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Research Article

REGENERATION POTENTIAL AND MAJOR METABOLITE ANALYSIS IN NOOTROPIC PLANT- *BACOPA MONNIERI* (L.) PENNELL

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ABSTRACT

OBJECTIVE: *Bacopa monnieri* (L) Pennell, commonly known in India as 'Brahmi' is an important nootropic plant. The study was mainly aimed at the mass multiplication of *B. monneri* and the effect of various growth regulator regimes for their active principle accumulation in regenerated plants.

METHOD: In this study, a protocol to produce high frequency of *in vitro* cultured multiple shoots / plantlets were established both by direct and callus based indirect regeneration using leaf and nodal explants. In direct regeneration, a single leaf grown on MS basal plant medium supplemented with 2mg/L kinetin gave large number of multiple plantlets. On the other hand the callus based regeneration using leaf as explant grown on MS basal plant medium supplemented with 1mg/L Benzyl aminopurine (BAP) and 0.5mg/L IAA produced large number of side shoots / multiple plantlets. The successfully acclimatized plants were screened for major metabolites /active principles using HPLC.

RESULT: Influence of Cytokinins and supplementation with auxins for the production of multiple shoots was investigated . 2mg/L kinetin alone showed highest number of shoots whereas the combination of Kin / BAP or Kin / IAA showed less number of shoots. Bacopaside content of regenerated shoots varied with hormonal treatments.

CONCLUSION: This study will facilitate the mass propagation and transformation of this nootropic plant. In addition the study can be used for commercial production of secondary metabolites like Bacopaside using different hormone combinations for elicitation.

Keywords: Secondary metabolites, Bacopasides, plant tissue culture, multiple shoots

INTRODUCTION

Bacopa monnieri (L) Pennell, commonly known in India as 'Brahmi' or 'the thinking person's herb' is an important medicinal plant belonging to family Scrophulariaceae [1] *Bacopa monneri* is a small, annual, succulent creeping herb with fleshy leaves. The plant grows in wet, damp and marshy areas. Brahmi is a reputed nervine tonic used for its ability to enhance memory, improve intellectual and cognitive functions, anti-inflammatory, analgesic, antipyretic, sedative and as antiepileptic agent [2] . It is also known to improve the working and reference memory by restoring the alterations in cellular oxidants and antioxidant enzymes [3]. In Ayurveda, *B. monnieri* has been classified under medhyarasayana, i.e., medicinal plants rejuvenating intellect and memory. Ayurvedic treatises, viz., Charak samhita, Susruta samhita, and Astanga hrdaya, have prescribed *B. monnieri* for the promotion of memory, intelligence, and general performance [4]. *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 [5,6] . The active principle constituents, reported in *Bacopa monnieri* are alkaloids; Brahmine , Herpestine and saponins. The memory- enhancing effects have been attributed to the presence of saponins, Bacopaside A and Bacopaside B [7] . Pharmacological activities of *Bacopa monnieri* are attributed to saponin compounds present in the alcoholic extract of the plant [8]. The principle active factors that have been reported are two saponins, Bacopaside A, Bacopaside B and two sapogenins Jujubogenin , pseudojujubogenin and Bacogenin A_4[9] (The other chemical constituents of the plant include Bacopasides, hersaponin, betulinic acid, stigmasterol, β-sitosterol and stigmastenol [10] . With an increasing world-wide demand for plant-derived medicines, there has been a concomitant increase in the demand for raw material .Novel approaches have to be developed to ensure the continuous availability of raw material of a consistent quality from regular and viable sources [6,8] . Plant *in vitro* tissue culture provides an alternative to customary agricultural processes for producing valuable phytochemicals. Stability testing of botanical drugs is challenging because of physico-chemical complexity and their active substance profiling which integrates the holistic approach of this plant [8]. This study was mainly aimed at the mass multiplication of *B. monnieri* , to assess the response of explants to different growth regulators and metabolite profiling of regenerated plants for the analysis of the metabolites particularly Bacopaside I and II [11] .

MATERIALS AND METHODS

Explants used for *in vitro* regeneration were collected from wet damp area around R V College of Engineering, Bangalore. Leaf and nodal segments between the 2nd and 6th node were selected as explants. The explants were washed with mild detergent under slow running tap water for 15 min followed by wash in sterile distilled water. The explants were transferred to laminar airflow then surface sterilized with 70% ethanol for 30 secs followed by 10 min wash with 2% sodium hypochlorite. The explants were surface sterilized with 0.1% mercuric chloride for 2 min and washed thoroughly with sterile distilled water to remove any traces of mercuric chloride. The nodal segments were then excised under sterile conditions using sterile scalpel blade. The leaves and nodal segments were inoculated on Murashige and Skoog, (MS) basal medium supplemented with plant growth regulators [12] . The cultures were then incubated at 24 ± 2°C under fluorescent tubes.

