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Induction of somatic embryogenesis in *in-vitro* cultures of *Bacopa monniera* (L.) Pennell

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Abstract
In the present study a simple protocol for induction of somatic embryogenesis and plantlet regeneration in *Bacopa monniera* (L.) Pennell is developed. The leaf explants showed extensive callus proliferation on MS medium containing 5 µM NAA in combination with 1 µM 2,4-D. The established calli derived from leaf explants were transferred on to ½ strength and full-strength MS medium lacking and containing auxins and cytokinins individually and in the ratio >1, <1 and =1. Among all these media, maximum number of somatic embryo formation was observed on MS media containing 20 µM 2,4-D in combination with 20 µM Kin (12.0 ± 0.3 per 100mg callus). Somatic embryo induction was also observed in ½ strength and full-strength MS medium lacking auxins and cytokinins. The visual observation and histological studies of the embryogenic callus showed the different developmental stages of the somatic embryogenesis, viz. globular, heart and torpedo stage.

Keywords: *Bacopa monniera*, somatic embryogenesis, plant growth regulators

1. Introduction

Bacopa monniera (L.) Pennell, (Scrophulariaceae) is an important plant in the Ayurvedic system of medicine, mainly used for treating age-related brain disorders and for improving cognitive processes. Among the 12 tritepenoids reported from *B. monniera*, bacoside-A is the major constituent believed to be responsible for its cognitive effects [1, 2, 3]. The plant is widely used as a nervine tonic, cardio tonic and diuretic in Indian traditional medicine [4]. Many brain tonics containing *B. monniera* extract are available in market. In the light of various medicinal properties of *B. monniera*, there is increasing demand for this plant. However, its collection from natural wetlands is ecologically damaging and therefore controlled by state regulation. Improper harvesting from natural resources, seedling death at the 2-leaf stage and specific habitat requirement has lead to rapid depletion of *B. monniera* from its natural habitat. The submerged shoots of *B. monniera* can hardly ramify to attain the required growth and multiplication. Therefore, it is necessary to develop and standardize the large-scale multiplication and secondary metabolite production of *B. monniera* by applying *in vitro* techniques.

Somatic embryogenesis is the process of a single cell or a group of cells initiating the developmental pathway that leads to reproducible regeneration of non-zygotic embryos capable of germinating to form complete plants. Occurrence of somatic embryogenesis depends on co-ordained behavior of a cell or cells to establish polarity as a unit. Age, physiological stage, genotype and orientation of the explant in contact with medium influence the induction of somatic embryogenesis. Somatic embryogenesis has proved to be useful for micropropagation and the production of mutants, artificial seeds and materials for use plant genetic engineering [5]. In the present investigation a protocol has been developed to initiate somatic embryogenesis from different explants in embryogenic callus of *Bacopa monniera* (L) Pennell.

2. Materials and Methods

2.1 Induction and Maintenance of cultures

Callus cultures were established from leaf and stem explants cultured on Murashige and Skoog (MS) medium [6] supplemented with 3% sucrose, 0.8% agar (w/v) and various concentrations of cytokinins (BA, Kin and TDZ) alone and in combination with auxins (IAA, NAA and 2,4-D). Maximum callus formation and proliferation from leaf explants was observed on MS + 5 µM NAA + 1 µM 2,4-D. The leaf calluses were maintained by subcultring on fresh parental medium at an interval of 4 weeks. After continuous subculturing for 6 months green patches were observed on the callus. To investigate the embryogenic potential, the calli showing green patches were subjected to different treatments of plant growth regulators.

The callus derived from leaf explants were inoculated on MS medium containing high concentrations (10 – 20 µM) of 2,4-D in combination with BA (10 – 20 µM) or Kin (10 – 20 µM). To check the effect of nutrients and sucrose, some embryogenic callus was cultured on full strength MS medium containing 1.5%, 3% or 4% sucrose as well as half strength MS medium containing 1.5%, 3% or 4% sucrose without plant growth regulators.

All the cultures were maintained under 40 µmol $m^{-2} s^{-1}$ light (provided by Phillip's cool white fluorescent tube lights) with 8 h light period at 25±2ºC. The cultures from different treatments were harvested after 4-week incubation period. The growth rate of callus was determined by recording fresh and dry weight.

2.2 Histology

For histological observation the procedure described by Gamborg and Phillips [7] was followed. The sections were mounted in DPX and analysed using an Olympus trinocular research microscope with a Magnus CCD camera for photomicrography.

2.3 Statistical analysis
All the experiments were setup in completely randomized design, conducted at least thrice with minimum 14 replicates per treatment. Observations were recorded at the intervals of eight days and wherever necessary daily observations were recorded. Means and standard error, ANOVA was carried out using Duncan's Multiple Range Test (DMRT) [8] at 5% probability level. Variability in data has been expressed as mean ± standard error.

