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Induction of Variations in Two Cultivars of *Bacopa monnieri* by Gamma Irradiation of *In Vitro* Cultures

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ABSTRACT

In vitro cultures of nodal segments and calli of two cultivars of *Bacopa monnieri* viz., 'Pragyashakthi' and 'Calcutta Local' were subjected to gamma irradiation (30 Gy to 100 Gy for nodal segments and 30 Gy to 80 Gy for calli) with a view to induce variations and to assess the sensitivity of the cultivars to the mutagen treatment. The mutagenic treatments generated substantial variability in the morphological characters of the regenerated plantlets. Variation in leaf morphology induced by the mutagen was observed in plantlets of both the cultivars, while distinctly dwarf plantlets were observed only in case of 'Calcutta Local' with a high gamma ray dose of 100 Gy. The results indicate that both cultivars respond differently towards mutagenic treatments, visible from the frequency of mutations induced. Nodal segments of 'Calcutta Local' showed a higher mutation frequency while, in the case of calli, 'Pragyashakthi' was more sensitive to gamma irradiation. Such variation between cultivars depending on the type of explant used has previously never been reported. Variation was also observed with regard to the bacoside (active principle) content of the plantlets derived from the gamma-irradiated explants. Variants with high content of bacoside (to a maximum of 3.03%) could be isolated from the fourth subculture in the case of both the cultivars. The encouraging results obtained from the present investigation emphasize the efficiency of *in vitro* mutation induction for the improvement of this important medicinal plant.

Keywords: bacoside, calli, cultivar sensitivity, gamma rays, *in vitro* mutation, nodes
Abbreviations: BAP, 6-Benzyl amino purine; **2,4-D**, 2,4-Dichlorophenoxy acetic acid; **Gy**, Gray; **HPTLC**, High Performance Thin Layer Chromatography; **LD**, Lethal Dose; **MS**, Murashige and Skoog

INTRODUCTION

Plant tissue culture has proved to be a practical tool in paving a new avenue for the application of biotechnology in agriculture and horticulture. The growing realization of the potentialities of plant cell and tissue culture for plant propagation and breeding has itself provided a substantial impetus for research. The development of efficient *in vitro* culture methods has facilitated the use of mutation techniques for the improvement of many vegetative propagated crops, where this may be the only effective method for plant improvement (Novak 1991). *In vitro* produced tissues overcome the problems of material availability, reproducibility and poor uptake of chemical mutagens. In addition, because of the rapid formation of axillary shoots under *in vitro* conditions, periclinal and homohistont structures can be obtained more rapidly than under *in vivo* conditions.

Bacopa monnieri, commonly known as brahmi, is an important medicinal plant used since ancient times as a brain tonic. The pharmacological effects of the plant have been attributed mainly to the presence of saponins called bacosides. The most important of these are the effects on the cognition and memory promoting functions, their anxiolytic effects, and their role in the management of convulsive disorders. This could be attributed to the antioxidant and increased free radical scavenging effects of the bacosides (Tripathi *et al*. 1996). Recent findings as an anticancer drug enhance the importance of this plant in pharmaceutics (Elangovan *et al*. 1995). Although the plant occurs abundantly in marshy areas, its bacoside content is found to be very low (0.2% in dried plant). The existing knowledge on the genetic diversity of brahmi is also minimal (Darokar *et al*. 2001). This necessitates the need for further improvement of the crop mainly in terms of its secondary metabolite content. *B. monnieri*, being a vegetatively propagated crop, is highly suitable for *in vitro* induction of variations.

The present investigation focuses on induction of variability by the use of gamma rays in two cultivars of brahmi and the assessment of difference in cultivar sensitivity to mutagen treatment.

