ISSN-2249-5746

International Journal Of Ayurvedic And Herbal Medicine 1:3 (2011)51:67

Journal Homepage http://interscience.org.uk/index.php/ijahm

Effects of antioxidants and gelling agents on regeneration, *in vitro* conservation and genetic stability of *Bacopa monnieri* (L.) Pennell.

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Abstract

Shoot regeneration of *Bacopa monnieri* was achieved, on Murashige and Skoog (MS) medium supplemented with 4.4 µM benzyl aminopurine (BA) from different explants. Nodal explants initiated 75-80 shoots, leaf explants 55-65 shoots and internodal explants 50-55 shoots within 40 days of culture. The initiated shoots were multiplied in MS medium containing 2.2 µM BA. Ascorbic acid (50 mg/l) and citric acid (25 mg/l) increased the number of shoots and conservation period of the cultures. Isabgol, when used as gelling agent provided consistent multiplication of cultures compared to microbiology grade agar and cultures were conserved in medium containing 3% (w/v) isabgol for a period of 190 days as against an average of 120 days in 1% (w/v) agar containing media. The conservation period was increased to a period of 200 days in medium gelled with 3% (w/v) isabgol containing 50 mg/l ascorbic acid and 25 mg/l citric acid and more than 80% of the cultures survived in this medium. Rooting was achieved *ex vitro* by culturing the isolated shoots in autoclaved soilrite moistened with 1/10 strength MS salts. Rooted plantlets were established in soil. Genetic stability assessment with random amplified polymorphic DNA (RAPD) primers showed no variation in the banding pattern of the cultures grown in different concentrations of growth regulators, agar or isabgol and additives containing media. These studies provide a feasible *in vitro* multiplication and conservation method and genetic stability assessment of *B. monnieri* cultures.

Key Words *Bacopa monnieri*, Ascorbic acid, Citric acid, Isabgol, RAPD, Genetic stability.

Introduction

Medicinal plants are widely exploited worldwide for their active ingredients. This has created a wide gap between the production and exploitation of most of the medicinal plants, resulting in complete loss or gradual reduction of important plant species. Biotechnology based methods have been utilized for multiplication, conservation and International exchange of large number of medicinal plants (Mandal et al. 2000).

Brahmi (*Bacopa monnieri),* also known as *Medhya Rasayana* used in ayurvedic medicines for enhancing memory, reduces stress induced anxiety, improves intellectual and cognitive functions that are attributed to the saponins and bacoposide A and B (Deepak and Amit 2004). Natural regeneration of this herb is hampered by death of seedlings at two-leaved stage and specific habitat (marshy areas) requirements. *Bacopa* is a poor competitor and can colonize only in open spaces. Urbanization and subsequent destruction of natural habitat created a decline in their natural population (Pandey et al. 1993). According to National Medicinal Plant Board (NMPB), Govt. of India, annual demand of *Bacopa* during the year 2004-2005 was 6621.8 tons with an anticipated 7% annual growth rate. This requirement is rising enormously in view of the popularity of the *Bacopa* based drugs, like "Memory Plus" in the market, resulting in enormous overexploitation of the natural populations of this plant to meet the present requirement. Because of ruthless exploitation for its active principles, the International Union for Conservation of Nature and Natural Resources lists this plant as a threatened species (Pandey et al. 1993). Technology, Information, Forecasting and Assessment Council (TIFAC, DST, GOI) has identified 45 medicinal plant species for conservation and specifically recommended 7 plants for immediate attention and *B. monnieri* is one among them. Hence, there is an urgent need to conserve the wild stocks of *B. monnieri* for posterity, by assessing the natural populations, developing feasible protocols for micropropagation, *in vitro* conservation and genetic stability of the *in vitro* conserved germplasm.

Ascorbic acid and citric acid affect the number and quality of multiple shoots regenerated in tissue culture (Arya and Shekhawat 1986). The positive effect of citric acid on preventing tissue necrosis in tissue culture is reported (Anthony et al. 2004). Synergistic action of these compounds along with growth hormones has positive effects on the establishment and multiplication of cultures in various plant species (Yusuf 2005). Gelling properties of agar is extensively utilized in tissue culture, the exclusive properties of agar being its stability, clarity and inertness in culture. However, some doubts were raised against the inertness and non-toxicity of agar in tissue cultures (Jain and Babbar 2005). The cost of tissue culture medium is affected to a great extent by the quality and price of agar, and cheap alternative sources of agar can reduce the cost of tissue culture medium substantially. Additionally, agar contains a large amount of Na as an impurity that could act as an inhibitory factor in tissue culture and its maintenance for longer periods (Deberg 1983). In these contexts, some alternative gelling agents were explored and successfully used in tissue culture media (Tyagi et al. 2007).

Genetic stability of tissue culture raised plants is a major concern as the plants conserved for longer duration in the same media and stress pertaining to exposure of the plant tissues to various chemicals contributes to changes in their genetic make up. RAPD is successfully used as a cost-effective, easily reproducible technique for determining the genetic variations among plant species including *B. monnieri* (Darokar et al. 2001; Martins et al.2004; Chaudhuri et al. 2007).

In the present study, initiation of cultures from different explants, the effects of antioxidants, gelling agents on conservation of cultures were tested. The genetic stability of cultures maintained and conserved in different media was evaluated by RAPD.