3. Results & Discussion
3.1 Influence of plant growth regulators
The effects of plant growth regulators on somatic embryo production are presented in Table 1 and Fig. 1 A-E. The leaf explants showed extensive callus proliferation on MS medium containing 5 µM NAA in combination with 1 µM 2,4-D. The calli formed on this media showed green patches after continuous subculture on same parental media for 6 months. The embryogenic callus thus obtained was cultured on MS media supplemented with high concentration of 2, 4-D together with high concentration of BA or Kin. The calli continue to proliferate and subsequently changes its color from pale yellow to pale green. In the 3rd and 4th week, green colored nodular callus was observed in the cultured test tubes. Microscopic observations of the calli revealed the presence of globular, heart and torpedo stage somatic embryos. Maximum proliferation and mean number of embryo production per gram was observed on MS media supplemented with 20 µM 2,4-D together with 20 µM Kin (12.0 ± 0.3 embryo per 100g of callus). Decreasing concentration of 2,4-D or Kin resulted in decrease in mean number of embryo production. As compare to Kin, incorporation of BA in 2,4-D containing MS media was less effective for somatic embryo induction.

Occurrence of somatic embryogenesis in *B. monniera* callus cultures has been previously reported [9]. However, the authors observed somatic embryo formation for calli derived from node explants and did not mention the response of leaf derived callus for somatic embryogenesis. In accordance with the present results, positive influence of BA and 2,4-D on somatic embryogenesis from *in vitro* derived leaf explants of *B. monniera* has been reported [10]. Synergistic effects of 2,4-D in combination with other auxin (BA or Kin) on somatic embryo formations has been reported in many medicinal plants like *Centella asiatica* [11], *Cephaelis ipecacuanha* [12], *Festuca arundinacea* [13], *Ophiorrhiza porstrata* D. [14] and *Azadirachta indica* [15]. Thus, the results of present investigation previous research reports indicate that, like several other plant species, inclusion of 2,5-D together with Kin was most effective treatment for somatic embryo formation in callus cultures of *B. monniera*.

3.2 Effect of nutrients and sucrose treatment
With the aim to enhance the embryo production, the embryogenic calli was transfer to full and half strength MS media containing varied levels of sucrose and devoid of plant growth regulators (Table:1). Removal of plant growth regulators from culture media supports the development of somatic embryos. The embryogenic calli cultured on MS medium containing 3% sucrose showed formation of pale yellow colored nodular sectors in 2 week of cultured incubation. In 3rd week translucent globular structures were seen in the calluses. Decrease in sucrose concentration slightly increases the embryo formation. 6.1 ± 0.1 embryos per culture were noted from the MS basal + 1.5% sucrose. While increase in sucrose concentration (4%) increases the callus proliferation but somatic embryo formation was not observed on this medium

Decrease in nutrient supply also showed positive influence on somatic embryo development (Table 1). The calli cultured on ½ strength MS media containing 3% sucrose produced 6.5 ± 0.3 embryos per 100 g of fresh callus. However, combined treatment of decreased nutrient supply and declined sucrose concentration was comparatively more effective for somatic embryo production. The calli cultured on said media produced 7.3 ± 0.3 embryos per 100 g of fresh callus. Increase in sucrose level (4%) in half strength MS medium resulted in proliferation of calli, but it suppressed somatic embryo formation.

The regulatory role of sucrose on organogensis and embryogenes has been reported in many plant species [16, 17, 18]. In the present study showed that slight decrease in sucrose concentration leads to increase in somatic embryo production. Similar increase in somatic embryogenesis was observed with decreasing nutrient strength of medium coupled with decrease in sucrose concentration. It may be due to the stress created by reduction in energy source. The involvement of stress in induction of somatic embryogenes is previously reported in many plant species [18, 19, 20].

Table 1: Effect of plant growth regulators, media strength and sucrose concentration on somatic embryogenesis leaf derived callus of *B. monniera* (L.) Pennell.

Treatments			Callus response	Embryos per 100mg callus
Full strength MS medium + 3% Sucrose + Growth regulators (µM)				
2,4-D	Kin	BA		
10	10	-	GC	0
	-	10	GC	0
	20	-	EC	10.9 ± 0.4 b
	-	20	EC	1.7 ± 0.0 h
20	10	-	EC	3.2 ± 0.1 f
	-	10	EC	1.8 ± 0.1 g
	20	-	EC	12.0 ± 0.3 a
	-	20	EC	3.1 ± 0.1 f
Treatments with media strength and sucrose concentration				
MS basal + 1.5% sucrose			EC	6.1 ± 0.1 de
MS basal + 3% sucrose			EC	5.8 ± 0.2 d
MS basal + 4% sucrose			GC	0
½ MS basal + 1.5% sucrose			EC	7.3 ± 0.3 c
½ MS basal + 3% sucrose			EC	6.5 ± 0.3 d
½ MS basal + 4% sucrose			GC	0

Data scored after 4 weeks of culture incubation; GC: Green Non embryogeneic callus; EC: Embryogeneic callus. Mean values within a column followed by the same letters did not differ at 5% level of probability by DMRT.

4. Conclusions
In the present study, we have demonstrated a protocol for induction somatic embryogenesis in leaf derived callus of *B. monniera*. The maximum rate of somatic embryo formation in *B. monniera* can be achieve by using high concentration of 2, 4-D in combination of Kin followed by using half strength MS media with reduce sucrose concentration.

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6. References

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