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High Frequency Regeneration through Somatic Embryogenesis in *Bacopa monnieri* (L.) Wettest, an Important Medicinal Plant

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ABSTRACT

Bacopa monnieri is well known in the Indian system of medicine or Ayurveda as brahmi or water hyssop and has been using as a brain tonic and to cure mental disorders. An efficient *in vitro* protocol for high frequency regeneration has been developed via somatic embryogenesis from the leaf explants of two months old *Bacopa monnieri* (L.) Wettest. plants grown under greenhouse conditions. A high frequency of embryoids formed after the transfer of fleshy, nodular, leaf-derived callus onto Murashige and Skoog (MS) medium supplemented with 0.5 mg l^{-1} 2,4-D (2,4-dichloro-phenoxy acetic acid). A mean of 42.20 ± 2.20 embryoids developed per callus mass mean of 1.09 ± 0.06. An increase in the concentration above 1.5 mg l^{-1} of 2,4-D reduced the embryonic potentiality of the callus. Fluorescent staining of the embryogenic callus with auramine O stain revealed the occurrence of the embryogenic callus and facilitated the observation of the occurrence of globular, cordate and torpedo embryos and the subsequent differentiation of vasculature in the embryoids. The isolated cotyledonary embryoids matured and induced multiple shoots and roots on both MS basal medium and MS medium fortified with 1 to 1.5 mg l^{-1} 6-benzylaminopurine, i.e., BAP. Each plantlet obtained from an embryoid, could further produce multiple shoots on MS basal medium from which a mean of 28.6±1.37 plantlets were harvested. Most regenerants (98%) were successfully hardened-off and transferred to the field and maintained for the evaluation of variation. The present system of somatic embryogenesis of *B. monnieri* could be a viable system for the improvement of this medicinal crop.

Keywords: brahmi, brain tonic, fluorescence, medicinal herb, plant regeneration, somatic embryo

Abbreviations: 2,4-D, 2,4-dichlorophenoxy acetic acid; **BAP**, 6-benzylaminopurine; **FDD**, Flora of Davanagere District; **MS**, Murashige and Skoog

INTRODUCTION

Bacopa monnieri (L.) Wettest. (Scrophulariaceae) is a well-known medicinal herb, in the Indian system of medicine (Ayurveda) as Brahmi (Sanskrit) and Indian water hyssop. The plant is commonly found in wet, damp and marshy areas and is widely distributed in tropical regions of the world. Indian *Materia Medica* (1500 AD) cites the uses of this plant as a brain tonic, which is effective in maintaining vigor and intellect (Anonymous 1998). The compounds which are responsible for the memory-enhancing effects of *B. monnieri* is a triterpenoid saponin called 'bacosides'. Bacosides enhance the efficiency of transmission of nerve impulses by strengthening memory and cognition (Bhattacharya and Ghosal 1998). They are also used as a laxative and curative for ulcers, inflammation, anemia, scabies, leucoderma, asthma (Dar and Channa 1997) and epilepsy (Martis and Rao 1992). Anticancer and antioxidant properties of this plant were also found (Elangovan *et al.* 1995). The bacoside content of the plant was found to be very low (0.2% in dried plants), which necessitates the requirement of a large amount of plant material for drug extraction (Tejavathi and Shailaja 1999). It has been estimated that in the herbal medicine market the requirement of this herb is about 10 Lac kg (Ahmad 1993). A crude brahmi powder of 50 g costs around $9.99 in 2006 (Xtreme Herbs, Australia). In India, Himalaya, product code HH40301, are sold as 60 capsules with 250 mg per capsule. Many pharmaceutical companies in India, Australia and the United Kingdom are engaged in drug production from this plant.

