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High frequency of shoot regeneration on leaf explants of *Bacopa monnieri*

Aruna G. Joshi*, Ashutosh R. Pathak, Asha M. Sharma, Swati Singh

Department of Botany, Faculty of Science, The Maharaja Sayajirao University of Baroda, Vadodara 390 002, India

*Corresponding author, E-mail: arunajoshi@gmail.com

Abstract

Plants of *Bacopa monnieri* were regenerated from entire leaf explants on Murashige and Skoog's basal medium supplemented with different concentrations (2 – 12 μM) of cytokinin 6-benzylaminopurine. The largest numbers of adventitious shoot buds were induced in 100% cultures from the explant when 6-benzylaminopurine was used at a concentration of 6 μM. The rate of adventitious shoot bud regeneration was affected by the type of wounding and placement of the explant on the medium. The square leaf lamina produced the maximum number of shoots within eight weeks. Rooting was achieved in microshoots on ½ strength basal liquid medium supplemented with sucrose (1%) and indole-3-butyric acid (2 μM). The obtained plantlets could be successfully acclimatized *ex vitro*.

Key words: leaf lamina explant, nodular callus, regeneration *in vitro*, shoot buds.
Abbreviations: BAP, 6-benzylaminopurine; IBA, indole-3-butyric acid; MS, Murashige and Skoog's medium.

Introduction

Bacopa monnieri (L.) Wettst., a species of the family Scrophulariaceae, is placed second in the priority list of Indian medicinal plants (Anonymous 1997). In 1990, the annual requirement of this plant was 12.7×10^6 kg of dry biomass at a value of $34 million (Ahmad 1993). The entire requirement is fulfilled from the natural population and the collection of wild material has lead to a rapid depletion of this plant from its natural habitat. The International Union for Conservation of Natural and National Resources has a long time ago listed *Bacopa monnieri* as a threatened species (Pandey et al. 1993). A number of studies have been carried out on shoot regeneration from different explants, like leaf, internodal segments and nodal segments of *B. monnieri* (Tiwari et al. 1998; 2000; 2001; 2006). However, considerable variation has been reported regarding the capacity of regeneration.

Success of regeneration depends not only on the type of the explant chosen, but also the way explants are placed on the culture medium (Duzyaman et al. 1994). Inflicting wounds to an explant after excision can often increase morphogenesis, which occurs at the site of the wounds. Wounding the explants may promote the transfer of endogenous hormones to the location of the scarring, causing a more suitable level of growth promoters for morphogenesis (Park, Son 1988).

The aim of the present study was to evaluate the effect of different concentrations of 6-benzylaminopurine (BAP) on regeneration capacity and to determine the role of wounding in regenerative competence of *Bacopa* leaf.

Materials and methods

Plant material and cultivation conditions

Explants were collected from young healthy vegetative plants growing in the Botanical garden of The Maharaja Sayajirao University of Baroda. Entire leaves were used as explants to establish cultures on Murashige and Skoog's (1962) medium (MS). Apical portions of healthy twigs bearing leaves up to the fourth node were excised, kept in running tap water (1 h), then washed with teepol (1 to 2 drops) and finally rinsed with water till the detergent was thoroughly removed. Further treatments were carried out in a laminar air flow cabinet. Explants were surface sterilized with 0.1% $HgCl_2$ (w/v) for 3 min and later thoroughly washed with sterile distilled water (three to four times) to remove traces of $HgCl_2$. Entire leaves were excised from stem in sterile Petri dishes and placed abaxially on the medium.

MS medium supplemented with sucrose (3%) along with different concentrations of BAP (2 to 12 μM) was used to establish cultures.

Effect of wounding on shoot bud induction

The following treatments were imposed to the leaf explants: (i) the entire leaf was inoculated without wounding (C); (ii) the entire leaf was fully wounded by piercing three times with a scalpel (E); (iii) the leaf was cut into two halves i.e. upper (distal) half of lamina (UL), lower (proximal) half of lamina (LL) and a square lamina (SL) and wounded by piercing three times with a scalpel. Explants for all three treatments were placed in abaxial orientation on MS

medium with sucrose 3% and supplemented with BAP (6 μM).

