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Multiple shoot regeneration and tissue culture studies on *Bacopa monnieri* (L.) Pennell

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Abstract Adventitious shoot buds were induced from leaf and stem explants of *Bacopa monnieri* on Murashige and Skoog medium supplemented with benzyladenine or kinetin. The source of the explants as well as different gelling agents in the medium were found to influence shoot induction and eventual shoot growth. The best response was obtained in leaf explants taken from shoot cultures grown in medium supplemented with 2 μ*M* benzyladenine and gelled with 0.2% gelrite. A transverse section of the leaf explant incubated in this medium showed several shoot primordia emerging from the leaf surface. This system exhibited a potential for repeated harvesting of the shoots from the original leaf explant as the latter continued to expand and regenerate new shoots, upon repeated periodical subculturing onto fresh medium. However, the callusing response of the plant was very low. Qualitative TLC studies of the regenerated shoots revealed a phytochemical profile similar to that of the field grown-plants.

Key words Callus · Explant source · Multiple shoot-bud induction · Gelrite · Shoot cultures

Abbreviations *BA* Benzyladenine · *2,4-D* 2,4-dichlorophenoxyacetic acid · *IAA* indole-3-acetic acid · *KN* kinetin · *NAA* α-naphthaleneacetic acid · *RM* regenerative medium

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Introduction

Bacopa monnieri (L.) Pennell (Scrophulariaceae), popularly known as 'Brahmi' in India, is one of the sources of the *medhya rasayan* drugs (that counteract stress and improve intelligence and memory) of Ayurveda. It is prescribed for a variety of therapeutic indications including epilepsy, insanity and memory enhancement (Satyavati et al. 1976). The drug is included in several Ayurvedic formulations such as *Brahmighrtam* and *Sarasvataristam* (Anonymous 1 1978; Sivarajan and Balachandran 1994) and found to be effective in cases of anxiety neurosis (Singh et al. 1979). In a recent study conducted on Indian medicinal plants (Anonymous 2 1997), *B. monnieri* was placed second in a priority list of the most important Indian medicinal plants evaluated on the basis of their medicinal importance, commercial value and potential for further research and development.

B. monnieri is a weak, creeping and somewhat succulent herb (Fig. 1) that can be found in damp and marshy areas throughout India. The principle active factors that have been reported are two saponins, bacoside A and bacoside B (Chatterji et al. 1963). Bacoside A yields bacogenins A_1, A_2, A_3 and A_4 upon hydrolysis (Chatterji et al. 1965). The other chemical constituents of the plant include bacoside A_1, hersaponin, betulinic acid, stigmasterol, β-sitosterol and stigmastenol (Chatterji et al. 1963; Jain and Kulshreshtha 1993).

With an increasing world-wide demand for plant-derived medicines and formulations (Parnham 1996), there has been a concomitant increase in the demand for raw material. Hence, there is a need to develop approaches for ensuring the availability of raw material of a consistent quality from regular and viable sources.

During the investigation presented here, we developed a tissue culture system for adventitious shoot-bud induction in *B. monnieri*. We also established suitable conditions for multiple shoot-bud regeneration and shoot culture, which have the potential of providing a

