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Original Article

Optimization of protocol for *in vitro* polyploidization in genetic improvement of *Bacopa monnieri* L.

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Abstract

Leaf and internodal segments of *Bacopa monnieri* was cultured on MS medium supplemented with eighteen combinations of growth regulators involving BAP, KN, 2,4-D, NAA and IAA to optimize suitable culture condition for callus induction and plant regeneration. MS medium supplemented with KN and IAA (0.5 mg/l each) induced the highest number of shoots per explant with significant quantity of callus to become the suitable medium for callus induction and shoot regeneration. Pretreatment of leaf and internodal explants with colchicine (0.1%) for different durations (1h, 3h, 6h, 12h and 24h), followed by initiation of primary culture on MS medium supplemented with KN and IAA (0.5 mg/l each) for 45 days resulted in optimization of requisite conditions for polyploidization. Pretreatment of explants with colchicine for shorter duration (1 – 3h) did not affect callus proliferation and subsequent regeneration of shoots. However, further increase in duration of colchicine pretreatment from 6 – 24h reduced the callus proliferation significantly and high incidence of browning coupled with development of weaker shoots was observed. *In vitro* response of leaf and internodal stem explants against different duration of colchicine pretreatment was studied and found that leaf tissues are more tolerant to colchicine than internodes as evidenced by high incidence of browning with failure of shoot regeneration in internodes than leaf explant. The utility of present findings in genetic improvement of *B. monnieri* is discussed.

Keywords: shoot regeneration, anti mitotic agent, polyploidization, genetic improvement.

Introduction

Bacopa monnieri is the most important medicinal herb in India used for a variety of herbal products in Ayurvedic and Siddha system of medicines (Tiwari *et al*., 1998; 2001; 2006; Shrivastava and Rajani 1999 and Rastogi *et al*., 1994). Despite ever increasing demand for its raw material in herbal industries of India, cultivation of this medicinal herb is restricted only to certain agroclimatic conditions in wetlands and muddy shores. Extended cultivation of this medicinal herb in drought prone areas for large scale production of raw materials is one of the major constraints owing to its high sensitivity to drought. Therefore, cultivation of *B. monnieri* is restricted only to certain agroclimatic condition and become the major limiting factor for overall production of raw materials (Ahmad, 1993; Chand *et al.,* 1997). Development of new variety of *B. monnieri* by conventional method is extremely difficult due to its very tiny flower size with complex genetics. There is only one report so far on development of new variety of *B. monnieri* for improving the ornamental value by enlarging the flower size through polyploidization. However, there is no continued effort to improve other agronomic features by biotechnological approaches.

Genetic improvement through polyploidization has been achieved in a number of plant species to improve agronomic values such as drought resistance in *Coccinia palmate* and *Lagenaria sphaerica* (Ntuli and Zobolo, 2008), improve oil contents in *Ocimum basilicum* (Omidbaigi, 2010), vegetative vigour in *Morus alba* (Chaicharoen *et al*., 1995) and improvement of flower size for a esthetic value in *B. monnieri* (Escandon *et al*., 2006). Colchicine is an antimitotic agent used for doubling the chromosomes for developing polyploids (Ramsey and Schemske, 1998; Dhooghe *et al*., 2011). *B. monnieri* is a small herb with tiny leaves containing bacoside A and B. These two phytochemicals are proven to be having wide range of medicinal properties (Mathur *et al*., 2002). Genetic improvement of *B. monnieri* for

enhancing its drought tolerance coupled with improvement of its medicinal property through polyploidization was emphasized. Thus, the present study was undertaken to optimize the protocol for inducing polyploids as well as to develop suitable culture conditions for regeneration of plantlets utilizing leaf and intermodal explants. The present finding and its possible utility in genetic improvement of *B. monnieri* is discussed.

Materials and Methods

Plant materials and Maintenance

Wildly grown healthy plants of *Bacopa monnieri* was collected from agricultural field of Pondicherry in southern part of India and maintained in the pots containing soil, sand and manure (6:2:1) in the botanical garden of Sri Paramakalyani Centre for Environmental Sciences, Manonmaniam Sundaranar University, Alwarkurichi. Plants were nourished well by frequent application of vermicompost under optimal soil moisture. Leaves and internodes collected from these plants were used as explants.