Materials and methods

Initiation of cultures

In vitro shoot cultures of *B. monnieri* were established from three explant sources, *viz.* leaf, internodes and nodal segments collected from plants grown in the marshy areas near Kengeri, Bangalore, India. The leaf segments (1cm^2), internodes (3-5 cm), and nodal portion containing at least two axillary buds were dissected from the plants and carried to the laboratory in two separate flasks, one containing distilled water and the other containing an antioxidant solution of ascorbic acid, 50 mg/l and citric acid, 25 mg/l, whose pH was adjusted to 6.0. Explants kept in water and antioxidant solution were washed separately with diluted (1:10 v/v) solution of Tween-20 containing 1% (w/v) Bavistin for 12 to 15 min, and washed thoroughly with distilled water or with antioxidant solution separately 4 to 5 times. The explants were surface sterilized with 0.1% (w/v) mercuric chloride prepared in distilled water or in antioxidant solution for 3-5 min, and were washed with distilled water or antioxidant solution to remove traces of mercuric chloride. The sterilized explants were cultured onto MS (Murashige and Skoog 1962) medium supplemented with different concentrations of BA (1.1, 2.2, 4.4 and 11.0 µM) or kinetin (1.15, 2.3, 4.6 and 11.5 µM) with 0.7% (w/v) agar.

Multiplication of cultures and in vitro conservation

The regenerated shoots were excised and cultured on MS medium containing BA (2.2 µM). Three different concentrations of agar (0.7, 1.0 and 1.5% w/v) and isabgol (1.0, 3.0 and 5.0 % w/v) were used as gelling agents to study their effects on plant regeneration and conservation. Isabgol powder was dispensed in water at room

temperature (25-27°C) to avoid clumping and poured into the media. The pH of the media was adjusted to 6.0 and autoclaved at 121°C and 1.06 Kg cm^{-2} for 20 min. After autoclaving, the media were supplemented with filter sterilized ascorbic acid (25, 50, 75 and 100 mg/l) and citric acid (10, 25, 50 and 100mg/l), by using Millipore™ filters (0.2 microns) and dispensed into the medium inside a laminar airflow. Approximately 20 ml of the medium was dispensed into culture tubes (25 x 150ml, Borosil, India). The culture tubes were covered with polypropylene caps and the edges were sealed with parafilm. Cultures were incubated at 25±2°C and a light intensity of 40µmol m^{-2}S^{-1}, provided by white fluorescent lamps (Philips,India) at 16-h photoperiod. After 21 days, the cultures were subcultured on fresh media.

Ex vitro rooting and hardening of plantlets

The shoots were dissected from the clumps and cultured in autoclaved soilrite (Kelpelrite, Bangalore), moistened with 1/10 strength MS nutrients and kept in culture room conditions. The caps of the bottles were gradually removed and the plantlets were exposed to ambient conditions after 25days. As the plantlets grew to a length of 12-15 cms., they were removed from the soilrite mixture and planted in soil.

Genetic stability assessment

The regenerated plant samples from all the treatments were subjected to RAPD analysis using source plant as control and the RAPD profiles of plants grown in different cultural conditions were documented and compared.

DNA extraction

Total genomic DNA was extracted from the 250 mg of leaves from two or three plants of the same experiment using the protocol described by Dellaporta et al. (1983), with modifications. 5 µl each of RNase (2mg/ml) and Proteinase K (20 mg/ml) were added to the buffer at the time of adding the powdered tissue sample. The quantity and quality of the purified DNA was estimated by using a spectrophotometer. The DNA samples were made into a final concentration of 10ng/µl with TE buffer and stored at -80°C in a deep freezer for further RAPD analysis.

RAPD analysis

Polymerase Chain Reactions (PCR) were performed in a Corbett Palm-Cycler™, High Performance Thermal Cycler (Corbett Research, Australia). Each 12.5 µl reaction mixture received 10X PCR reaction buffer (10mM Tris-HCl pH 8.8, 50 mM KCl, and 0.1% gelatin), 100 mM dNTPs, 2.5mM $MgCl_2$, 0.5 U Taq DNA Polymerase (Genei, Bangalore, India) and 0.5 µM oligonucleotide primer (Operon Technologies Inc. Alameda, California, USA) and 25 ng of template DNA. The control received equal quantity of distilled water in place of genomic DNA. Forty random decamer primers were tested for RAPD analysis. The conditions for the PCR reactions were standardized as follows; initial denaturation at 94°C for 5 min and a 40 cycles of denaturation at 94°C for 1 min, annealing at 37°C for 1 min and extension at 72°C for 1 min., followed by final extension at 72°C for 5 minutes. The PCR products were separated on 1% (w/v) agarose gel in 0.5X TAE buffer and stained with ethidium bromide and documented by using gel documentation system (Alpha Innotech Corporation, U.S.A) under UV light. The reactions were repeated thrice to ensure reproducibility of banding pattern. The bands were scored as present and absent to compare the donor plants and tissue culture raised plants.

Data recording and statistical analyses

Tissue culture experiments contained a set of 10 cultures and were repeated at least thrice. The experiments were conducted in a completely randomized design in the culture room. Observations were recorded at a periodic interval of 21 days for initiation and multiplication experiments. However, cultures for the conservation experiments were kept in respective media until shoot regeneration as well as the growth of the cultures ceases and the cultures show the symptoms of degeneration. Cultures containing a minimum of 3-4 green shoots were considered as surviving and the survival percentage was recorded. Means and standard deviations were determined and were subjected to analysis of variance (ANOVA). The differences among the treatment means were tested using Duncan's multiple range test (DMRT) (Gomez and Gomez 1984) at a 1% probability level ($P<0.01$).

