γ – rays and EMS treatments.

(ii) Effect of mutagens on adventitious shoot induction and bacoside A content

The individual leaves were exposed to γ – radiation at Bhabha Atomic Research Centre, Mumbai, India. The γ – radiation was derived from a Cobalt-60 (^{60}Co) source with a measured dose rate of 4.07 Gy min^{-1}. The leaves were treated with γ – radiation at 10, 20, 40 and 80 Gy. The alkylating substance EMS (Himedia, India) was used as a chemical mutagen. 0.5% EMS solution in phosphate buffer (0.1 M, pH 7) was prepared just before use. The solutions were filter-sterilized with sterile millipore 0.45 µm membrane filter in the laminar air flow chamber. The leaves were treated with 0.5% EMS for 0.5, 1.0, 1.5, 2.0 and 2.5 hours (h). After the EMS treatment the leaves were washed 4 to 5 times with sterile distilled water to remove the mutagen. A

single control was maintained for both γ and EMS treatments. The treated explants were transferred on to fresh MS medium supplemented with 2% sucrose (w/v) and 2 mg l^{-1} KIN with 0.8% agar at pH 5.8. All cultures were incubated in the growth chambers at 25 ± 1°C, with a 16 h photoperiod (40 μ mol m^{-2} s^{-1}) provided by 40-W fluorescent lamps (Philips, Kolkata, India). After 2 months period, the explants were assessed in terms of shoot number, fresh weight, dry weight (DW) and bacoside A content was determined.

Data in each treatment were derived from the means of 20 replicates and all the 20 replicates were used for bacoside A content analysis. Results were analyzed by SPSS 17.0 for MS Windows (SPSS Inc. Chicago, USA), using analysis of variance (ANOVA). The mean values were calculated and compared according to Duncan's multiple range tests at $P \leq 0.05$ levels.

(iii) Extraction and HPLC analysis

Extraction and HPLC analysis of bacoside A were carried out by following the method of Murthy et al.[16]. Thirty milligram of powdered plant material were extracted in 25 ml of 70% methanol by heat refluxing and filtered through 0.45 μm membrane filters. The bacoside fractions were analyzed using 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 0.05 M sodium sulphate buffer pH 2.3 and acetonitrile (68.5: 31.5, v/v) at flow rate of 1 ml min^{-1} 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 *Bacopa monnieri* extracts.

RESULTS AND DISCUSSION

In the present study it was found that the treatment of *Bacopa monnieri* leaves with mutagens (γ – rays and 0.5% EMS) affected the survival rate, adventitious shoot growth and bacoside A content after 2 months of *in vitro* culture.

(i) Effect of γ- radiation on adventitious shoot induction

The γ – rays with doses at 0, 10, 20, 40, and 80 Gy had effects on the survival of explants after 2 months of *in vitro* culture. It was found as a general trend in the treatment with γ – rays, that as radiation dosage increased, the frequency of regenerable explants decreased and the frequency of mortality and irregenerable explants increased (Table 1). Necrosis of explants commenced at 20 and 40 Gy of γ – rays with mortality rate of 4% and 8% respectively. Under the γ – rays treatments, the explants were green in colour but unable to produce shoots, which was 4% at 10 Gy, 8% at 20 Gy and increased 12% with the 40 Gy dose. The number of regenerable explants ranged from 96% to 0% as the γ – rays doses increased (Table 1). Similar results were found in *Wasabia japonica* when treated with ionizing radiation[9]. Gavidia and Perez-Bermudez[10] reported, γ – radiation treatment on *Digitalis obscura* shoot tips decreased the survival rate as the doses increased. Similarly, treatments of explants *in vitro* with γ – rays have been shown to reduce the multiplication rate of *Alpinia purpurata* irradiated at 15 Gy dose onwards[17], to decrease the production of microtubers of *Solanum tuberosum* at a dose from 10 Gy[18] and to decline to 50% in the survival rate of *Nelumbo nucifera* when irradiated at 2 Krad[19]. Xantha mutants were observed with irradiation at 10, 20 and 40 Gy (Fig. 1a, 1b, and 1c respectively). Chlorophyll-deficient phenotypes were observed in γ – rays treated *in vitro* culture of *Tillandsia fasciculata* Swartz var. fasciculata[20]. Deformity in leaves occurred with irradiation at 40 Gy, but not with

other doses (Fig. 1d). The irradiation dose of 80 Gy (γ – rays) caused the highest mortality rates at 84% and 16% of irregenerable explants therefore this dose was not selected to assess the growth and quality of the explants.

Table 1

Effect of γ – radiation and EMS treatment on the survival of Bacopa monnieri leaf explants after two months of culture on MS medium supplemented with 2 mg l^{-1} KIN at pH 5.8.