MATERIALS AND METHODS

Two cultivars of *B. monnieri* viz., 'Pragyashakthi' and 'Calcutta Local', were used for the mutation induction studies (the salient features of the cultivars are given in **Table 1**). Nodal segments 1 to 1.5 cm long, excised from *in vitro* derived plantlets were cultured on basal Murashige and Skoog (MS; Murashige and Skoog 1962) medium, supplemented with 2mg/l 6-Benzyl amino purine (BAP), 3% sucrose and 0.8% agar (bacteriological grade) and these cultures were subjected to gamma irradiation treatments. Calli, used for the treatments, were initiated from excised leaves of *in vitro* grown shoots. The calli were subcultured and maintained on MS medium with 1 mg/L 2,4-Dichlorophenoxy acetic acid (2,4-D) to ensure an adequate mass of calli for imposing the treatments. Small pieces of calli (c.a. 1.5 g) were used for the mutagenic treatments. The cultures of nodal segments and calli, in the culture vessels, were subjected to gamma irradiation from a ^{60}Co source at a dose rate of 2.5 Gy per minute at the Rashmi Radiation Sterilization Plant attached to Kidwai Hospital, Bangalore, Karnataka, India. Based on the LD_{50} values determined earlier (50-80 Gy), the nodal segments were subjected to gamma irradiation doses of 30, 50, 60, 80, 90 and 100 Gy while calli were subjected to 30, 50, 60, and 80 Gy (since the survival percentage of calli was less than 10% at 90Gy and 0% at 100 Gy, these irradiation doses were excluded for calli).

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Table 1 Salient features of the cultivars of brahmi used for mutation induction experiment.

Salient features	'Pragyashakthi'	'Calcutta Local'
Origin	Orissa	Calcutta
Stem	Pale green stem with light shade of anthocyanin pigmentation	Pale green stem with more of anthocyanin pigmentation
Leaves	Greyish green, obovate	Green, broader leaves
Dry herb yield per harvest	65 q/ha	-
Bacosides	118 kg/ha	4.0% (Farooqi, pers. comm)

Immediately after irradiation, the plant material (designated as the C_0 generation) was transferred to fresh MS media and incubated in a growth room at a temperature of 25 ± 2°C under a 16 h photoperiod. Thirty days after treatment, each treated plant material was subcultured separately on plain MS media to get the C_1 (first clonal) generation. Subculturing was continued in a similar manner three more times to get the C_2 (second clonal), C_3 (third clonal) and C_4 (fourth clonal) generations. In each of these generations, the plantlets were subcultured individually. The frequency of viable morphological mutations (changes in leaf morphology and plant stature) in the C_1 and C_4 clonal generations was estimated by the formula:

$$\text{Mutation frequency (\%)} = \frac{\text{Number of mutations}}{\text{Total number of plants scored}} \times 100$$

The plantlets of both cultivars showing the highest mutation frequency were evaluated under field conditions for their vegetative growth and total bacoside content. A scoring method was followed to quantify the variation in the vegetative growth of these plants based on the plot area covered by the vegetative spread of the plants in four months.

Total bacosides were estimated using the dried shoot portion of the plantlets by High Performance Thin Layer Chromatography (HPTLC) equipped with Densitometer (Shimadzu Flying Spot Densitometer CS9301), applicator (CAMAG-Linomat IV), developing chamber (CAMAG twin trough 10 x 10 cm) at Natural Remedies Pvt. Ltd., Bangalore, Karnataka, India. 100 mg of powdered sample was dissolved in 50 ml of HPLC grade methanol. Standard Bacoside (77% total Bacoside) preparation (5, 10, 15 and 20 µl) was applied in four different tracks. Samples (10 and 15 µl) were applied in another two tracks and the plate was allowed to develop in a solvent system (Mobile phase: ethyl acetate:methanol:water in 70:20:10; adsorbent – silica gel, $60F_{254}$ (Merck Al. Sheets-1.05554). It was dried in an air current and sprayed with ANS reagent (0.5% anisalidehyde in glacial acetic acid: methanol:concentrated sulpuric acid in 10:85:5). The chromatograms of standard and samples were recorded and a calibration curve was plotted and the content of bacoside was calculated.

