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RESEARCH ARTICLE

In Vitro Micropropagation of *Bacopa monnieri* and Detection of Bacosides from Secondary Callus

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Abstract

Callus and shoot induction was observed from leaf, node and internode explants of *Bacopa monneiri* when grown in MS media amended with varying concentrations of auxin and cytokinin. Concentration at 0.2 mg and 0.4 mg of 2,4-D showed 20 and 33% of callus formation from leaf explant. *In vitro* callus was induced from nodal explants in media amended with napthyl acetic acid (NAA) (0.2 mg) and 6-benzyl amino purine (BAP) (0.1 mg) wherein the growth response was 33.3%. A 50% callus induction was observed in BAP (0.1 mg) and Kinetin (KIN) (0.2 mg). Shoot induction was recorded from *in vitro* generated nodal callus after 10 d. From internodal explants, 33.8 and 50% callus formation was observed in indole acetic acid (IAA) (0.2 mg), BAP (0.1 mg) and NAA (0.1 mg) and BAP (0.3 mg) respectively. Shoot induction was recorded from 15-18 d. Regeneration of secondary callus was achieved in NAA (0.3 mg) and BAP (0.1 mg) with a growth response of more than 50%. Methanol extract of the secondary callus was resolved on TLC in toluene, ethyl acetate, glacial acetic acid and methanol (3:4:1:3) and bacoside was detected as a pink coloured spot after spraying with 20% H_2S0_4 in methanol.

Keywords: Callus, shoot induction, *Bacopa monneiri*, auxin, cytokinin, 6-benzyl amino purine, bacoside.

Introduction

Bacopa monnieri L. Penn. of Scrophulariacea family, commonly known in India as 'Brahmi', is an important ancient medicinal plant used in the ayurvedic system of medicine. Reports of Shakoor *et al*. (1994) support the ayurvedic uses of Bacopa on anxiety, epilepsy, bronchitis and asthma, irritable bowel syndrome and gastric ulcers. It also has anti-inflammatory, analgesic, antipyretic, insanity, anticancer and antioxidant activities (Pandiyan and Selvaraj, 2012). *Bacopa monnieri,* besides containing saponins (Bacosides A and B) and triterpenoids as major chemical constituents, bacosides A1 and A3, betulic acid, monnierin, alkaloids bramine and herpestine also has flavonoids, luteolin-7-glucoside, glucoronyl-7-apigenin and glucoronyl-7-luteonin as minor constituents (Sundriyal *et al.,* 2013). Recent research has been focused primarily on Bacopa's cognitive-enhancing effects especially, in autistic children. Bacoside, a natural memory enhancer from Bacopa, acts on CNS where it improves grasping power, memory, intellect and speech, and corrects aberrations of emotions, mood and personality of an individual (Roodenrys *et al.*, 2002). The *in vitro* propagated medicinal plants furnish a ready source of biochemical characterization and identification of phyto-constituents (Banerjee and Srivastava, 2006). Production of metabolites of medicinal value from callus cultures through plant tissue culture paves an efficient way in intervening with the depletion of natural resources thereby, leading to the conservation of endangered plants. Moreover, bacosides do not exert any side effects as proven by clinical studies in animal models and human volunteers. These features render bacosides immensely important in pharmacology. Based on the above mentioned importance, a sincere effort is made in micropropagating *Bacopa* explants on MS medium amended with various concentrations of NAA, BAP and KIN and detecting bacosides from the secondary callus of *Bacopa monnieri*.

Materials and methods

Chemicals: Analytical grade chemicals from Hi-Media, Merck and Sigma were used throughout the study.

Collection of plant material: Healthy and disease free plant was collected from Anna University, Taramani campus, Chennai, TN, India, in the month of Feb 2014 (Fig. 1).

Fig. 1. *Bacopa monnieri.*

Table 1. Effect of plant growth hormones on callus formation.