Explant preparation

Murashige and Skoog's (1962) medium supplemented with sucrose (3%) was used as basic media. The medium was further augmented with eighteen different combinations of growth regulators involving BAP, KN, 2,4-D, NAA and IAA. The pH of the medium was adjusted to 5.8 before gelling with 0.7% agar (Hi-media, India). All the chemicals used in the present study were of analytical grade (British Drug House, Sigma, Merck, and Hi-media). Molten medium was dispensed into 200-ml screw-capped glass jars or 150-ml Erlenmeyer flasks (Borosil, India). The culture vials containing the media were autoclaved at 104 kPa and 121°C for 20 min. Whole leaves and internodes measuring 1.5 cm were rinsed with water for 10 - 15 min depending upon their maturity and then disinfected with 0.1% $HgCl_2$ (wt/vol) for 5 - 8 min. Disinfected explants were thoroughly rinsed 4 - 5 times with sterile distilled water. All the cultures were maintained at 25±1°C and grown under16h photoperiod. Number of explants cultured in each treatment was varied from 60 – 90 depending upon the experimental design.

Standardization of culture conditions

Whole leaves and internodes measuring about 1 cm were implanted on MS medium fortified with varying concentrations of two commonly used cytokinins (BAP and KN) either alone or in combination with three auxins (2, 4-D, NAA, IAA). Eighteen different combinations were experimented to optimize the suitable media for callus induction and shoot regeneration. Leaf and internodes cultured in different combinations of media were maintained for 45 days. Response of explants with regard to callus proliferation and shoot regeneration was recorded and subjected to statistical analysis to identify the optimal culture conditions.

Optimization of colchicine pretreatment

Leaf and internodes of *B. monnieri* was subjected to treatment with colchicine (0.1%) for different durations (0 hr, 1 hr, 3 hrs, 6 hrs, 12 hrs and 24 hrs) and inoculated on full strength MS medium supplemented with KN and IAA (0.5mg/l each). Explants were maintained on the same media for 45 days without any subculture. After 45 days of primary culture, leaf and internodes were subjected to observations. Parameters such as browning of explants, callusing response, rate of callus proliferation and nature of shoots produced etc. were recorded in each treatment and subjected to statistical analysis in order to select the optimal duration of treatment for utilizing the protocol in development of polyploids.

Results and Discussion

Leaf and internodal explants of *B. monnieri* cultured on various combinations of auxins and cytokinins exhibited varying response with regard to callus and shoot regeneration (Table 1 and Figure 1). The percentage of response of leaf explants in various combinations of media was ranging from 18 - 68.7% depending upon the types and hormonal combinations. Of the various combinations of auxins and cytokinins tested, KN in combination with 2,4-D (0.1mg/l each) induced the highest response with 68.7%. However, shoot multiplication was significantly reduced with 11.5 shoots per explant. Although KN in combination with 2,4-D (0.1mg/l each) induced maximum response for callus and shoot regeneration during primary culture, this combination was not preferred in the subsequent experiments due to lesser number of shoots per explants. When leaf explants were cultured on medium containing KN and IAA (0.5mg/l each), highest number of healthy shoots could be obtained. Shoots obtained on this media was found to have broader leaves with healthy root system. Therefore, use of KN and IAA (0.5mg/l each) was found to be an effective combination for regeneration of healthier shoots from leaf and internodes.

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Table- 1: Effect of auxin and cytokinin on callus induction and shoot regeneration from cultured leaf tissues of *Bacopa monnieri*. Explants were cultured on MS medium supplemented with various combinations of growth regulators for 90 days.