RESULTS

Initiation of cultures

The explants treated with antioxidant solution remained green and responded faster. However, explants kept in distilled water, showed necrosis within three days in culture. Nodal explants treated with antioxidants

produced 15-20 shoots within 14 days of culture in MS medium containing BA 4.4 µM. Lower concentrations of BA (1.1 and 2.2 µM) induced 6-8 shoots from the nodal explants. Increased concentration of BA (11.0 µM), induced green rounded structures from the nodal regions and numerous small shoots emerged from this structures within 20-25 day of culture (Table-1).

Table-1.Shoot regeneration from antioxidant treated explants of *B.monnieri* ; number of shoots per culture on cytokinins supplemented MS media.

Growth regulator		Nodal segments	Internode	Leaf
BA (µM)	1.1	6.2[d]	2.8[d]	0
	2.2	8.1[c]	4.2[c]	11.4[c]
	4.4	18[b]	24.0[b]	54.2[b]
	11.0	25[a]	28.0[a]	72.0[a]
Kinetin (µM)	1.15	1.8[b]	2.1[b]	5.0[b]
	2.30	2.1[a]	3.0[a]	4.8[a]
	4.60	0	0	0
	11.50	0	0	0

Data collected after 14 d. of culture. Values superscripted with different letters in the same column are significantly different at 1% level using DMRT. F-values are highly significant.

However, the nodal explants without antioxidant treatment kept in all the concentrations of BA produced two or three shoots, irrespective of the hormone concentration. Kinetin at lower concentrations (1.15 and 2.30 µM) induced one or two shoots from the antioxidant treated nodal explants, at higher concentrations (4.6 and 11.5 µM) induced green hard callus from the basal portion of the explant. Non-treated explants cultured in all the concentrations of kinetin did not evoke any morphogenic response and the explants dried after 12-14 days in culture.

Antioxidant treated internode segments bulged at the ends within 6 days of culture on MS medium containing all tested concentrations of BA, however, their shoot regeneration capacity varied with concentration of growth regulator. BA, 4.4 µM initiated 20-25 shoots within 14 day of culture (Fig-1a). In lower concentrations of BA (1.1 and 2.2 µM) the number of shoots induced were less, however, their length varied. In higher concentrations of BA (11.0 µM) the bulged internodal regions produced small rounded structures and this structures initially produced small leaves and elongated into numerous shoots. Irrespective of the explant source and treatment, kinetin at lower concentrations (1.1 and 2.2 µM) induced 2-3 small shoots from

the antioxidant treated internode explants, and at higher concentrations (4.4 and 11.5 µM) induced callus with differential intensity. Untreated internode explants did not respond to kinetin treatment.

Fig-1.Stages of micropropagation, *in vitro* conservation and hardening of *B. monnieri*.