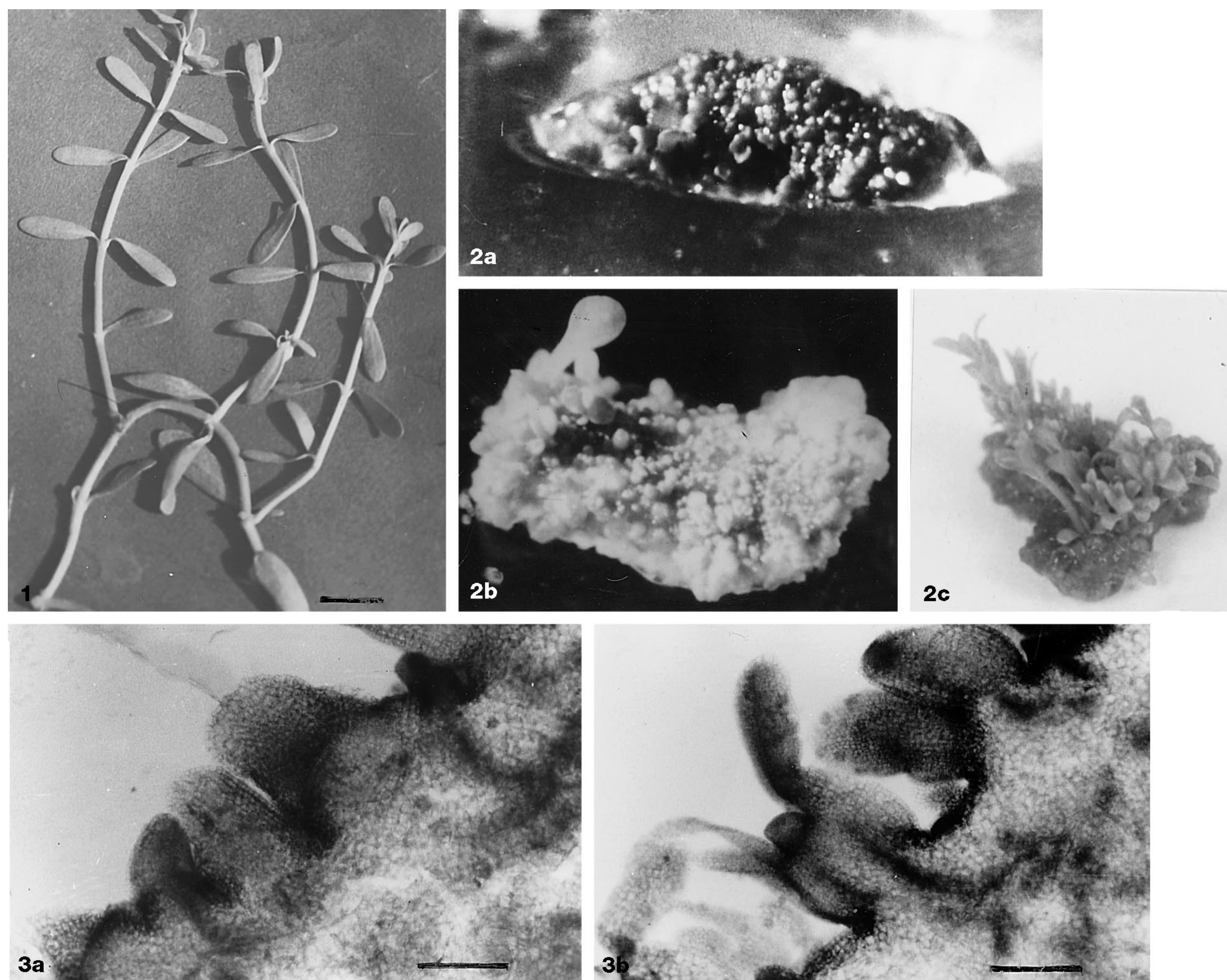

Fig. 1 A twig of a field-grown *B. monnieri* plant. *Bar*: 1 cm

Fig. 2a–c Shoot buds regenerating in RM from stem explant after 4 weeks of incubation (**a**), from leaf explant after 3 weeks of incubation (**b**) and from leaf explant after 4 weeks of incubation (**c**)

Fig. 3a,b Transverse section of leaf explant showing shoot primordia in various stages of development after 2 weeks of incubation in RM. *Bar*: 10 μm

source for a continuous supply of shoots at periodic intervals.