The pH of all the media was adjusted to 5.8 using 0.1 N NaOH or 0.1 N HCl as requirement. The medium was solidified with agar (0.8%) and dispensed into culture vessels. All cultures were sterilized by autoclaving at 121 °C for 20 min at 15 atm. The aseptic manipulations were carried out in a horizontal laminar air flow cabinet; the working table was cleaned with dettol, followed by 70% ethyl alcohol. All of the sterilized instruments and medium were irradiated with UV light for 20 min. The inoculated cultures were transferred to a culture room, where they were maintained at 25 ± 2 °C under 16 h photoperiod at 40 μmol m^{-2} s^{-1} provided by cool-white fluorescent lights. In all experiments, 15 replicates were maintained and the experiment was repeated twice.

The experiment was monitored for eight weeks and data for number of shoots per explants was recorded. Standard error of the mean for each value was calculated.

Rooting and hardening

Root induction of *in vitro* shoots was performed on MS ½ strength liquid medium supplemented with 2 μM of IBA. Shoots (4 to 5 cm) were transferred to tubes containing a filter paper bridge with the arms of bridge dipping in liquid (20 mL) medium. A single microshoot was inserted in the center of the bridge and the lowest node dipped in the medium. For the two initial weeks the lower portion of the tube was covered with aluminum foil and kept in the culture room. The rooted plants were hardened in sand/soil (3:1, v/v) mixture, and then transferred to greenhouse conditions.

Results and discussion

Leaf explants were found to be suitable for *in vitro* propagation of *B. monnieri*. MS basal medium with sucrose (3%) and supplemented with individual cytokinin BAP was efficient for shoot regeneration from entire unwounded leaf explants. Adventitious shoot bud regeneration was observed in all the combinations evaluated, but there was variation in the induction of shoot buds from the explants.

Table 1. Effect of 6-benzylaminopurine on shoot bud regeneration from leaf explants of *Bacopa monnieri* after eight weeks of cultivation. Means from 15 replicates ± SE are shown

BAP concentration (μM)	Total number of shoots per explant	Average length of shoot (cm)	Relative response (%)
2	3.60 ± 1.25	1.28 ± 0.15	24
4	19.23 ± 1.16	4.50 ± 0.46	84
6	30.00 ± 1.33	6.70 ± 0.34	100
12	10.60 ± 0.99	5.43 ± 0.25	74

When the medium was supplemented with 2 μM BAP, many shoot buds differentiated, but only two or three per explant developed in to healthy shoots. Further increase in BAP concentration to 4 μM evoked a better response, reaching an optimum at 6 μM BAP. At this concentration, shoot buds differentiated within two weeks from the proximal and distal ends of the leaf explants (Fig. 1A) and on average 30 shoots per explant were formed by the end of eight weeks, with length of microshoots reaching 6.7 cm in 100% of cultures (Table 1). A further increase in cytokinin concentration did not enhance the number of buds and resulted in profuse callusing.

In *B. monnieri*, BAP at 6 μM was the optimum concentration for shoot bud differentiation from leaf explants. Cytokinins are known to be very effective in promoting shoot proliferation and their role in shoot organogenesis is well established (Evans et al. 1983). Similar findings have also been reported in *Pterocarpus santalinus* and *Pterocarpus marsupium* (Anuradha, Pullaiah 1999) and *Prunus armenica* (Tornero et al. 2000).