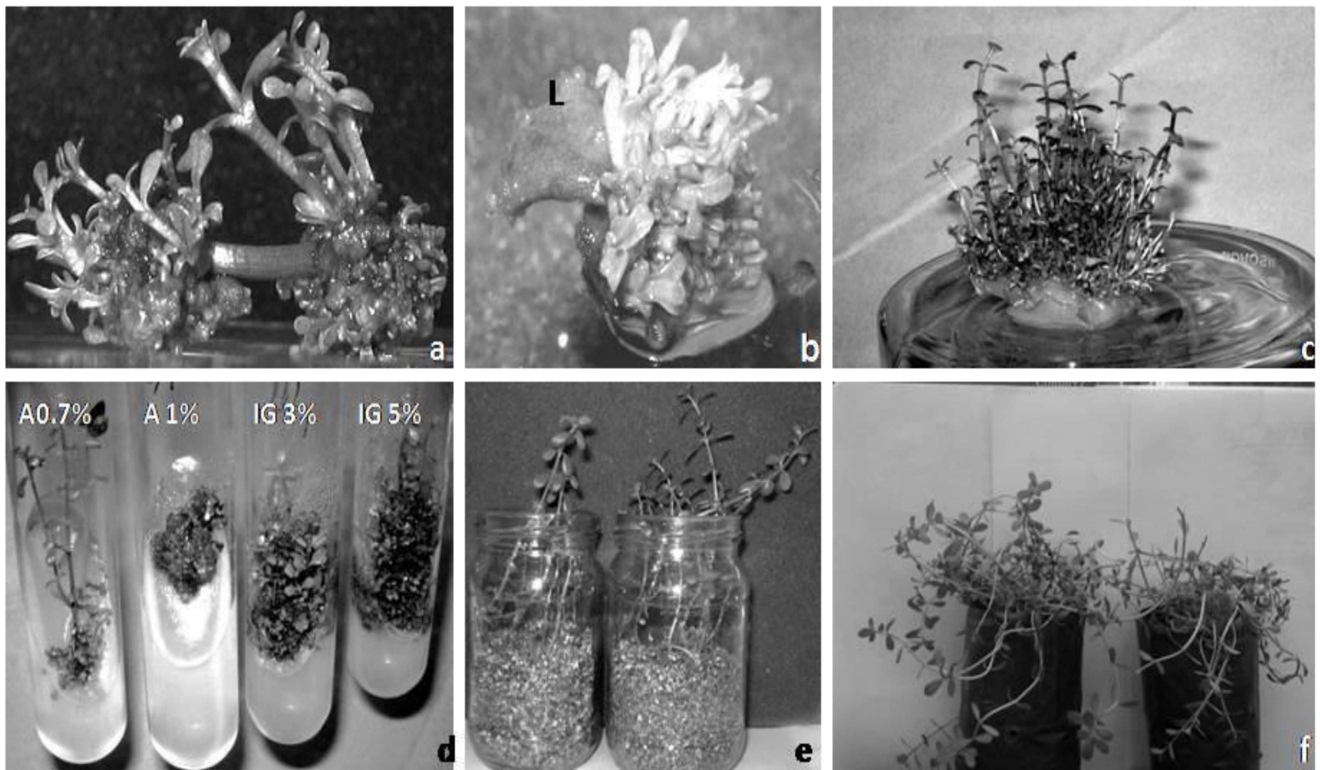


Fig.1-a. Initiation of shoots from the intermodal segments of *B. monnieri* on MS medium containing 4.4 µM BA

Fig.1-b. Shoot initiation from the leaf explants of *B.monneiri* on MS medium containing 4.4 µM BA

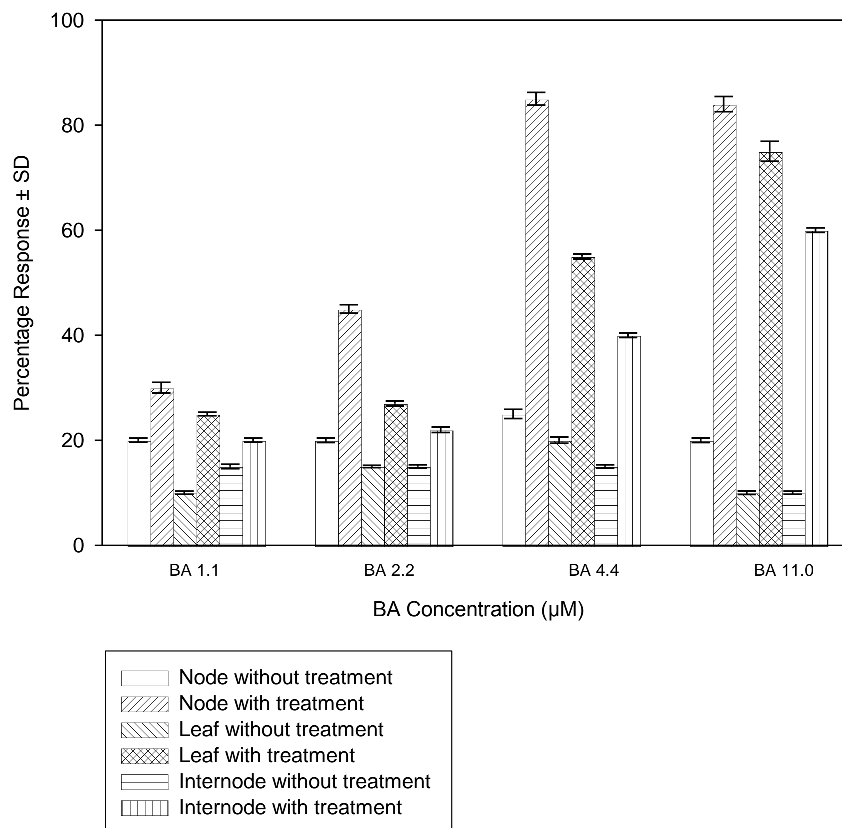
Fig.1-c.Shoot clusters of *B.monneiri* developed on MS medium containing 2.2 µM BA

Fig.1-d. Conservation of *B.monneiri* cultures in MS medium containing 2.2 µM BA + agar 0.7%, 1% and Isabgol 3% and 5%.

Fig.1-e &f. Rooting, hardening and pot transfer of tissue culture raised plants *of B. monneiri*

Leaf segments treated with antioxidants, cultured in MS medium containing 4.4 µM BA with adaxial surface facing the medium showed the first symptom of initiation with bulged cut ends, which developed into 50-60 small shoots from all over the surface within 21 days of culture (Fig.1-b). Lower concentrations of BA

Fig.2 Effects of antioxidant treatment on explant response in *B.monnieri* cultured in MS medium containing different concentrations of BA



(1.1µM) did not evoke any response as the leaf segments dried within 21days of culture. BA, 2.2 µM induced 10-12 shoots from the edges of the leaves in contact with the medium. Higher concentration of BA (11.0 µM) induced large number of small shoots from all over the surface of the explant, however, these shoots were clubbed together and were very difficult to isolate. Antioxidant treated leaf segments cultured in MS medium containing kinetin (1.15 and 2.3 µM) produced 4-5 shoots from the edges of the leaves, however, at higher concentrations (4.6 and 11.5 µM) the explants bulged from the bases and induced green friable callus from the whole surface of the explant. The percentage response of all the explants treated with antioxidant solution in different concentrations of BA was better than untreated explants (Fig.2).

Multiplication of cultures and in vitro conservation

The initiated shoots were multiplied in MS medium containing BA (2.2 µM) along with 50 mg/l ascorbic acid and 25 mg/l citric acid. The cultures initiated 70-80 shoots from a single explant and all the shoots were of uniform length and were green in colour (Fig-1c). This medium supported the growth of shoot clumps for a period of 140-150 days (Fig-3).