Materials and methods

The present tissue culture study included the following experiments:

1) adventitious shoot-bud induction from leaf and stem explants from field-grown plants
2) the influence of the source of leaf explants, i.e. from field-grown plants and from in vitro shoot cultures, on shoot regeneration
3) the influence of gelling agents on shoot regeneration
4) callus induction from leaf and stem explants

For experiments 1 and 2 explant material was obtained from *B. monnieri* plants growing in the medicinal plant garden on campus. New healthy branches were collected from the field-grown plants in the morning. These were surface-sterilized with 0.1% mercuric chloride (w/v) for 2 min and rinsed thoroughly with sterile distilled water. Leaves about 0.75 cm^2 in area and internodal portions of the stems about 1.5 cm in length were used as explant material. Leaves and stems between the second and sixth nodes from the apex were taken to avoid the selection of very old and very young explants. For experiments 2, 3 and 4, explants were excised aseptically from 5- to 6-week-old shoot cultures obtained in experiment 1.

MS (Murashige and Skoog 1962) basal medium supplemented with 30 $g \cdot l^{-1}$ sucrose and 0.7% agar (Bacteriological, Qualigens, India) or 0.2% gelrite (Kelco, USA) was used for all experiments. The medium was supplemented with different concentrations of plant growth regulators: BA (1, 2, 4 and 6 μ*M*), KN (1, 2, 4 and 6 μ*M*), IAA (2, 4, 6 and 8 μ*M*), NAA (2, 4, 6 and 8 μ*M*), 2,4-D (2, 4, 6 and 8 μ*M*), singly or in combination. The pH of the medium was adjusted to 5.7 prior to the addition of the gelling agent. Aliquots (25 ml) of the medium were distributed in 100-ml Erlenmeyer flasks and autoclaved at 121 °C for 20 min. The cultures were incubated at 26 ± 0.2 °C, either under cool-white fluorescent light for 16 h for shoot regeneration or in the dark for callus initiation. Leaf explants were placed with their adaxial surface in contact with the medium, and stem explants were placed horizontally in the medium.

For experiments 2 and 3, the shoots along with the continuously regenerating base were taken out of the flask after 6 weeks of incubation and the fresh weight was recorded. The sample was

then dried at 50 °C in a hot-air oven to a constant weight and the dry weight was recorded.

Each treatment consisted of six replicates with one explant per flask. Experiments 1 and 3 were repeated three times, and experiments 2 and 4 were repeated twice. The values were recorded as means ± standard deviation. For experiment 1, the area covered by the profusely regenerating shoot buds was scored arbitrarily as seen by the naked eye and given in terms of percentage area of the explant covered by shoot buds. The effect of different treatments on multiple shoot-bud induction was compared in a two-way analysis of variance (ANOVA) and Tukey's multiple range test ($\alpha=0.05$). Results of experiments 2 and 3 were analysed by Student's *t*-test ($\alpha=0.05$).

For preliminary qualitative phytochemical evaluation by thin-layer chromatography (TLC), shoots from both the field-grown plants and the 5- to 6-week-old shoot cultures were dried in an oven at 55 °C and passed through a 40-mesh sieve. The powder that resulted was extracted in 50% methanol for 24 h; the extract was concentrated under vacuum and then evaporated to dryness. The residue was dissolved in a solvent system of ethylacetate:methanol:water (16:2.5:1.6), spotted on precoated silica gel G TLC plates (E. Merck) and developed in the same solvent system. The spots were visualized by spraying the plates with anisaldehyde reagent (Wagner et al. 1984).

Results and discussion

Induction of adventitious shoot buds

Multiple shoot buds, without an intervening callus phase, were induced at the cut ends of stem and leaf explants (Fig. 2a-c). The emergence of the shoot buds was observed visually on the 9th day of incubation in all replicates, in media containing BA/KN (1, 2, 4 and 6 μ*M*). After a period of 3–4 weeks a thick mat of shoot buds spread over 90–100% of the explant surface (Table 1) in the presence of 2 μ*M* BA (in both stem and leaf explants) and in the presence of 4 μ*M* KN (in stem explants). Each explant was transformed into a dense mass of profusely regenerating shoot buds which made it impossible to count the number of shoot buds per unit area. There was an increase in the extent of shoot regeneration with an increase in the concentration of BA from 1 to 2 μ*M* and KN from 1 to 4 μ*M* from the 2nd week of incubation onwards. However, a further increase in the levels of either BA or KN resulted in a decrease in the extent of shoot regeneration. Further, the shoot induction response of the leaf explant was qualitatively better than that of the stem explants at all concentrations of BA tested, while the reverse was true at all the concentrations of KN tested. It was observed that there was no difference in shoot-bud induction response between the media gelled with gelrite and agar.