Metabolite Extraction and HPLC analysis

One gram of *B. monnieri* powdered plant material was weighed into a 100 ml round bottom flask, added about 30 ml of 70% (v/v) methanol (Qualigens) and refluxed on a water bath for 30 min. Filtered and the extraction was repeated twice (2X30 ml) with 70% (v/v) methanol, combined all the alcoholic fractions and made up to 100 ml with 70% (v/v) methanol and filtered through 0.45 μ membrane filter.

The fractions were analyzed by Shimadzu HPLC System equipped with pinnacle DB C18 column 5 μm (4.6mm x 250mm) column from Restek , LC 10 AT VP lamps , SCL-10 AVP system controller , SIL-10

AD VP autoinjector , SPD-M10 AVP photodiode array detector. The mobile phase was gradient of solvent A and B . Solvent A was a mixture of potassium dihydrogen orthophosphate and orthophosphoric acid in HPLC grade water (Milli-Q) and solvent B being Acetonitrile (Qualigens) gradient with a flow rate of 1.5 ml / min, column temperature at 30^0 C. The detection of wavelength was at 205nm , with injection volume being 20 µl. The chromatography system was equilibrated initially by mobile phase.

STATISTICAL ANALYSIS

The results were expressed in terms of mean values ± standard deviation (SD) with three replicates . The effect of different treatments on shoot regeneration was compared to detect the significance of differences among the treatments using ANOVA at a 5 % probability level. The difference between the treatments was considered to be statistically significant when p values ≤ 0.05.

RESULTS AND DISCUSSION

The regeneration potential and metabolite analysis in *Bacopa* has been dealt in this manuscript. From the study we have observed that *Bacopa monnieri* leaf / nodal explants grown on MS medium supplemented with cytokinins are required for multiple shoot induction. For callus based indirect regeneration, explants were inoculated in MS basal medium supplemented with combination of auxins and cytokinins.

Table 1: Effect of plant growth regulators on the shoot induction from stem explants of *B. monnieri* and Bacopasides content in regenerated plants. Numbers with different letters are significantly different at *P* <0.05.

Plant growth regulators mgl^{-1}			Number of shoots/ explant*	Amount of Bacopasides content $mgg^{-1}DW$	
KIN	BAP	IAA		Bacopaside I#	Bacopaside II#
2	-	-	126.5a	8.92	10.64
1	0.5		98.2 ab	7.54	4.21
0.5	2		69.7a	5.32	1.41
	1	0.5	31.2 b	11.46	9.21
0.5		0.5	77.1 bc	3.24	0.23
2	1	0.5	84.5 c	8.79	4.59

* *n* = 20.

Values represent the mean ± S.D. (*n* = 3).

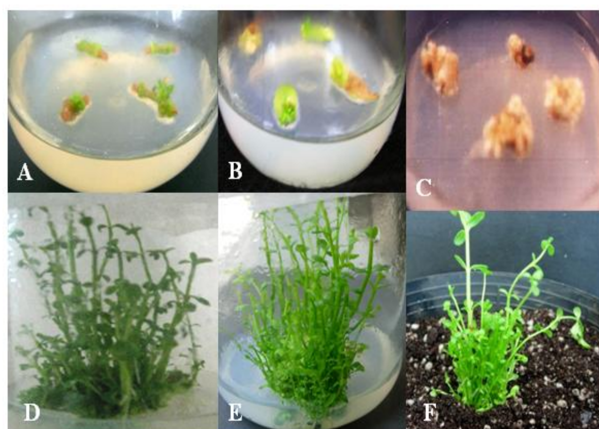

Fig 1 A) Nodal segment as explant; B) leaf as explant; C) Callus Induction in MS media supplemented with 2,4D (1mg/L) cultures from the leaf explants after a month; D) Nodal explants showing complete regeneration observed by direct regeneration 45 days grown on MS medium with 2mg/L Kinetin: E) Plantlets maintained on MS basal medium before hardening; F) Hardening of the regenerated plant in the soilrite.