Further studies were conducted to understand whether polarity of leaf explant and wounding affected shoot regeneration. All the explants in this experimental study were placed abaxially, and wounded on this surface (except C explants), to ensure contact with the medium, as this is known to promote efficient regeneration in many herbaceous plants (Bhatia et al. 2005). The observations revealed that all of the different sized explants responded quickly, but there was variation in the number of shoot buds being produced. Direct shoot bud formation started within the second week in all the different sized explants, and the best response was observed in square shaped leaf laminas (SL) which were cut from all sides. These explants immediately swelled two to three days after inoculation and shoot bud induction started from the cut surfaces as well as from the sites of wounded regions of the lamina, quickly covering the entire upper surface (Fig. 1E). Simultaneously there was formation of greenish nodular callus from the gashed regions of the lower surface, which proliferated up to the margins. A maximum number of 63.12 shoots (Fig. 1F) per explant was formed by the end of eight weeks

Table 2. Effect of different sizes of leaf explants and wounding on shoot bud regeneration in *Bacopa monnieri* after eight weeks of cultivation. C, control without wounding; E, wounding of entire leaf; UL, wounding of upper (distal) half of lamina; LL, wounding of lower (proximal) half of lamina; SL, wounding of square lamina. Means from 15 replicates ± SE are shown

Type of explant	Number of shoots per explant
C	30.00 ± 1.33
E	55.24 ± 1.33
UL	47.20 ± 1.25
LL	28.63 ± 0.99
SL	63.12 ± 1.16