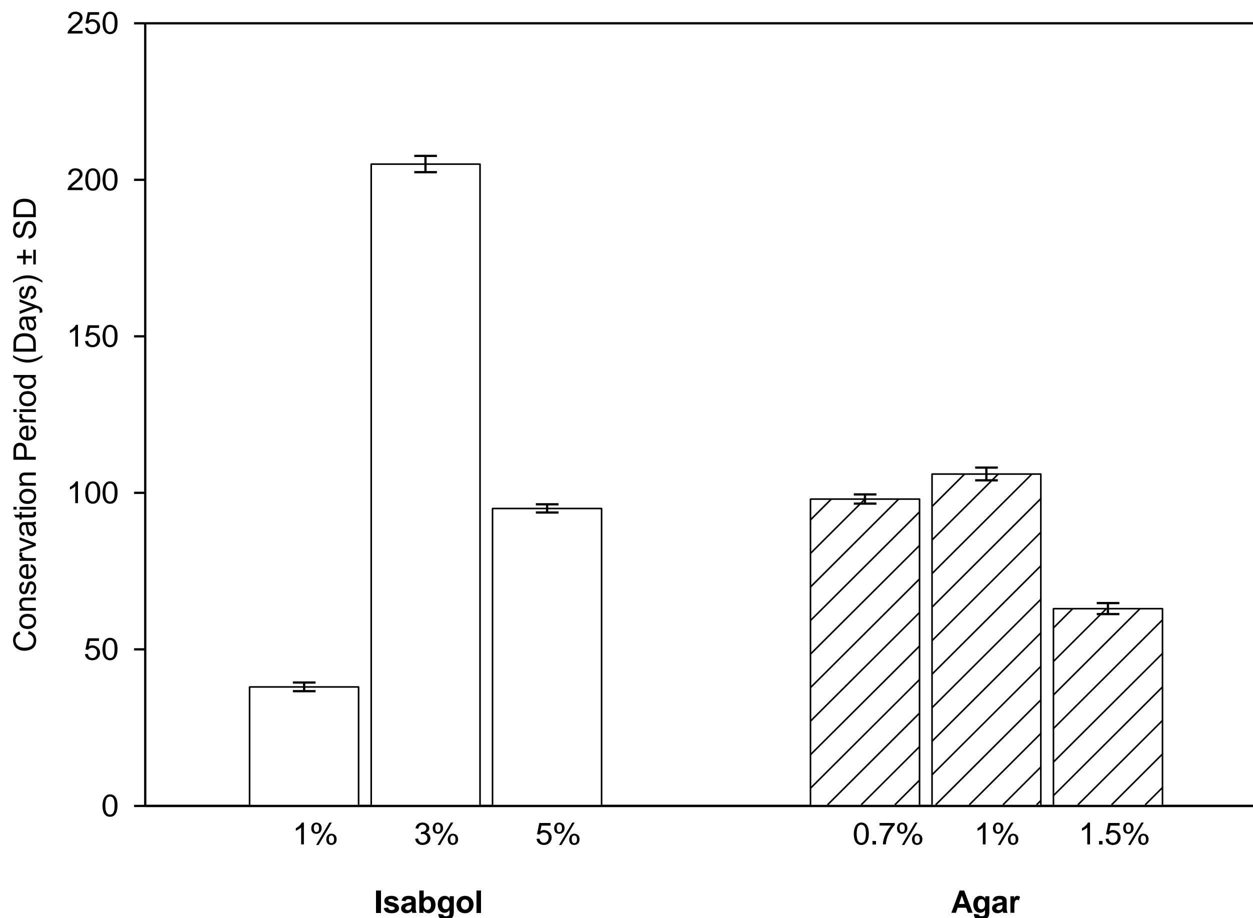
The addition of ascorbic acid (25 mg/l) alone into the medium containing 2.2 µM BA did not augment shoot multiplication, however, the subcultured shoots produced green rounded bodies from the surface and remained as such for a period of 25-30 days and dried out. Ascorbic acid (50 mg/l) along with BA 2.2 µM induced friable callus along with green shoot tips, which did not regenerate into complete plantlets. Higher concentrations of ascorbic acid (75 and 100 mg/l), induced only callus, which remained green and no regeneration was observed.

Citric acid (10 and 25 mg/l) along with MS medium containing 2.2 µM BA produced 2-3 shoots and produced large number of hairy roots from the shoot above the medium, however, these shoots showed yellowing after 25 days of culture. Citric acid (50 mg/l) in the same medium produced 8-10 small shoots and green hard callus induced from the base of the shoots. These cultures remained healthy for a period of 40-45 days in culture. Higher concentrations of citric acid (100 mg/l), developed small shoots along with callus from the surface of the explant.

Combination of ascorbic acid and citric acid provided better results than used independently along with BA 2.2 µM in MS medium. Ascorbic acid (25 mg/l) along with different concentrations of citric acid (10, 25, 50 and 100 mg/l) showed no advantageous results in shoot bud induction and most of the shoots cultured to these media induced callus which turned brown. However, higher concentration of ascorbic acid (50 mg/l) along with citric acid (10 mg/l) produced 4-5 shoots from the nodal region; however, these shoots were not healthy. Higher concentrations of citric acid (50 and 100 mg/l) produced green rounded structures from all over the surface of the explants and these structures turned brown after 35-40 days without developing into complete shoots. Higher concentrations of ascorbic acid (75 and 100 mg/l) along with all the concentrations of citric acid did not show any advantageous effects on shoot regeneration from the explants, and they showed common response of developing green compact callus initially from the cut ends and later from the whole surface of the explant.