Among the levels of KN and BA tested the best shoot-bud induction response, as represented by the rapid regeneration of shoot buds over at least 80% of the explant surface area was obtained with leaf explants in medium containing 2 μ*M* BA (hereafter, regenerative medium, RM) after a 2-week incubation. A transverse section of the leaf explant, after 2 weeks of incubation in RM showed several shoot primordia at different stages of growth arising from the surface of the leaf (Fig. 3a,b). Subsequent experiments on shoot-bud induction were then restricted to leaf explants.

Influence of source of leaf explant on shoot bud regeneration

Multiple shoot regeneration response of leaf explants from field-grown plants (LF) and from shoot cultures (LSC) was tested on the RM. The emergence of shoot buds was observed on the 9th day of incubation in both

Table 1 Effect of cytokinins on multiple shoot-bud initiation from leaf and stem explants of *B. monnieri*

KN/BA (μ*M*)	Explant	Shoot-bud initiation (percentage of the surface area of the explant covered with shoot buds)							
		After a 3-week incubation				After a 4-week incubation			
		KN		BA		KN		BA	
		A[a]	G[a]	A	G	A	G	A	G
1	Stem	51.1 ±4.71 j[b]	50.5 ±4.16 j	19.4 ±5.11 t	24.7 ±2.69 s	60.5 ±4.16 i	69.4 ±3.79 h	30.0 ±2.97 q,r,s	30.8 ±3.53 p,q,r
2	Stem	71.1 ±4.39 f,g,h	70.5 ±4.17 f	89.7 ±4.36 b	95.2 ±2.08 a	80.0 ±2.97 c,d	81.1 ±4.71 c	99.1 ±2.57 a	99.4 ±1.61 a
4	Stem	81.3 ±4.13 c	90.0 ±4.85 b	49.4 ±4.16 j,k	50.5 ±4.16 j	99.1 ±2.57 a	99.4 ±1.61 a	50.5 ±4.16 j	51.1 ±4.71 j
6	Stem	30.5 ±3.79 p,q,r	25.0 ±2.97 s	19.7 ±3.19 s,t	24.7 ±3.19 s	51.1 ±4.71 j	50.5 ±4.16 j	34.7 ±2.08 o,p,q	39.4 ±4.16 n,o
1	Leaf	41.1 ±4.71 l,n	46.1 ±2.13 j,k,l	39.4 ±4.16 n,o	35.2 ±3.19 o,p	40.5 ±4.16 m,n	44.7 ±3.62 k,l,m	41.1 ±4.71 l,n	40.5 ±4.16 m,n
2	Leaf	61.6 ±4.85 i	61.6 ±4.85 i	99.7 ±1.17 a	100.0 ±0.00 a	74.7 ±4.01 e,f,g	75.5 ±1.61 d,e,f	100.0 ±0.00 a	100.0 ±0.00 a
4	Leaf	70.5 ±4.16 f,h	70.0 ±4.85 g,h	69.7 ±4. 01 g,h	60.0 ±4.85 i	79.7 ±3.19 c,d,e	80.5 ±3.79 c,d	69.4 ±4.16 h	70.5 ±4.16 f,h
6	Leaf	28.3 ±3.42 r,s	30.0 ±2.97 q,r	40.5 ±4. 16 m,n	30.0 ±2.97 q	49.4 ±4.16 j,k	50.5 ±4.16 j	49.4 ±4.16 j	51.1 ±4.71 j