Commercial exploitation from natural resources has resulted in the dwindling of the population of this species; furthermore, many pharmaceutical industries are the facing threat of unavailability of genuine Brahmi plants. The plant *Centella asiatica* is being used as an adulterant with the same name, brahmi. By using conventional breeding methods two cultivars namely 'Pragyashakthi' and 'Calcutta local' have been developed. Earlier investigators (Bose and Bose 1931; Shastry *et al.* 1959; Pal and Sarin1992; Rastogi *et al.* 1994; Tejavathi and Rao 1998; Tiwari *et al.* 1998) cultured stem, leaf and flower bud explants *in vitro* for the regeneration of plantlets. This paper reports on the high frequency regeneration of plantlets via somatic embryogenesis from leaf explants of *Bacopa monnieri* cultivar 'Pragyashakthi'.

MATERIALS AND METHODS

Plant material and sterilization

The cultivar 'Pragyashakthi' used for induction of somatic embryogenesis was collected from the Medicinal garden, Division of Horticulture, and confirmed by the Herbarium collection (FDD 248) in the University of Agricultural Sciences, G.K.V.K. Campus, Bangalore, Karnataka, India. Tender leaf material were collected from two months old plants grown in greenhouse conditions (22°C and 70% relative humidity) leaf materials were thoroughly washed in running tap water for 25-30 min and then rinsed in the surfactant, Tween-20 (two drops in 100 ml solution) for 5 min and washed five times with distilled water. The explant was treated with the fungicide Bavistin 1% (w/v) for 20-30 min, washed five times with distilled water followed by a rinse with 0.1% (w/v) $HgCl_2$ so-

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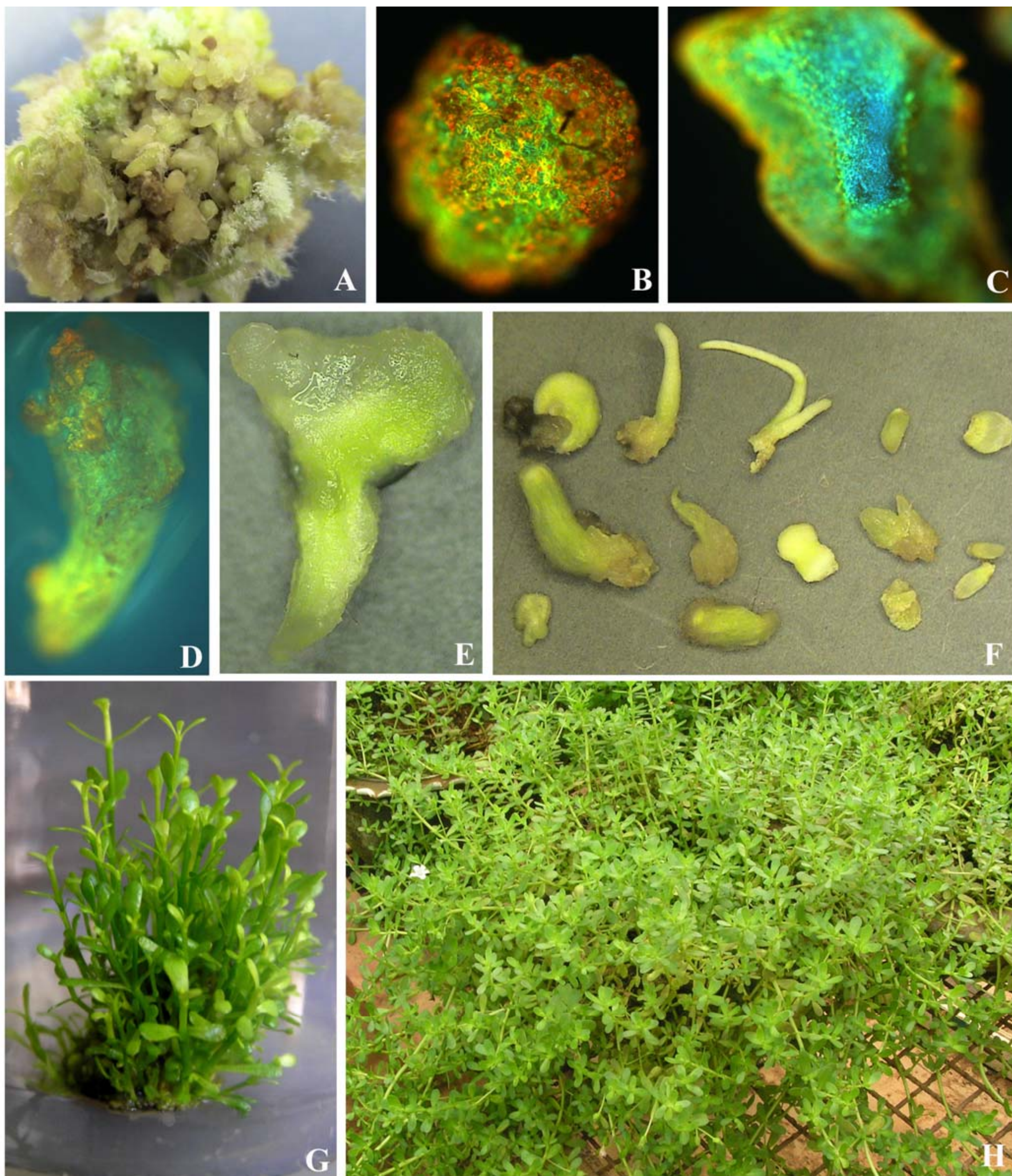