Mutagen	Mortality rate (%)	Survival rate (%)	
		Irregenerable explants	Regenerable explants
Control	0	0	100
γ radiation dose (Gy)			
10	0	4	96
20	4	8	88
40	8	12	80
80	84	16	0
EMS (0.5%) treatment in h			
0.5	28	8	64
1.0	8	0	92
1.5	20	0	80
2.0	0	0	100
2.5	36	0	64

(ii) Effect of EMS on adventitious shoot induction

EMS (0.5%) treatment for 0, 1.0, 1.5, 2.0 and 2.5 h had effects on the survival of explants after 2 months of *in vitro* culture (Table 1). EMS (0.5%) treatment at 0, 1.0, 1.5, 2.0 and 2.5 h showed mortality rate of 0%, 28%, 8%, 20%, 0% and 36% respectively. In EMS, lower and higher treatment timing showed highest mortality rate. The occurrence of 8% irregenerable explants were observed at 0.5 h EMS (0.5%) treatment and found 0% irregenerable explants in other EMS (0.5%) treatments. The number of regenerable explants ranged from 100% to 64% (Table 1). EMS (0.5%) treatment at 2.0 h showed 100% regenerable explants and 0.5 h and 2.5 h showed 64% regenerable explants. The γ – radiation appeared to have a greater influence on the *in vitro* growth of *Bacopa monnieri* leaf explant than EMS treatment (Table 1). Unlike γ – rays, in EMS treatment there was no xantha mutants and deformity of leaves were observed.

Number of adventitious shoots was affected by the γ – rays and EMS (0.5%) treatment (Table 2). The γ – rays at 40 Gy doses and EMS (0.5%) at 0.5 h treatment showed significantly reduced number of shoots (21.00 and 35.55 respectively), when compared with control which produced 69.50 number of shoots/explants. In γ – radiation treatment number of shoots decreased as doses increased. Hung and Johnson[9] reported in *Wasabia japonica* as the γ – rays doses increased the number of shoots decreased. In *Digitalis obscura* the number of shoots decreased as the doses of γ – radiation increased[10]. In EMS (0.5%) treatment the number of shoots increased with the increase in the time and reached optimum of 53.25 numbers of shoot at 1.5 h and thereafter there was decrease in the shoot number with increase in the time (Table 2).

Table 2

Effect of γ – radiation and EMS treatment on the in vitro growth and bacoside A content in treated leaves of Bacopa monnieri after two months of culture on MS medium supplemented with 2 mg l^{-1} KIN at pH 5.8.*

Mutagen	Mean number of shoots/explants	Mean fresh weight in g	Mean dry weight in g	Mean bacoside A content mg g^{-1} DW
Control	69.50 ± 3.265a	2.632 ± 0.188a	0.159 ± 0.010a	13.750 ± 0.180a
γ radiation dose (Gy)				
10	42.90 ± 3.275b	2.365 ± 0.228ab	0.149 ± 0.011a	12.718 ± 0.502ab
20	33.35 ± 3.892b	1.965 ± 0.190b	0.135 ± 0.010a	11.900 ± 0.444b
40	21.00 ± 3.945c	0.908 ± 0.116c	0.084 ± 0.007b	11.729 ± 0.686b
EMS (0.5%) treatment in h				
0.5	35.55 ± 1.553c	1.930 ± 0.115b	0.132 ± 0.005b	11.134 ± 0.137b
1.0	46.20 ± 2.344b	2.301 ± 0.084ab	0.151 ± 0.004ab	11.122 ± 0.075b
1.5	53.25 ± 3.066b	2.362 ± 0.113a	0.160 ± 0.005a	10.992 ± 0.164b
2.0	48.85 ± 3.705b	2.305 ± 0.120ab	0.158 ± 0.005a	10.771 ± 0.194b
2.5	44.55 ± 3.774b	2.270 ± 0.109ab	0.150 ± 0.006ab	11.121 ± 0.207b

Data were collected after two months of culture. Values represent the mean ± S.E. Each experiment consisted of twenty replicates. Mean values following the same letter within columns are not significantly different, according to Duncan's multiple range (P ≤ 0.05) test.

(iii) Effect of mutagens on the biomass production

Fresh and dry weight of two months *in vitro* cultured *Bacopa monnieri* leaf explants were significantly reduced as γ – radiation doses increased (Table 2). Fresh weight of *Wasabia japonica* was decreased as γ – radiation doses increased[9]. Both fresh and dry weight were increased in EMS (0.5%) as treatment time increased and reached highest (2.362 g fresh weight and 0.160 g dry weight) at 1.5 h, thereafter there was decrease in the fresh and dry weight with increase in the time (Table 2). EMS (0.5%) treatment at 1.5 h showed optimum dry weight (0.160 g), which was similar to that of the control (0.159 g).