Explant type	Media conditions (MS media) Hormone (GR) (mg/L)					pH	Growth (%)	Nature of morphogenetic response
	2,4-D	2,4-D	NAA:BAP	BAP:KIN	IAA:BAP			
Leaf	0.2	0.4	-	-	-	5.7	25 and 33	Callus induction
Node	-	-	0.2:0.1	0.1:0.2	-	5.7	33 and 50	Callus and shoot induction
Internode	-	-	0.1:0.3	-	0.2:0.1	5.7	33 and 50	Callus and shoot induction
In vitro grown callus	-	-	0.3:0.1	-	-	5.7	>50	Callus induction

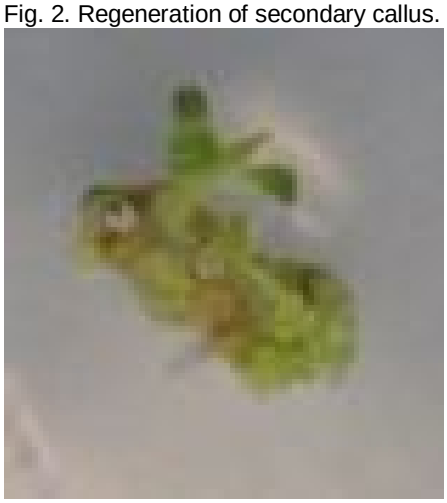
Callus and shoot induction: Well grown and healthy leaves, nodes and internodes were collected and washed under tap water for 10 min. Subsequently these were washed with 0.1% Tween-20 followed by immersion for 2-3 min in sodium hypochlorite (0.5%) and 1-2 min in mercuric chloride (0.5%) solution. The explants were finally washed 5 times with sterile distilled water, cut into small pieces (0.5-1.0 cm) and inoculated on MS medium supplemented with 0.2 mg and 0.4 mg of 2,4-D and sucrose (30 g/L) for leaf explant; NAA (0.2 mg), BAP (0.1 mg) and sucrose (30 g/L); BAP (0.1 mg), KIN (0.2 mg) and sucrose (30 g/L) for nodal explant and IAA (0.2 mg), BAP (0.1 mg), sucrose (30 g/L) and NAA (0.1 mg), BAP (0.3 mg) and sucrose (30 g/L) for internodal explants respectively. The inoculated culture bottles were incubated under dark condition at 25°C for 3 d and then transferred to 16/8 h light/dark period.

Thin Layer Chromatography (TLC): Secondary callus from *in vitro* generated callus obtained from shoot explants was extracted with methanol in 1:4 ratio for 24 h. The extract was concentrated and resolved along with wild plant methanolic extract on precoated Silica gel 60 GF254 E Merck plates. The plate was developed using toluene: ethyl acetate: glacial acetic acid: methanol (3:4:1:3 v/v) in mobile phase (Monica *et al.*, 2013) and 20% sulphuric acid in methanol was used as the spraying reagent. The plates were kept in hot air oven at 120°C for 10 min for visualization. Standard bacoside A was used as a marker for TLC evaluation.

Results and discussion

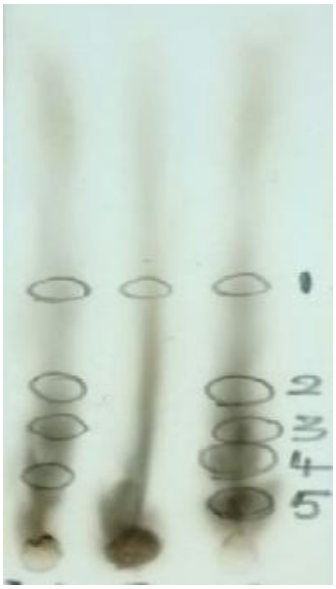
In vitro callus was induced from leaves, nodal and internodal explants in media amended with various growth regulators. After a week, the callus growth from leaf explants was 20 and 33% in 0.2 mg and 0.4 mg of 2,4-D respectively. Rout *et al.* (2011) have recorded the highest 58±0.98% callusing rate in *Bacopa monnieri* on 1.5 mg L^{-1} KIN, where a single concentration of BAP (2.0 mg L^{-1}) along with different concentrations of NAA or IAA were applied, the maximum callusing rate of 71±2.2% was observed on 2.0 mg L^{-1} BAP + 0.5 mg L^{-1} NAA which was more than the 2.0 mg L^{-1} BAP. The role of auxin alone or in combination with cytokinin for callus proliferation is well documented (Hammerschlag *et al.,* 1985; Jain *et al.,* 1988; Verhagen and Wann, 1989; Roy and De, 1990).