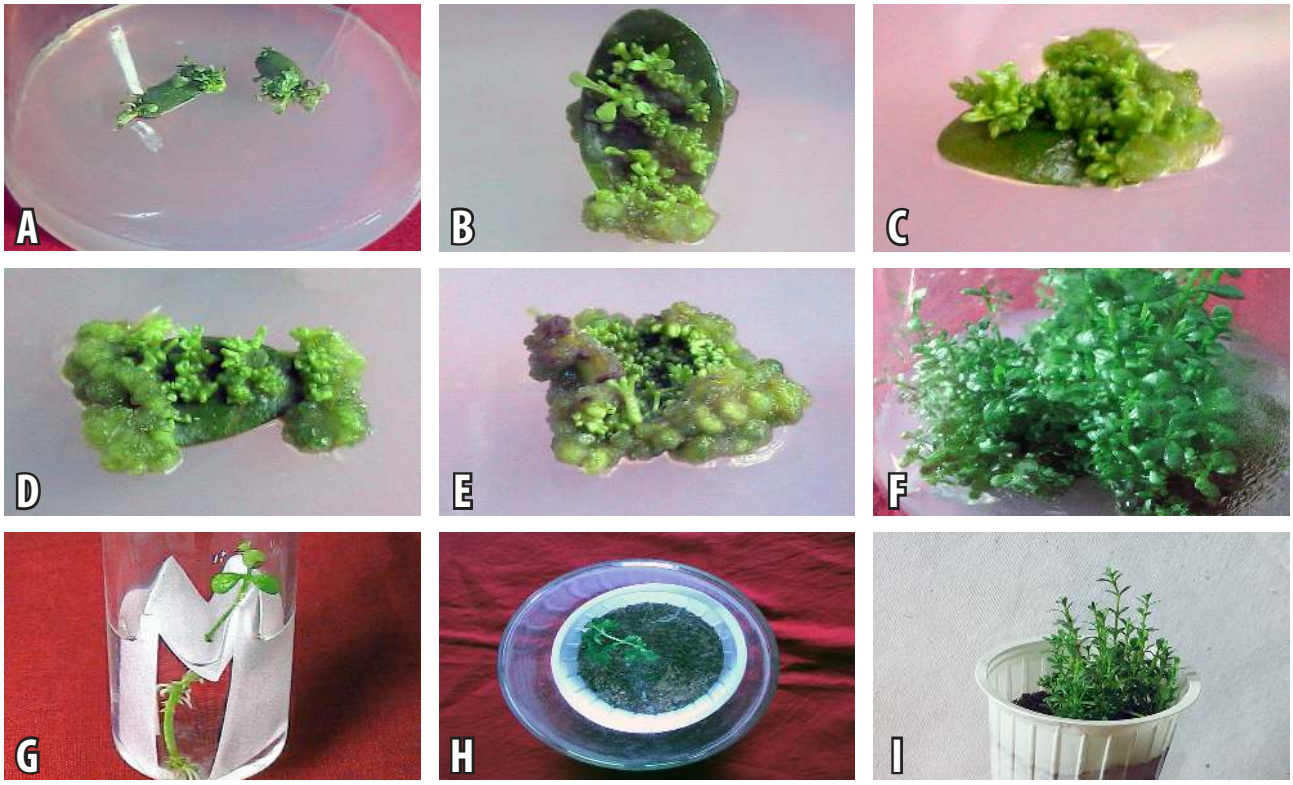

Fig. 1. Regeneration of *Bacopa monnieri* from leaf explants. A, entire unwounded leaf with the formation of shoot buds at the proximal and distal ends. B, entire wounded leaf with the formation of shoot buds all over from upper surface. C – E, shoots along with nodular callus in UL, LL and SL explants respectively. F, large number of micro shoots after eight weeks of cultivation. G, root induction in ½ strength MS + S (1%) + IBA (2 µM) liquid medium with a filter paper bridge. H, hardening of *Bacopa* microshoots in sand/soil (1:1) substrate. I, acclimatized *Bacopa* microshoots.

(Table 2). The microstructure of a cultured leaf showed a large number of round meristematic regions in the form of nodular callus, which differentiated into shoot buds. The entire lamina (E) was the second best in response, as shoot bud induction was observed on the entire surface (Fig. 1B). The lamina explants that were cut into two halves, a distal upper half (UL) and a proximal lower half (LL), differentiated shoot buds from the adaxial surface and from the greenish nodular callus formed at the cut margins of the respective explants. The UL explants followed in their response after SL explants and the least number of differentiated buds was observed in the LL explants (Table 2). In UL explants, shoot bud induction started from the upper surface and along the cut margin of lamina. Simultaneously, nodular callus was induced from the lower surface towards the cut margin and within four weeks it migrated to the upper surface (Fig. 1C). In the lower half of lamina (LL), similar results were observed but in this case, the formation of shoot buds started on the upper surface, followed by nodular callus along the cut margin and lamina base (Fig. 1D). The nodular callus over-powered the growth of shoot buds and became profuse. The potential of leaf tissue to form shoot buds was influenced by both the tissue polarity and the size of the explants, which was similar to observation made on sugarcane (Lakshmanan et al. 2006). Thus, a dual type of direct and indirect regenerative pattern of shoot bud formation was observed for *B. monnieri*, in respect to orientation of the explants on medium.

Control explants (C; entire and without wound) were were slow in regeneration, compared to entire explants (E) with wounds on lamina surface. The injury that the explants experience during inoculation may influence morphogenic response, and inflicting a further wound to an explant after excision can often increase morhogenesis (George 1993). The present study showed that wounded square leaf explants (lamina with cut surface on all sides) and, had the largest number of shoots. Similar results have been observed in tomato where cotyledon explants placed abaxially on medium differentiated into the highest number of shoots (Bhatia et al. 2005). In *Begonia* also the frequency of adventitious bud formation was dependent on the position of leaf explant on medium (Shimada et al. 2007). Our study revealed that abaxial orientation of leaf explant on medium can induce fast shoot bud regeneration, similar to that reported in tomato (Duzyaman et al. 1994).

The regenerated shoots were rooted in half strength MS medium with sucrose (1%), supplemented with IBA (2 µM). Similar results were reported in *Vitex* (Balarju et al. 2008) and *Pappea capensis* (Mngomba et al. 2007). It was observed that, within two weeks, root formation started

from the nodes of the shoots dipped in the medium (Fig. 1G). By the end of four weeks the roots had grown long enough, and hence the plantlets were transferred to plastic cups containing a mixture of planting substrate sand/soil (1:1). To maintain high humidity, initially the cups were covered with glass beakers for two weeks after transfer (Fig. 1H). New shoots developed by the end of four to five weeks (Fig. 1I) and these regenerated plantlets were successfully acclimatized and then transferred to greenhouse conditions.

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