Fig. 1 Organogenesis and somatic embryogenesis in *B. monnieri*. (**A**) Emryogenic callus showing globular, cordate, torpedo and cotyledonary-shaped embryoids. (**B**) The cordate- and torpedo-shaped embryoids showed the presence of a distinct meristematic layer of cells when treated with the fluorochrome Auramine O. (**C**) The torpedo-shaped embryoid showing the differentiation of vascular elements at its center. (**D**) Yellow fluorescence was observed at the root meristematic zone of the cotyledonary embryoids. (**E**) Stereomicroscopic observation of the isolated embryoids showed the formation of a prominent shoot and root primodium. (**F**) Some malformed embryoids were also noticed. (**G**) A plantlet obtained from each embryoid further produced multiple shoots and roots. (**H**) Acclimatized plantlets in pots.

lution for 3 min. The disinfectant was removed by rinsing the material with sterile, cooled distilled water 5-6 times. Under asceptic condition the leaves were carefully inoculated onto callus- and somatic embryo-inducing medium.

Culture media

The culture medium consisted of MS salts (Murashige and Skoog 1962) augmented with 3% (w/v) sucrose and gelled with 0.8% (w/v) agar. The callus- and somatic embryo-inducing medium included the synthetic auxin 2,4-dichlorophenoxy acetic acid (2,4-D) from stock containing 100 mg in 100 ml or 1 mg l^{-1} (Himedia Laboratories Ltd., Mumbai, India.; 99.0% purity) at a range of 0.25 to 1.5 mg l^{-1}. The pH of the medium was adjusted between 5.7-5.8 and autoclaved at 15 p.s.i. (1.06 kg/cm^2) and at 121°C for 20 min. Thirty ml of medium was dispensed into sterilized culture bottles (Varsha Storage Racks, Bangalore, India) closed with polypropylene caps. The cultures were incubated at 28 ± 2°C in a growth room with a 16/8 photoperiod and a light intensity of 50 µmol m^{-2} s^{-2}. Each bottle was inoculated with five explants with ten rep-

licates per concentration. The explants were inoculated on media adaxial surface facing up. Embryonic callus formed after 60 days. The mean number of somatic embryoids per callus mass was calculated. The cotyledonary embryoids were easily distinguishable, they were aseptically isolated and transferred onto MS basal medium without plant growth regulators but fortified with 1 to 1.5 mg l^{-1} BAP for maturation and multiplication. Each treatment consists of 10 replicates and a complete randomized design was used in all experiments. Analysis of variance was carried out using Duncan's Multiple Range Test (Duncan 1955). Statistical significance was determined at the 5% level.