For callus induction the explants were inoculated on MS Medium supplemented with 1mg/l 2,4- Dichlorophenoxyacetic acid (2,4-D) and after 30 days the callus cultures were transferred to MS medium supplemented with Indole acetic acid (IAA) and Benzyl amino purine (BAP) of varied concentration. Shoot induction was evaluated on MS medium with combinations of IAA and BA or Kinetin alone using nodal and leaf explants (Fig. 1A&B). After 45 days of culture, we succeeded to induce shoot formation from all combinations of plant growth regulators. The best result concerning the shoot number per explant (126 shoots +/ explant) was obtained in MS medium supplemented with 2 mg/l kinetin after 45 days of culture (Table I, Fig. 1D). This result showed that supplementation with auxin and cytokinin in the medium reduced the number of shoots per explant (Table I). The number of shoots per explant using a low concentration of BAP (0.5 mg/l) and Kinetin (1 mg/l) was high compared with combinations of IAA and BAP. Multiple shoots were elongated and rooted after being transferred to culture on MS medium without hormones for 15 days (Fig. 1E).

The callus cultures started regenerating within 15 days and complete regeneration (shooting and rooting) was observed within 30 days. The plants were moved to soil rite (Mixture of coco brick, cocopeat perlite and vermiculite) for acclimatization. From the study we have observed that *B. monnieri* leaf / nodal explants grown on MS medium supplemented with Cytokinins are required for multiple shoot induction. Cultures grown on MS Medium with 2mg/L kinetin gave vigorous multiple shoots without intervening callus phase. On the other hand callus cultures developed multiple shoots with combination of both cytokinin and auxin, 1mg/L BAP and 0.5mg/L IAA. *B. monnieri* has a high morphogenic potential, and the explants readily responded to cytokinins and auxins in the culture medium and raised multiple shoot buds.

The employment of plant tissue culture offers new opportunities for *in vitro* production of plant secondary metabolites. Data on quantitative analysis of Bacopaside I and II is presented in the table 1. The amount of Bacopasides content of regenerated shoots varied with hormonal treatments; Bacopaside I content in the shoots developed without growth regulators was 2.61 mg g^{-1} Dry Weight (DW) (data not shown), whereas it was 8.92 mg g^{-1} DW in the shoots regenerated on the medium supplemented with 2 mg l^{-1} KIN. Thus 3.5-fold higher Bacopaside I was accumulated in the shoots grown on KIN supplemented medium. All samples gave the similar HPLC fingerprints. Three peaks were detected Bacopaside I, II and Bacopaside A3. The dominant compound was Bacopaside I or Bacopaside II.

Role of growth regulators and metabolite profiling varies with the change in the type and nature of growth media [13]. In the present study Kinetin stimulated the production of bacoside I and II in the tissue cultured shoot of *B.*monnieri. The amount of Bacopaside I was highest in the shoots regenerated in MS media supplemented with BAP 1mg/L and IAA 0.5mg/L (11.46 mg g^{-1} DW). For the Bacopaside II trend remains the same, KIN rich media has highest concentration where as the least concentration was observed in media supplemented with 0.5 mgl^{-1} KIN and IAA. Similar results have been observed in *Centella*, using BAP and IBA [14,15,16]. Coconut milk rich in zeatin riboside enhanced the bacopaside level in *B. monnieri* [17] . Results obtained from these experimental data has an immense congruence with the use of cytokinin (BAP) proved antagonistic [6] where as with auxin and Kinetin proved synergistic [18,19]. Addition of BAP resulted in the increase in number of shoots, mean shoot length and number of roots/explants [14,15,17]. Alterations in metabolite profiles is considered to be cellular consequence of environmental changes . It has been reported that, concentration of growth regulators is often a crucial factor in secondary metabolite accumulation in cell and organ cultures [20] and several elicitors like Methyl Jasmonate have been utilized for enhanced production of Bacoside A in shoots [21] and *Agrobacterium rhizogenes* transformation for increased accumulation of secondary metabolites in Bacopa [22] . This study provides relevance of how different growth regulator combinations could be synergistically acting as elicitors by stimulating the accumulation of secondary metabolites in tissue cultured plants.

CONCLUSION

This study was aimed at determining the influence of auxin , cytokinins and in combination, on the morphogenetic potential of the important nootropic plant *B. monnieri* (L.) . The results exhibited

the profuse variability in the form of normal multiple shoots. The major bioactive saponins which were analyzed in the regenerated plants showed that different growth regulators could be utilized as elicitors for enhanced accumulation of secondary metabolites in tissue culture plants and offers a platform to look into the regulation of genes responsible for bacopaside biosynthesis.

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