BAP	KN	NAA	2,4-D	IAA	% of Response	Quantity of callus		No. of explants Shoots/	Length of longest shoot(mm)
						FW	DW		
0.0	0.0	0.0	0.0	0.0	40.2^{egf}	1.08^{j}	0.03^{j}	30.50^{b}	50.5^{bc}
0.1	-	0.1	-	-	28.7^{i}	1.41^{ij}	0.14^{i}	11.0^{e}	56.0^{bc}
0.5	-	0.5	-	-	41.2^{ef}	3.18^{g}	0.27^{f}	20.7^{cd}	29.0^{efg}
1.0	-	1.0	-	-	39.2^{fg}	5.08^{d}	0.65^{c}	23.5^{c}	22.7^{hi}
-	0.1	0.1	-	-	18.0^{j}	1.65^{hi}	0.15^{i}	13.0^{e}	52.5^{c}
-	0.5	0.5	-	-	26.0^{j}	4.37^{e}	0.55^{d}	21.5^{cd}	32.5^{de}
-	1.0	1.0	-	-	43.0^{def}	6.27^{ab}	0.75^{b}	28.7^{b}	20.7^{i}
0.1	-	-	0.1	-	33.7^{h}	1.96^{h}	0.26^{fg}	12.7^{e}	53.5^{c}
0.5	-	-	0.5	-	44.7^{de}	4.01^{f}	0.55^{d}	13.7^{e}	32.7^{de}
1.0	-	-	1.0	-	53.2^{c}	6.17^{ab}	0.85^{a}	23.2^{c}	23.7^{hi}
-	0.1	-	0.1	-	68.7^{a}	1.68^{hi}	0.19^{ghi}	11.5^{e}	58.7^{b}
-	0.5	-	0.5	-	46.5^{d}	3.30^{g}	0.49^{de}	18.5^{d}	30.2^{ef}
-	1.0	-	1.0	-	52.5^{c}	5.60^{c}	0.76^{b}	24.2^{c}	23.2^{hi}
0.1	-	-	-	0.1	21.0^{j}	1.65^{hi}	0.24^{fgh}	13.0^{e}	53.2^{c}
0.5	-	-	-	0.5	44.0^{def}	3.37^{g}	0.43^{e}	22.7^{c}	35.2^{d}
1.0	-	-	-	1.0	63.5^{b}	5.90^{bc}	0.85^{a}	31.0^{ab}	25.0^{ghi}
-	0.1	-	-	0.1	33.7^{h}	1.37^{ij}	0.16^{hi}	18.2^{d}	71.0^{a}
-	0.5	-	-	0.5	43.7^{def}	4.47^{e}	0.85^{a}	33.5^{a}	51.7^{c}
-	1.0	-	-	1.0	26.0^{i}	6.35^{a}	0.66^{c}	22.2^{cd}	26.5^{fgh}

Data represents the mean values of 20 explants in each treatment with three replications and analysis was made using Duncan's new multiple range test. Figures with the same superscript are not differing significantly.

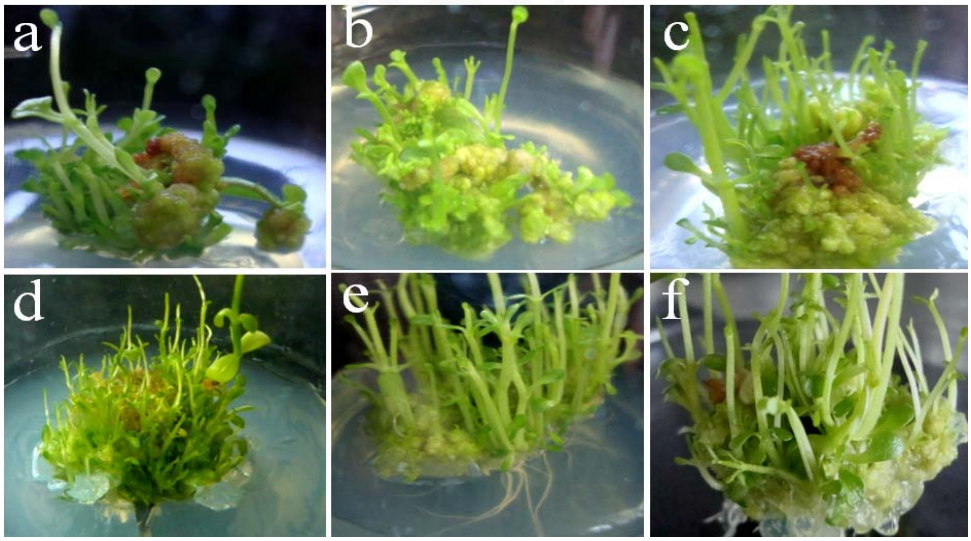

Figure1: Effect of auxins and cytokinin on callus induction and shoot regeneration. a) Leaf tissues with minimal response on MS basal medium, b) Profuse callus with weaker shoot development in presence of BAP, c and d) High frequency shoot proliferation with profuse callusing, e and f) Development of healthy shoots on medium with KN and IAA (0.5mg/l).