In vitro rooting and acclimatization

The shoots in the multiplication media formed roots simultaneously in the same media. Cultured rooted, intact plantlets recovered were washed with running tap water and the agar sticking to the roots was removed. Plantlets with fully expanded leaves and well developed roots were first transferred to plastic cups containing sterilized soil. The regenerated plantlets were hardened for two weeks by covering with a thin perforated transparent polythene bag to maintain humidity. The brahmi plant in the field roots easily and develops laterally expanding adventitious roots at nodal points. The plantlets were hardy and showed 100% survival in all the hardening media.

Fluorescent microscopic studies

The embryonic nodules containing sequential stages of embryonic development i.e., globular, cordate and torpedo were aseptically isolated and treated with 0.001% (w/v) fluorescent dye auramine O for 10 min. The material was rinsed with distilled water to remove excessive stain, and examined under a Fluorescent microscope (Olympus model QG2-32, Ver. V 3.1.0) using a UV filter. Specimens were photographed.

RESULTS AND DISCUSSION

Fleshy compact leaves induced calli on MS medium supplemented with 2,4-D at a range of 0.25 to 1.5 mg l^{-1} further induced somatic embryos. 2,4-D at 0.5-mg l^{-1} induced the highest frequency of somatic embryogenesis from leaf callus and the existence of globular-, cordate-, torpedo- and cotyledonary-shaped embryoids were verified (**Fig. 1A**). At the optimum concentration (0.5 mg l^{-1}) a mean of 42.20 ± 2.20 organized embryoids formed. A higher concentration of 2,4-D in the medium hindered the embryogenic potency of the callus (**Table 1**). After 45 days of incubated culture, torpedo and cotyledonary embryoids were prominent. One of the interesting features observed was the retention of embryogenic potency of the callus on 2,4-D-supplemented medium for more than two subcultures. When nodular embryogenic masses (1.09 ± 0.06) were subcultured onto fresh medium the callus increased in size and the embryogenic nodules were also well formed. Generally this synthetic auxin favored callogenesis and hindered caulogenesis from the explants or calli. In reports by Ammo *et al.* (2005) and Chandrashekar *et al.* (2006) in *Tylophora indica* (Burm.f.) Merril., 2,4-D at a lower concentration (1.5 μM 2,4-D along with 0.5μM TDZ) could induce somatic embryogenesis from calli in leaf explants. At a higher concentration of 2,4-D (above 1.5 mg l^{-1}) we did not observe somatic embryogenesis, simply callus formation from the explants. In as early as 1978 Murashige stated that an increase in the level of 2,4-D would result in a rise in the production of ethylene in cultures. High ethylene production leads to increased activity of the enzyme cellulase and pectinase, which causes fragmentation of callus. In the present study also at higher concentration of 2,4-D embryogenic potency of callus was suppressed and it became brown.

Embryogenic callus from the 45-day-old culture were aseptically isolated and observed under a stereomicroscope and a fluorescent microscope. After treating with fluorochrome auramine O the cordate- and torpedo-shaped embryoids showed orange-yellow cells indicating the presence of a distinct meristematic layer of cells (**Fig. 1B**) and the torpedo-shaped embryoid showed the differentiation of vascular elements at its center, which was indicated by blue coloured patches (**Fig. 1C**). The use of auramine O as a fluorochrome provides an externally rapid method for the direct observation of components of the cellular endomembrane system. The staining reaction occurs with greatest intensity in cells with a high metabolic activity. The wavelengths of emitted fluorescence vary depending on tissue type, so that yellow fluorescence is observed in lignifying cells of vascular bundles, whilst orange fluorescence occurs in secretary cells of nectaries (Harris and Gates 1984). In the present study also yellow fluorescence was observed at the root meristem of the cotyledonary embryoid (**Fig. 1D**). Auramine O binds with two important chemical constituents, lignin and cutin and fluoresces yellow when excited under a UV filter. The stereomicroscopic observation of the isolated embryoids showed the formation of a prominent shoot and root primordium (**Fig. 1E**). However, in addition to normal embryoids some malformed embryoids were also noticed (**Fig. 1F**), also reported by Philip and Ammirato (1977) who used caraway (*Carum carvi*) in suspension culture but did not use 2,4-D.