(iv) Effect of mutagens on the bacoside A production

HPLC analyses were performed for individual treatments (20 replicates) of γ – radiation for bacoside A content and data were represented in Table 3. There were 5 lines which produced maximum bacoside A content than the control. The D15 (40 Gy) line produced maximum amount of bacoside A (22.567 mg g^{-1} DW) followed by B11 (10 Gy) line which yielded 21.051 mg g^{-1} DW, C18 (20 Gy), D17 (40 Gy)

and B6 (10 Gy) lines produced 19.320, 17.163 and 14.133 mg g^{-1} DW respectively when compared to the control that produced 13.750 mg g^{-1} DW of bacoside A content (Table 3). There were no significant lines producing high content of bacoside A in the individual treatments (20 replicates) of EMS (Data not shown). The effects of γ – radiation and EMS (0.5%) treatment on bacoside A content were evident following 2 months *in vitro* culture, with all treatments producing explants with significantly lower levels of bacoside A content than the control when the data were pooled (Table 2). In γ – radiation treatment, there was decrease in the accumulation of bacoside A content as the doses increased when compared to control. Control showed highest accumulation of bacoside A (13.750 mg g^{-1} DW) followed by 10, 20 and 40 Gy (12.718, 11.900 and 11.729 mg g^{-1} DW respectively). *In vitro* culture of *Wasabia japonica* showed decreased allyl isothiocyanate (AITC) content as the γ – radiation doses increased[9]. In EMS (0.5%) treatment, the bacoside A content decreased with increase in the treatment duration except at 2.5 h (Table 2).

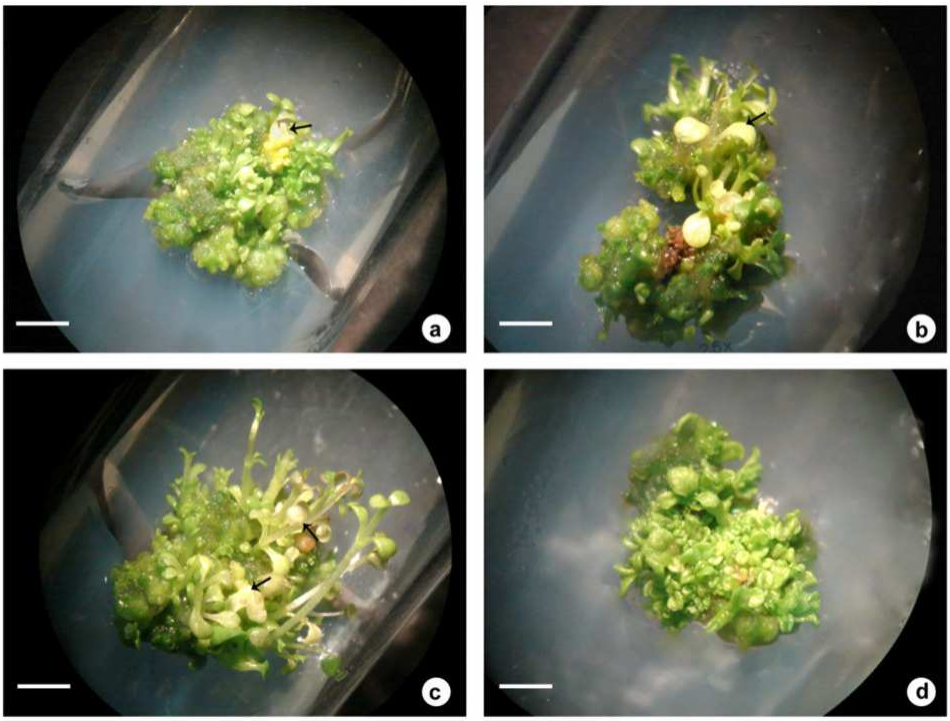
Table 3.

The γ – radiation which produced five high yielding bacoside A content lines of Bacopa monnieri after two months of culture on MS medium supplemented with 2 mg l^{-1} KIN at pH 5.8.

γ radiation dose (Gy)	Number of shoots/explants	Fresh weight in g	Dry weight in g	Bacoside A content mg g^{-1} DW
Control	69.50	2.632	0.159	13.750
10 Gy (B)				
B6	28	0.901	0.060	14.133
B11	11	0.347	0.031	21.051
20 Gy (C)				
C18	10	0.362	0.038	19.320
40 Gy (D)				
D15	11	0.298	0.033	22.567
D17	5	0.472	0.061	17.163

Figure. 1

Effect of γ – radiation on shoot regeneration from leaf explant of Bacopa monnieri on MS medium supplemented with 2 mg l^{-1} KIN (a) Xantha mutant at 10 Gy (Bar = 3.6 mm). (b) Xantha mutant at 20 Gy (Bar = 2.6 mm). (c) Xantha mutant at 40 Gy (Bar = 3.6 mm). (d) Deformity of leaves at 40 Gy (Bar = 2.6 mm).



CONCLUSION

From the present study it can be concluded that both the mutagen treatments suppressed the survival rate of explants, number of adventitious shoots formed, fresh and dry weight of two months *in vitro* cultured leaf explants. The γ – radiation treatment at 10, 20 and 40 Gy induced five high yielding lines of bacoside A content, where as EMS (0.5%) treated leaf explants did not show any significant results. Xantha mutants were observed in the γ – radiation treatments, but no such mutants were found in the EMS (0.5%) treatments. Further it can be concluded that γ – radiation was beneficial when compared with the EMS treatment with respect to the bacoside A content.

ACKNOWLEDGEMENTS

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

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