Leaf and internodes of *B. monnieri* cultured on MS medium supplemented with KN and IAA (0.5mg/l each) after exposure to colchicine resulted in optimization of colchicine pretreatment for inducing polyploids in *B. monnieri*. The results of this experiment was presented in Table 2 and 3 and compared with Figure 2. Both the type of explants exposed to colchicine (0.1%) for 1h and 3h did not show any

visible sign of phytotoxicity and response with regard to callus proliferation and shoot regeneration was more or less comparable with control. These shoots were found healthier with shorter internodes as observed in control. Increase in duration of colchicine treatment for 6h - 12h resulted in decline in response with regard to percentage of survival, callus proliferation and shoot regeneration. However, in few of the cultures, callus proliferation followed by shoot regeneration was occurred during primary culture and subsequently, many of the shoots turned brown and dead possibly due to longer exposure to colchicine. Interestingly, in several of the cultures, new shoots were regenerated from the clump of dead multiple shoots after prolonged culture period (Fig. 2e).

Visible sign of phytotoxicity against colchicine pretreatment was observed only from 6h – 24h treatment as evidenced by significant level of reduction in callus proliferation, browning of leaf explants and weaker shoot development. Increase in duration of treatment with colchicine for 12h and 24h resulted in high incidence of morality as revealed by highest percentage of browning and death of microshoots even during primary culture (Fig. 2f). Therefore, the present experiment indicated that treatment of leaf and internodes with colchicine for 6h is optimal for inducing polypoids since significant effect of colchicine was noticed only at 6h treatment without largely affecting the regeneration.

Escandon *et al*., (2006) have reported only 18.37 and 17.04 shoots per explants in presence of 0.2mg/l and 0.5mg/l BAP respectively. In our study, combination of KN and IAA (0.5mg/l each) induced a maximum 33.5 shoots per explant. Shoots regenerated on this media was healthier even after exposure of original explants to colchicine. Tiwari *et al.*, (2001) have reported that use of BAP alone was not beneficial in regeneration of more number of shoots in *B. monnieri*. In support of this finding, a combination of BAP and KN or with any one of the auxins, namely 2,4-D, IAA and NAA was better for high frequency shoot regeneration than the use of BAP alone. Colchicine is a potential antimitotic agent used for developing polypoids to improve various agronomic traits in fruits, vegetables and medicinal plants. Although, a large number of research has been reported about polyploidization in various species such as *Gossypium herbaceum* and *Gossypium arboretum* (Omran *et al*., 2008), *Solanum* spp. (Chauvin *et al*., 2003), citrus (Wu and Mooney, 2002), pomegranate (Shao *et al*., 2003), *Allium* spp. (Jakse *et al*., 2003) and azaleas (De Schepper *et al*., 2004). Banana (Baziran and Ariffin, 2002), Grapes (Notsuka *et al*., 2000), Blueberry (Lyrene and Perry, 1982), Potato (Hermsen *et al*., 1981), and Sugarcane (Heinz and Mee, 1970), research on genetic improvement of *B.monneri* through development of polyploids was very scanty and there was only one report on induction of polypoids in this species (Escandon *et al*., 2006).

Table-2: Effect of colchicines on leaf explants of *Bacopa monnieri* cultured on MS medium supplemental with KN and IAA (0.5 mg/l each). Leaf tissues were treated with 0.1% colchicine for different duration from 1 - 24 h before culturing the explants on nutrient medium.

Colchicine Treatment (hrs)	No. of shoots/ Explant	Length of longest shoot (mm)	Length of internodes (mm)	No. of roots/ culture	Root length (mm)
Control	31.25^{bc}	35.00^{c}	1.17^{bc}	00.00^{c}	00.00^{c}
01	22.50^{c}	22.50^{d}	1.57^{ab}	00.00^{c}	00.00^{c}
03	63.75^{a}	53.75^{a}	0.57^{a}	17.50^{a}	01.42^{a}
06	36.25^{b}	42.50^{ab}	0.95^{bc}	12.75^{ab}	00.92^{b}
12	15.00^{c}	28.75^{bc}	0.90^{c}	11.25^{b}	00.62^{bc}
24	23.25^{c}	27.50^{cd}	0.62^{c}	11.25^{b}	00.65^{b}

Data represents the mean values of 20 explants in each treatment with three replications and analysis was made using Duncan's new multiple range test. Figures with the same superscript are not differing significantly.