[a] *A*, Agar (0.7%); *G*, gelrite (0.2%)

[b] Means followed by same letter do not differ significantly at $\alpha=0.05$ ($n=18$)

the explants. However, the fresh and dry weights of the shoots regenerated from LSC (7.02 g ± 0.38, 0.34 g ± 0.015 per explant, respectively) were significantly higher ($P<0.05$) than those from the LF (5.42 g ± 0.35, 0.32 g ± 0.014 per explant, respectively) after 6 weeks of incubation on the same medium. Even though according to statistical analysis the differences turned out to be significant ($P<0.05$), they are not substantial enough, especially the difference in dry weights, from a practical point of view. However, we noticed certain important qualitative differences. After 4 weeks of incubation, LF started turning brown and soft and there was a reduction in the regeneration of new shoots, which finally stopped by the 6th week. However, the LSC remained fresh, firm and continued to differentiate shoot buds even at the end of the 6th week. It thus appears that the LSC have a greater tendency towards shoot regeneration and growth than LF.

Influence of gelling agent on the shoot culture

Since preliminary observations showed marked differences in the appearence of shoots regenerated from leaf explants in the presence of the two gelling agents, agar and gelrite, in the medium, the influence of agar (0.7%) and gelrite (0.2%) on shoot regeneration and shoot growth was further studied in the RM. Although both the time taken for and the extent of shoot-bud regeneration did not differ between the two gelling agents (Table 1), the fresh weight of the shoots regenerated from a single leaf explant after 6 weeks of incubation was significantly higher ($P<0.05$) in gelrite (4.29 g ± 0.49) than in agar (3.85 g ± 0.58), while there was no significant difference in the dry weight (0.218 g ± 0.015, 0.219 g ± 0.023, respectively). Like in earlier experiment, the difference does not appear to be critical from a practical standpoint. However, the shoots growing on media gelled with gelrite were bright green in colour, whereas those on agar appeared pale. These observations could be attributed to the increased water availability in the medium gelled with gelrite (Nairn et al. 1995), as a result of which *B. monnieri*, being a semiaquatic plant, may have shown a better morphological response in terms of the colour and general appearance of the shoots and fresh weight.

We undertook a preliminary experiment on the possibility of repeated periodical harvesting of the shoots from the same regenerating base (of LSC) in RM with gelrite (data not included). As the base continued to expand and regenerate new shoot buds, it was possible to cut it into two halves at the time of every subculture, which gave eight regenerative bases from one original leaf explant by the end of a 4-month incubation (four subcultures). When shoots about 6–8 cm long were harvested by trimming them near the base and the regenerating base transferred to fresh RM, the base continued to expand and regenerate a new crop of shoots which filled the flask and could be harvested again after a 3-week incubation (yield 3–4 g F.W. per flask).

Qualitative TLC studies revealed that the shoots regenerated from leaf explants incubated in RM gelled with gelrite have a phytochemical profile similar to shoots of field-grown plants. The quantification of bacosides and TLC fingerprinting studies of such shoot cultures are underway.

Callus initiation from leaf and stem explants

Observations on the callusing response of stem and leaf explants incubated with varying concentrations of NAA, IAA, and 2,4-D (2, 4, 6 and 8 μ*M*) individually and in combination with BA (1 and 2 μ*M*) are summarized in Table 2. The explants exhibited a tendency to regenerate shoots or roots rather than to form callus.

Lower levels of NAA (2 and 4 μ*M*) induced shoots (1–5 per explant), while higher levels (6 and 8 μ*M*) induced callus and roots in both explants within 2 weeks of initiation of incubation. In combination with BA, NAA induced roots and shoots along with very little callus from the cut ends of both explants.

IAA induced callus at the cut ends of both explants along with 1–5 shoots per explant, while in combination with BA it induced only shoots (1–10 per explant) within 2 weeks of incubation.