Both gelling agents supported multiplication and further maintenance of cultures, however, their survival rates varied. The concentrations of agar tested did not influence the conservation period. Agar (0.7% w/v) along with BA (2.2 µM), ascorbic acid (50 mg/l) and citric acid (25 mg/l), the cultures could survive for 90-100 days. Agar (1% w/v) along with the same concentrations of additives supported multiplication of cultures, and 10-12 shoots originated from a single explant. These cultures survived for a period of 100-110 days. In higher concentration of agar (1.5% w/v) the multiplication rate was reduced and the shoots showed tip burning. Isabgol (1% w/v) used as gelling agent along with BA (2.2 µM), ascorbic acid, 50 mg/l, citric acid, 25 mg/l produced 15-18 shoots. However, these shoots developed glassiness within 20-25 days of culture, and decayed. Cultures kept in higher concentrations of isabgol (3.0% w/v) multiplied profusely, produced a

Fig.4 Effects of different gelling agents on conservation period of *B.monnieri*



maximum of 60-70 shoots, with a high survival rate of 50-52% for a period of 180-210 days and was found to be ideal for

the

multiplication and conservation of cultures (Fig-4).

In media containing increased concentrations of isabgol (5.0% w/v), the shoots multiplied, however, they were very small and showed yellowing (Fig-1d). The cultures survived in this medium for a period of 90-100days.

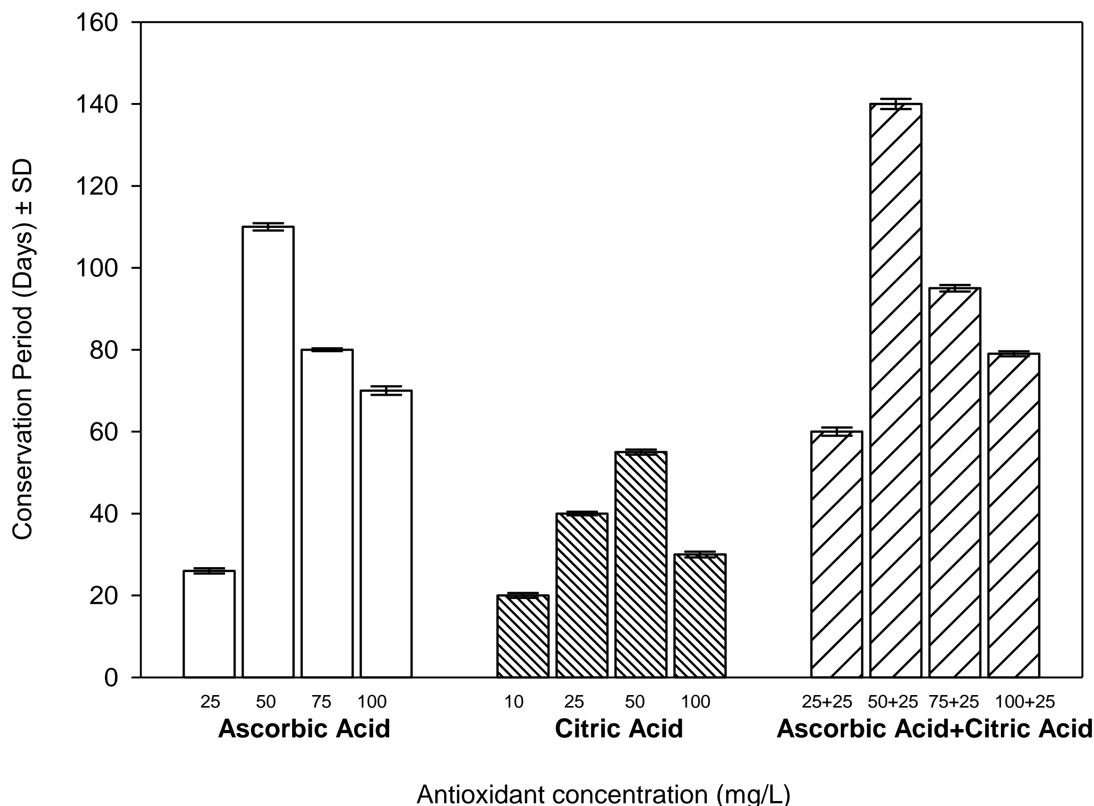
Viability of in vitro conserved plantlets

Nodal and apical potion of the *in vitro* conserved shoots readily regenerated into multiple shoots upon subculturing to fresh multiplication medium. The multiplication frequency of the shoots observed to be the same as the initial stages. These shoots initiated roots when transferred to rooting mixture proving that the shoots did not loose their regenerative potential after conserved in the media for longer durations.

Ex vitro rooting and hardening of plantlets

All the shoots kept for *ex vitro* rooting produced roots within a period of 14-20 days. All the plantlets transferred to the field survived the hardening process (Fig-1e and f).