A completely randomized design was adopted to lay out the laboratory experiment and each treatment was repeated five times. The field experiment was laid out according to Randomized Block Design and each treatment was replicated three times. Analysis of Variance (P=0.05) was performed for the data obtained by MSTAT.

RESULTS AND DISCUSSION

Mutagens have a remarkable possibility to cause variations in plants (Maluszynski *et al*. 1995) with regard to their quantitative and qualitative characters by altering the genetic architecture (Broertjes and van Harten 1978). Gamma rays are known to cause chromosomal breakages, the exposure of cells to gamma rays brings in ionization (Dubinin and Soyfer 1969). In addition to ionization, radiation also causes an indirect effect by producing free radicals, which react with DNA bases leading to genetic changes (Broertjes and van Harten 1978). The spectra of viable mutations are of most important consideration for isolating desirable mutant types (Sonnino *et al*. 1986; Ahloowalia 1990; Novak *et al*. 1990). In the present investigation, the effect of gamma irradiation of nodes was manifested in the leaf morphology and stature (plant height) of the regenerated plantlets. The variations included formation of shoots with two leaves per node in the same direction (**Fig. 1A**), three leaves from a node (**Fig. 1B**), four leaves per node (**Fig. 1C**), shoots with very short internodes and closely arranged leaves leading to short statured plantlets (**Fig. 1D**) (seen in 'Calcutta Local' with gamma irradiation of nodes with 100 Gy), and shoots with closely arranged leaves in a portion of the shoot (**Fig. 1E**) (seen in 'Calcutta Local' with gamma irradiation of nodes with 100 Gy). It was interesting to observe plantlets with very short internodes in the case of 'Calcutta Local', where nodes were irradiated with 100 Gy resulting in very short plantlets (**Fig. 2**). All the plantlets that had been derived from the nodes of 'Calcutta Local' in the C_1 generation, irradiated with 100 Gy exhibited this dwarf stature. The plantlets obtained from these variants by direct organogenesis from its leaf pieces and nodal segments also gave rise to short statured plantlets. This comparatively dwarf stature was exhibited in all the clonal generations with 98.62% of the plantlets exhibiting this dwarf stature in the C_4 generation. Such abnormalities following mutagen treatments might be due to chromosomal aberration or a change in the route of growth regulator synthesis, disturbance in the metabolism or the accumulation of free amino acids (Gupta *et al*. 1982). The reduction in plant height due to irradiation might be due to the suppression of mitotic activity (Muthusamy and Jayabalan 2000), inhibition of translocation of assimilates (Roy and Clark 1970), impairment of water uptake (Rageb and Mohamed 1983) and physiological and chromosomal damage caused by irradiation (Ram and Zaman 1972; Katoch *et al*. 1992). There are several earlier reports where gamma irradiation resulted in a reduction of plant stature, such as in cherry (Hedtrich 1990; Yang and Schmidt 1994). Similar variations in leaf shape and plant

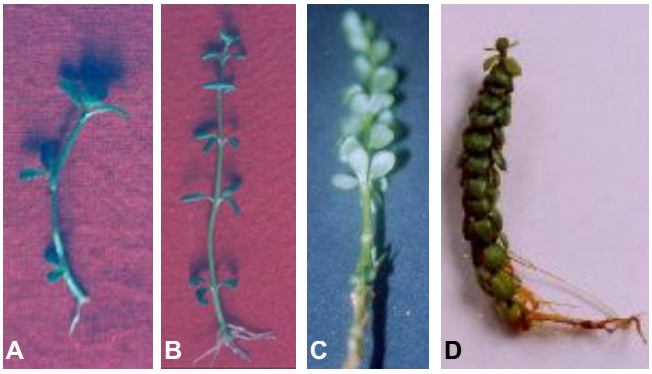

Fig. 1 Morphological abnormalities observed in plantlets obtained from gamma irradiated explants. (**A**) Shoot with two leaves in same direction from a node; (**B**) shoot with three leaves from a node; (**C**) shoot with four leaves from a node; (**D**) shoot with very short internodes and closely packed leaves.