2,4-D induced roots (6–15) from both explants at lower concentrations (2 and 4 μ*M*), while higher concentrations (6 and 8 μ*M*) were found to be toxic, with the explants turning brown after 2 weeks of incubation. The addition of 1 μ*M* BA along with 2,4-D induced a thin layer of granular callus after 4 weeks of culture. BA at 2 μ*M* induced rooting (1–10 per explant) along with a thin layer of callus, with 4, 6 and 8 μ*M* of 2,4-D. All the explants eventually turned brown to black at the base after 6 weeks of culture.

These results demonstrated that the three auxins singly or in combination with BA produced very little callus. Callus growth and the regeneration and growth of roots and shoots were very low in the presence of auxins. Subculturing the callus into fresh medium did not improve its growth.

Conclusion

Our results show that cytokinin is required for adventitious shoot-bud induction in *B. monnieri*, as has been reported earlier for *Vitis* species (Clog et al. 1990; Stamp et al. 1990). *B. monnieri* has a high morphogenic potential, and the explants readily responded to cytokinins in the culture medium and formed multiple shoot buds. Of the two cytokinins tried, we found BA to be more suitable than KN as the former resulted in a quicker and better response than the latter. While there are reports of a requirement for high concentrations of

Table 2 Effect of auxins and BA on callus (*C*) initiation from stem and leaf explants of *Bacopa monnieri* after 4 weeks of incubation. The results are represented by arbitrary scoring of C1–C5 for percentage area of the explant covered with callus (1 = 1–20%, 2 = 20–40%, 3 = 40–60%, 4 = 60–80%, 5 = 80–100%), 1–3 for number of shoots (*S*) and roots (*R*) per explant (1 = 1–5, 2 = 6–10, 3 = 11–15). (*NR* no response of any type)

BA (μ*M*)							
		0		1		2	
Auxin	μ*M*	Stem	Leaf	Stem	Leaf	Stem	Leaf
NAA	2	S1	S1	C1 + R1	C1 + R1	C1 + R1	C1 + R2
	4	S1	S1	C1 + R1	C1 + R2	C1 + R1	C1 + R2
	6	C2 + R1	C1 + R2	R1	R2	C1 + R1 + S1	C1 + R2 + S1
	8	C3	C1 + R1	R2	R2	C1 + R1 + S1	C1 + R1 + S1
IAA	2	C1 + S1	C1 + S1	S1	S1	S1	S1
	4	C1 + S1	C1 + S1	S2	S2	S2	S2
	6	C1 + S1	C1 + S1	S2	S2	S2	S2
	8	C1 + S1	C1 + S1	S1	S1	S1	S1
2,4-D	2	R2	R2	C5	C5	C5	C5
	4	R2	R3	C5	C5	C5 + R1	C5 + R1
	6	NR	NR	C5	C5	C5 + R1	C5 + R1
	8	NR	NR	C5	C5	C5 + R2	C5 + R2

BA (9–10 μ*M*) for shoot regeneration (Yang and Read 1997; Bhuyan et al. 1997), we obtained a high level of shoot-bud regeneration with just 2–4 μ*M* of BA, which stimulated the induction and continuous proliferation of shoot buds from the explants. Leaves taken from in vitro shoot cultures were found to be a superior explant material for shoot regeneration when compared to leaves from field-grown plants, as indicated by the higher biomass of shoots regenerated from the former during repeated subculture.

B. monnieri leaf explants continued to expand exponentially and regenerate new shoots up to 4 months of incubation in RM, with periodical transfer into fresh RM medium every 3 weeks. The system showed a potential for a continuous supply of shoots of *B. monnieri*. Further experiments are in progress to standardize the harvesting experiment, characterize the phytochemical profile of the shoots at each harvest and evaluate the potential of these shoot cultures as a source of herbal raw material.

B. monnieri leaf and stem explants showed a very poor callusing capability. Even 2,4-D, which is often included in tissue culture media for callus initiation and callus culture (Sunderland 1973), could not induce the formation of proliferating callus in *B. monnieri*.

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