Generally, cytokines suppress organogenesis of embryogenic cells but increase the rate of proliferation and promote the maturation of somatic embryoids (Sandeep *et al.* 2003). Reports also indicated that the maturation of somatic embryos takes place in *Psoralea corylifolia* Linn., when they were transferred onto a cytokinin-supplemented medium (Ashok and Suresh 2001). In the present study maturation and further multiplication of the torpedo and cotyledonary embryoids occurred on full-strength MS medium without plant growth regulators and the medium contained 1-1.5 mg l^{-1} BAP. Among these, MS basal medium was most suitable for the growth of the isolated cotyledonary embryoids into plantlets. On maturation medium shoot meristems initially became photosynthetic and turned to a luster green. On BAP-supplemented with 2 mg l^{-1} medium a plantlet obtained from each embryoid further produced multiple shoots and roots (**Fig. 1G**). In 45-day-old culture a mean of 52.90 ± 3.21 shoots were recorded from the isolated embryoids (**Table 2**). The spontaneous initiation of roots was also noticed from these shoots, which were in direct contact with the medium. The shoots in the multiplication media formed roots simultaneously in the same media. Brahmi plants in the field root easily and develop lateral, creeping roots from nodal points. This easy-to-root character of the plant was also exhibited in *in vitro* culture and did not require any special treatment for the induction of roots. The lower percentage survival in sand might be due to the fact that brahmi is a water-loving plant and is known to flourish in wet and marshy areas.

Hence, sand, due to its poor water holding capacity might not be a good media for its *ex vitro* establishment. The plantlets derived from somatic were successfully transferred to the soil. Most plantlets (98%) were acclimatized to field conditions and they exhibited similar morphology with the *in vivo* mother plant (**Fig. 1H**). The regenerates were maintained in a mist house for comparative analysis of the

Table 1 Effects of different concentrations of 2,4-D on somatic embryogenesis from leaf-induced calli of *B. monnieri*.

Concentration of 2,4-D (mg l^{-1})	% organized embryoids/callus clump	№ embryoids/callus clump (mean ± SD)
0.25	78	25.50 ± 1.35
0.5	96	42.20 ± 2.20
0.75	88	28.80 ± 1.13
1.0	74	15.90 ± 1.91
1.25	36	9.40 ± 2.06
1.5	32	6.30 ± 0.82
F-Value		22.09

The value of each concentration consisted of ± S.D. of 10 replicates.
The *F*-value is significantly different at 0.05%.
The initial mass of the clump was 1.09 ± 0.06 g.

Table 2 Effect of growth regulators on germination of cotyledonary somatic embryos of *B. monnieri*.

Medium + plant growth regulator	№ shoots per embryoid (Mean ± SD)	№ rooted plantlets harvested per embryoid (Mean ± SD)
MS basal	52.90 ± 3.21	28.6 ± 1.37
MS + BAP 1.0 mg l^{-1}	41.20 ± 2.40	21.2 ± 1.51
MS + BAP 1.25 mg l^{-1}	36.10 ± 1.87	15.3 ± 2.67
MS + BAP 1.5 mg l^{-1}	26.40 ± 1.82	11.7 ± 1.34
F-value	522.85	316.05

The value of each concentration consisted of ± S.D. of 10 replicates.
The *F*-value is significantly different at 0.05%.

bacoside content.

The regeneration of plants *in vitro* via somatic embryogenesis has some distinct features such as single cell origin, the consequent low frequency of chimeras and production of a high number of regenerates which are morphologically and cytologically uniform (Ammirato 1983; Sato *et al.* 1993; Gopi and Ponmurugan 2006; Wei *et al.* 2006). As somatic embryogenesis is a single-celled event, it is especially advantageous in the formation of plantlets in fewer steps with a concomitant reduction in labor, time and cost.

This technique of mass production is hope full for the large-scale propagation, and production and extraction of bacoside from *B. monnieri* (L.) Wettest.

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