Table -3: Effect of colchicine on internodal explants of *Bacopa monnieri* cultured on MS medium supplemental with KN and IAA (0.5 mg/l each). Internodes were treated with 0.1% colchicines for different duration from 1 - 24 h before culturing on nutrient medium.

Colchicine Treatment (hrs)	No. of shoots/ Explant	Length of longest shoot (mm)	Length of internode (mm)	No. of roots/ culture	Root length (mm)
Control	15.50^{c}	07.75^{c}	1.67^{ab}	0.00^{c}	0.00^{c}
01	11.0b^{c}	09.25^{bc}	1.15^{bc}	0.00^{c}	0.00^{c}
03	23.25^{a}	18.75^{a}	0.80^{c}	11.5^{a}	0.87^{b}
06	13.50^{b}	10.25^{b}	1.50^{ab}	10.5^{a}	0.97^{b}
12	06.00^{c}	05.00^{c}	1.90^{a}	4.75^{b}	1.67^{a}
24	05.50^{c}	04.25^{c}	1.95^{a}	5.72^{b}	0.00^{c}

Data represents the mean values of 20 explants in each treatment with three replications and analysis was made using Duncan's new multiple range test. Figures with the same superscript are not differing significantly.

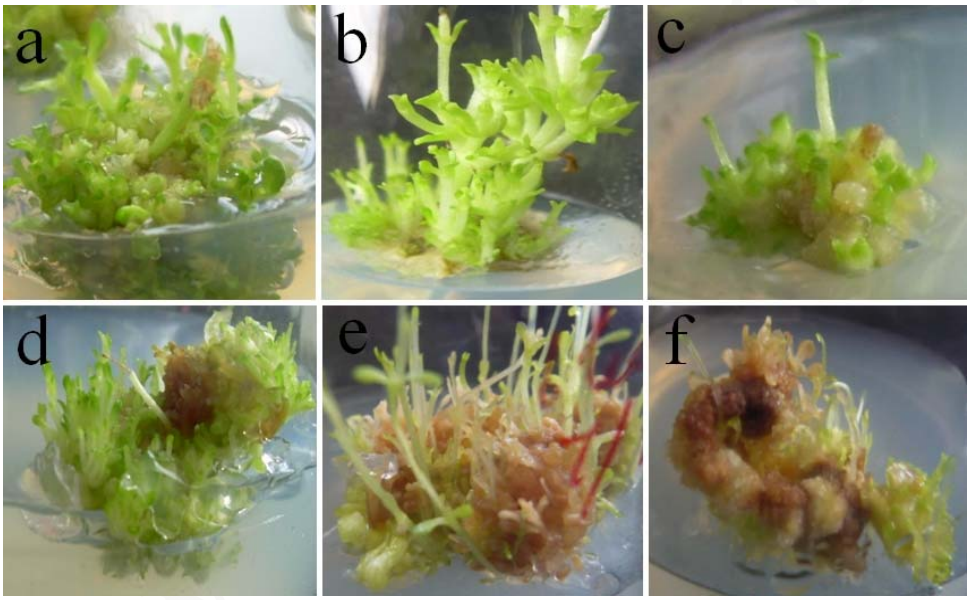

Figure 2: Effect of colchicine on cultured leaf tissues of *B. monnieri*. a) Healthy shoot development in control, b & c) Cultures showing callus and shoot generation after pretreatment of leaf tissues for 1hrs and 3hrs with colchicine (0.1%), d - f) Cultures showing phytotoxicity upon longer exposure of leaf tissues to colchicine as evidenced by increase in browning of tissues from 6 h – 24 h.

B.monnieri is primarily used as an ornamental plant in Argentina and its ornamental value could be improved by enhancing the flower size by polyploidization. Thus, it is possible to improve several other agronomic traits of *B. monneri* through polyploidization. Since colchicine is a strong antimitotic agent, polyploids can be generated in *B. monneri* to improve plant vigor as well as its medicinal properties by enhancing the quantity of Bacoside A, an important phytochemical having wide range of medicinal properties (Tiwari, 1998). The present study revealed that MS medium augmented with KN and IAA (0.5mg/l) is suitable for regeneration and exposure of leaf and internodes to colchicine pretreatment for 6h was found optimal for inducing polypoids. These findings are expected to be useful in genetic improvement of *B. monnieri* through polyploids.

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