Fig.3 Effects of different concentrations of antioxidants on conservation period of *B.monnieri*



Genetic stability assessment

Good quality DNA was isolated from the leaves of source plants and *in vitro* raised plants. Out of 40 primers used 25 primers gave good amplification and were used for further analyses. The cultures conserved in different concentrations of agar and isabgol produced a total of 125 reproducible bands providing an average

of 5 bands/primer, ranging in molecular weight between 0.5- 2.5 Kb (Fig-5a). In the case of cultures conserved in ascorbic acid (25,50,75 and 100 mg/l) and citric acid (25 mg/l) the RAPD profile provided a total of 140 bands; an average of 5.6 bands/primer (Fig-5b). In the case of RAPD profile of control plants compared with shoots regenerated from nodal, internodal and leaf explants cultured and maintained in BA (2.2 µM), ascorbic acid, 50 mg/l and citric acid, 25 mg/l produced a total of 155 bands for all the primers used, an average of 6.2 bands/ primer (Fig-5c). DNA used from all the different experimental conditions, did not show any variation from the mother plants in the RAPD profiles, showing that no variation occurred in the genetic constitution of the tissue culture raised plants and the plants conserved *in vitro.*

Fig.5- Representative RAPD profile from plants conserved in different concentrations of gelling agents, antioxidants and regenerated from different explants

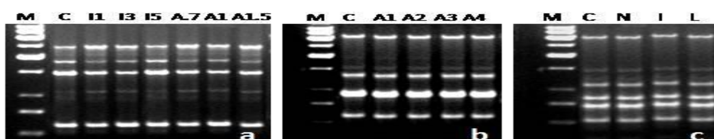


Fig.5-a. Representative RAPD profile with OPA4 Primer comparing plants cultured in different concentrations of isabgol (1%, 3% and 5%) in lanes I1, I3 and I5 and agar (0.7, 1.0 and 1.5%) in lanes A.7, A1 and A1.5. M represent the one–kb DNA ladder and C the control plant.

Fig.5-b. Representative RAPD profile with OPB12 Primer for plants grown in different concentrations of ascorbic and citric acid. Lanes A1, A2, A3 and A4 represent ascorbic acid (25, 50, 75 and 100mg/l respectively) along with citric acid (25 mg/l). M represent the one–kb DNA ladder and C the control plant.

Fig. 5-c. Representative RAPD profile with OPI 10 primer for nodal (N), internodal (I) and leaf (L) explants cultured in BA 4.4 µM BA. M represent the one–kb DNA ladder and C the control plant.

DISCUSSION

The response of nodal segments, leaves and internodal segments on shoot regeneration and conservation in different additives and gelling agents was studied. The effects of cytokinins on shoot bud induction from various explants are well documented for *B. monnieri* (Tiwari et al. 2001). The type of explants and the cytokinins used for multiplication influenced the regeneration potential of the explants in tissue culture of different plant species (Martin 2005; Bhattacharyya and Bhattacharyya 2001). However, all the explants regenerated into shoots, the capacity to develop into complete plantlet influenced by the exposure to hormones. Antioxidant treated explants responded better as compared to non-treated ones. The effectiveness of ascorbic acid and citric acid on protecting the plant cells from oxidative activity of phenolic substances were well-documented (Arya and Shekhawat 1986). These properties of the antioxidants may be helping the cultures to survive for more culture period, thereby enhancing the culture age. The gelling agents played an important role in the establishment of cultures and prolonging the culture age. The shoots cultured in medium containing isabgol, produced greener shoots as compared to plants grown in agar gelled medium. This may be due to the high availability of free water in the medium gelled with isabgol and upon hydrolysis during autoclaving of media, the breakdown products of isabgol may be playing a metabolic role in promoting the survival of cultures for longer period. The conservation period of cultures kept in isabgol containing media was more as the availability of nutrients and minerals to the plants were more as compared to agar containing media. The increased culture age has a positive effect on reducing the cost of tissue culture as the routine practices of preparation of media, subculturing and incidences of developing genetic chimeras can be avoided (Murrata 1989). The efficacy of isabgol as a gelling agent is due to the presence of mucilaginous husk in seeds. Mechanical milling or grinding of the outer layer of the seed yields approximately 25% by weight of mucilage. The grinded seed husk is a white fibrous material, which is hydrophilic. Chemical components reported are about 22.6% arabinose and 74.6% xylose (Fischer et al. 2004). Xylose and arabinose supported the induction and establishment of shoots and flowers *in vitro* in tobacco (Eberhard et al. 1989).

Ex vitro rooting of tissue culture raised plants has advantages over *in vitro* rooted plants. From the perspective of survival rate, the *ex vitro* rooted plants have a higher survival rate on field transfer compared to *in vitro* rooted plants (Paspisilova et al. 1997). *Ex vitro* rooting reduces the cost of tissue culture raised plants by one step as the media cost and labor cost before and after rooting the plantlets also can be avoided.

Genetic stability of plants developed from tissue cultures is a major concern. Molecular marker aided detection of genetic variations by using RAPD is most common due to its simplicity and cost effectiveness

(Martins et al. 2004). RAPD markers are used successfully in order to identify the genetic variability of *Bacopa* germplasm (Darokar et al. 2001). RAPD studies on cultures of *B. monnieri* regenerated in different gelling agents and antioxidants did not show any variation in the molecular profile for the primers tested. The plantlets developed through forced axillary branching without an intervening callus phase, mostly does not produce genetic variations as recorded for other species (Feyissa et al. 2007).

The results discussed in this paper provide an efficient protocol for *in vitro* regeneration and conservation of *B. monnieri* from different explants and use of different gelling agents for the conservation of germplasm in vitro and provide an insight on the genetic stability of cultures.