Fig. 2 Dwarf statured plantlets (right) of cv. 'Calcutta Local' regenerated from gamma irradiated (100 Gy) nodes compared with control (left).

Fig. 3 Stimulation of multiple shoots from nodes of cv. 'Pragyashakthi' treated with 90 Gy of gamma rays.

Fig. 4 High bacoside-containing variant of cv. 'Pragyashakthi' (derived from gamma irradiation of nodes with 90 Gy in field).

height consequent to gamma irradiation under *in vitro* conditions have also been reported in *Alpinia purpurata* (Fereol *et al*. 1996) and *Coleus forskohlii* (Srinivasappa *et al*. 2003).

Some of the nodal explants of cv. 'Pragyashakthi', on transfer to plain MS media soon after treatment with 90 Gy gamma rays resulted in a phenomenally high proliferation of multiple shoots in the C_1 generation (**Fig. 3**). Such a response was observed only in the case of shoots from eight nodal explants treated. The multiple shoots, when separated and grown on plain MS medium, grew normally.

The type of mutagen, plant genotype and physical state of the material are crucial factors which contribute to the difference in the frequency and spectrum of induced mutations (van Harten 1988; Reddy *et al*. 1993). The frequency of viable mutations were more with higher doses of gamma rays in the case of nodal explants of both cultivars, while the frequencies were quite high at lower doses itself in the case of calli. The difference in the mutation frequency between the explants might be due to the difference in the level of cellular organization, probably due to the less organized and undifferentiated state in a callus compared to a more organized and differentiated state in a nodal explant *per se*.

The extent of mutations was greater in the C_1 generation, but was comparatively less in subsequent generations. This presumably indicates that the meristematic cells of the original material were differentially affected and that the plants in the first generation are often grossly chimeric, perhaps with relatively high multiplication rates of the less affected cells leading to a gradual reversion to normal in the subsequent clonal generations (Fereol *et al*. 1996).

The mutation frequencies in both the cultivars did not exhibit an increasing trend with the increase in the dose of the mutagen. It could be due to the saturation in the mutational events and vigour of diplontic selection (elimination of the mutated cells due to competition from the normal diploid cells) in the biological material (Sharma and Sharma 1986).

Plantlets derived from gamma irradiated nodal segments of 'Calcutta Local' recorded a higher mutation frequency than 'Pragyashakthi' and in both cultivars, mutation frequencies were higher in the C_1 clonal generation (**Table 2**). In 'Calcutta Local', the range of mutation frequency was from 18.42% to as high as 98.62% while it was from 19.38% to 64.84% in 'Pragyashakthi'. A high frequency of mutation was recorded corresponding to 90 Gy for 'Pragyashakthi' and 80 Gy and 100 Gy for 'Calcutta Local'.

The mutation frequency was comparatively low in both cultivars, where plants had been derived from gamma-irradiated calli. 'Pragyashakthi', in general, was found to record a higher mutation frequency (3.87%) than 'Calcutta Local' (3.38%) (**Table 3**). A lower dose of 30 Gy recorded a higher mutation frequency (7.61%) in the case of 'Pragyashakthi', while it was higher with 50Gy (7.41%) in the case of 'Calcutta Local'.

The data on mutation frequency revealed a greater sensitivity of nodes of 'Calcutta Local' when compared to that of 'Pragyashakthi'. However, in the case of calli, 'Pragya-

Table 2 Mutation frequency in gamma irradiated nodes of brahmi cultivars.

Gamma ray dose	'Pragyashakthi'			'Calcutta Local'		
	First clonal generation	Fourth clonal generation	Mean	First clonal generation	Fourth clonal generation	Mean
30 Gy	0	0	0	0	0	0
50 Gy	20.67	0	10.34	38.89	0	19.44
60 Gy	21.00	0	10.50	56.08	18.42	37.25
80 Gy	32.52	21.29	26.90	77.69	64.63	71.16
90 Gy	64.84	51.59	58.22	71.97	57.93	64.95
100 Gy	21.80	19.38	20.59	97.03	98.62	97.82
Control	0	0	0	0	0	0
Mean	22.98	13.18	18.08	48.81	34.23	41.52

Table 3 Mutation frequency in gamma irradiated calli of brahmi cultivars.