REFERENCES

1. Anthony JM, Senaratna T, Dixon KW, Sivasithamparam K (2004) The Role of Antioxidants for Initiation of Somatic Embryos with *Conostephium pendulum* (Ericaceae) Plant Cell Tissue Org. Cult. 78:247-252. doi: 10.1023/B: TICU .0000 025661 .56250.b4
2. Arya HC, Shekhawat NS (1986) Clonal multiplication of tree species in Thar desert through tissue culture. Forest Ecol. and management. 16:201-208. doi:10.1016/0378-1127(86)90020-4
3. Bhattacharyya R, Bhattacharyya S (2001) High frequency *in vitro* propagation of *Phyllanthus amarus* Schum.&Thom. by shoot tip culture. Indian J. Exp. Biol. 39:1184-1187.
4. Chaudhuri RK, Pal A, Jha TB (2007) Production of genetically uniform plants from nodal explants of *Swertia chirata* Buch.-Ham.ex Wall.-an endangered medicinal herb. In Vitro Cell Dev. Biol.-Plant 43:467-472.doi:10.1007/s 11627-007-9095-9.
5. Darokar MP, Khanuja SPS, Shasany AK, Kumar S (2001) Low levels of genetic diversity detected by RAPD analysis in geographically distinct accessions of *Bacopa monnieri.* Genetic Resources and Crop Evolution 48: 6, 555-558. doi:10.1023/A:1013800101604
6. Debergh PC (1983) Effects of agar brand and concentration on tissue culture medium. *Physiol.Plant*. 59:270-276. doi:10.1111/j.1399-3054.1983.tb00770.x
7. Dellaporta SL, Wood VP, Hicks JB (1983) A plant DNA mini-preparation: version II. Plant Mol. Biol. Reptr. 1: 19-21. doi:10.1007/BF02712670

8. Deepak M, Amit A (2004) The need for establishing identities of 'bacoside A and B', the putative major bioactive saponins of Indian medicinal plant *Bacopa monnieri*. Phytomedicine.11: 264-268. doi:10.1078/0944-7113-00351
9. Eberhard S, Doubrava N, Marfa V, Mohnen D, Southwick A , Darvill A, Albersheim P (1989) Pectic cell wall fragments regulate tobacco thin-cell-layer explant morphogenesis. Plant Cell. 1: 747-755 doi:10.1105/tpc.1.8.747
10. Feyissa T, Welander M, Negarsh L (2007) Genetic stability, *ex vitro* rooting and gene expression studies in *Hagenia abyssinica*. Biol. Plant. 51: 15-21. doi: 10.1007/s10535-007-0004-1
11. Fischer MH, Yu N, Gary GR, Ralph J, Anderson L, Marlett JA (2004) The gel forming polysaccharide of psyllium husk (*Plantago ovata* Forsk). Carbohydr. Res. 339: 2009-2017 doi:10.1016/j.carres.2004.05.023
12. Gomez KA, Gomez AA (1984) Statistical procedures for Agriculture Research. John Willey & Sons, New York.
13. Jain R, Babbar SB (2005) Guar gum and Isabgol as cost effective alternative gelling agents for *in vitro* multiplication of an orchid *Dendrobium chrysotoxum*. Curr. Sci. 88:292-295.
14. Mandal BB, Tyagi RK, Pandey R, Sharma N, Agrawal A (2000) *In vitro* conservation of germplasm of agri-horticultural crops at NBPGR: an overview. In: Razdan MK, Cocking EC(eds.) Conservation of plant genetic resources *in vitro.* Vol.2: Applications and Limitations. Science Publishers Inc.USA. 297-307.
15. Martin KP (2005) Organogenesis on root, leaf, stem-disc and scape explants of *Eryngium foetidum* L., a rare medicinal plant. J. Herbs, Spices and Medicinal Plants. 11 (4): 9-17. doi:10.1300/J044v11n04_02.
16. Martins M, Sarmento D, Oliveira MM (2004) Genetic stability of micropropagated almond plants as assessed by RAPD and ISSR markers. Plant Cell Rep. 23:492-496. doi: 10.1007/s00299-004-0870-3
17. Murrata M (1989) Effects of auxin and cytokinin on the induction of sister chromatid exchange in cultured cells of wheat (*Triticum aestivum* L.). Theor. Appl. Genet. 78:521-524. doi:10.1007/BF00290836
18. Murashige T, Skoog F (1962). A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol.Plant.15:473-497. doi: 10.1111/j.1399-3054.1962.tb08052.x

19. Pandey NK, Tewari KC, Tewari RN, Joshi GC, Pande VN, Pandey G (1993) Medicinal plants of Kumaon Himalaya, strategies for conservation. In: Dhar U (ed.) Himalayan biodiversity conservation strategies. No.3. (pp.293-302). Himavikas Publication, Nainital, India.
20. Paspisilova J, Catsky J, Sestak Z (1997) Photosynthesis in plants cultivated in vitro. In: Pessarakli, M (ed.) . pp. 525-540. Marcel Dekker, New York-Basel-Hong Kong.
21. Tiwari V, Tiwari KN, Singh BD (2001) Comparative studies of cytokinins on *in vitro* propagation of *Bacopa monniera*. Plant Cell Tissue Org. Cult.66:9-16. doi: 10.1023/A:1010652006417
22. Tyagi RK, Agrawal A, Mahalakhsmi C, Hussain Z, Tyagi H (2007) Low-cost media for *in vitro* conservation of turmeric (*Curcuma longa* L.) and genetic stability assessment using RAPD markers. In vitro Cell. Dev. Biol.-Plant. 43:51-58.doi 10.1007/s11627-006-9000-y.
23. Yusuf A (2005) Clonal propagation of *Anogeissus sericea* var. *nummularia*- A tree of arid forestry. J. Sustainable Forestry 20:67-78.doi 10.1300/ J091v 20n 01_05.