Gamma ray dose	'Pragyashakthi'			'Calcutta Local'		
	First clonal generation	Fourth clonal generation	Mean	First clonal generation	Fourth clonal generation	Mean
30 Gy	11.67	3.55	7.61	9.02	0	4.51
50 Gy	8.00	2.76	5.38	9.92	4.90	7.41
60 Gy	9.84	2.90	6.37	7.20	2.76	4.98
80 Gy	0	0	0	0	0	0
Control	0	0	0	0	0	0
Mean	5.90	1.84	3.87	5.23	1.53	3.38

Table 4 Evaluation of the mutagen treated plants in the field.

Cultivar	Explant	Gamma ray Dose (Gy)	Vegetative growth (Score)	Bacoside content (% w/w)
'Pragyashakthi'	Node	90	++	3.03
'Pragyashakthi'	Callus	30	+++	2.60
'Pragyashakthi'	Node	Control (0)	++	1.60
'Calcutta Local'	Node	80	++++	1.63
'Calcutta Local'	Node	100	+	1.17
'Calcutta Local'	Callus	50	+++	2.61
'Calcutta Local'	Node	Control (0)	++	1.75

Scoring for vegetative growth

Score	Description
+	Poor growth, only 25% of the plot covered by vegetative growth of the plant
++	Good growth, 50% of the plot covered by vegetative growth of the plant
+++	Very good growth, 75% of the plot covered by vegetative growth of the plant
++++	Excellent growth, 100% of the plot covered by vegetative growth of the plant

shakthi' was more sensitive to mutagen treatment.

The vegetative growth of the variants evaluated were in general, comparable or significantly better than that of the respective control plantlets (**Table 4**), with the exception of plantlets of 'Calcutta Local', where nodes had been subjected to 100Gy. A wide variation was also observed with respect to the bacoside content between the variants, as identified by HPTLC. Plants of 'Pragyashakthi' derived from nodes, which had been treated with 90Gy of gamma rays (**Fig. 4**) were found to have the highest bacoside content (3.03%) (**Table 4**). The same dose of gamma rays had resulted in the highest mutation frequency also. Plantlets of 'Pragyashakthi' obtained from gamma irradiated calli also showed significantly higher bacoside content when compared to the control. While in the case of 'Calcutta Local', the dose that resulted in the highest mutation frequency (100 Gy) caused a drastic reduction in the bacoside content (1.17%). However, the variants of 'Calcutta Local' derived from calli irradiated at 50 Gy showed a significantly high bacoside content.

CONCLUSIONS

Gamma irradiation was thus effective in producing a wide range of variations in *B. monnieri*. The results elucidate a differential response of 'Pragyashakthi' and 'Cacutta Local' towards gamma irradiation treatments, as obvious from the frequency of mutations induced. It seems likely that these differences in sensitivity observed between the cultivars relate to the differences in their genetic make-up. The differrences in mutagen sensitivity among genotypes have also been reported earlier on several crops like wheat (Walther and Huang 1973), banana (Novak *et al*. 1990), chinese gooseberry (Shen *et al*. 1990), gerbera (Jerzy and Lubomski 1992), *Rosa hybrida* (Ibrahim *et al*. 1998) and pear (Predieri and Zimmerman 2001) while broader applications to ornamental breeding and all crop species has been reviewed in Teixeira da Silva (2006). It was also interesting to observe a difference in the sensitivity of the cultivars depending on the type of explant subjected to mutagen treatment. Thus, from the present study it was possible to isolate high bacoside containing variants of 'Pragyashakthi' and 'Calcutta Local', which hold a lot of promise in the crop improvement programme of this important medicinal plant.

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