Fig. 2. Regeneration of secondary callus.



Nodal explants in media containing NAA (0.2 mg), BAP (0.1 mg); BAP (0.1 mg), KIN (0.2 mg) and sucrose (30 g/L) showed 33.3 and 50% callus growth respectively along with shoot induction. Tiwari *et al*. (1998) has also reported prolific callus formation from nodal explants of *Bacopa* cultured on MS medium containing 0.5 mg/L 2,4-D. Thejavathi *et al.* (2001) has also used nodal explants for micropropagation studies of *B.monnieri* L. internodal explants in media containing NAA (0.1 mg), BAP (0.3 mg) and sucrose (30 g/L) exhibited callus growth of 33.8 and 50% respectively with one or two shoots. Regeneration of secondary callus was achieved in NAA (0.3 mg) and BAP (0.1 mg) with a growth response of more than 50% (Fig. 2). Rapid callus growth (90%) response was observed in the MS medium with 0.5 mg/L NAA, 0.5 mg/L 2,4-D and 2,4-D 0.5 mg/L with TDZ 0.15 mg/L, TDZ 0.05 + NAA 0.4 mg/L and TDZ 0.05 + NAA 0.5 mg/L in combinations of growth regulators (Vijaykumar *et al.,* 2010). The callus methanolic extract along with the standard bacoside was resolved on TLC using ethylacetate:methanol:toluene:water (4:1:1:0.5) as mobile phase. After drying, the TLC plate was sprayed with 20% sulphuric acid in methanol which revealed pink coloured bands with Rf value of 0.12 (5), 0.18 (4), 0.2 (3), 0.3 (2) and 0.49 (1) respectively (Fig. 3 and Table 2). The band with Rf value of 0.49 corresponds to the active principle bacoside. The chromatogram of callus extract exhibited a new band of high intensity with Rf value of 0.12 and 0.18 which was not found in the wild plant extract.

Fig. 3. TLC profile of callus extract and standard bacoside.



W–Wild plant extract; S–Standard bacoside, C–Callus extract
Solvent system-Ethylacetate:Methanol:Toluene:Water (4:1:1:0.5).

Table 2. TLC profile of wild and callus extract of *Bacopa monnieri.*

Spot	Rf value		
	Standard	Wild	Callus
1	0.49	0.49	0.49
2		0.3	0.3
3		0.2	0.2
4		0.15	0.18
5		-	0.12

Monica *et al.* (2013) have also reported the presence of novel bands from callus extract which were not shown by the natural plant system. Bacoside synthesis was detected mainly in the matured callus. Talukdar (2014) has reported enhanced production of bacoside from callus culture (1.53%) than that extracted directly from the field grown plants which was recorded at 1.02%. This proves that enhanced production of bacoside can be obtained by callus culture against that obtained from *in vivo* grown plants. Using *in vitro* cell suspension cultures of *B. monnieri*, 5-6 fold well grown over 40 d, Rahman *et al.* (2002) have recorded up to 1 g/100 g dry weight of bacoside. An increase in total saponin bacoside content of 166% obtained from suspension culture established from callus biomass was reported by Jain *et al.* (2013). For quantification of bacosides as well as active ingredients in plant extracts and cell cultures various researchers have adopted several methods like HPLC (Pal *et al*., 1998; Ganzera *et al.*, 2004), Spectrophotometry (Pal and Sarin, 1992) and HPTLC (Shrikumar *et al*., 2004).

Conclusion

The synthesis and detection of bacoside in the secondary callus of *Bacopa monneiri* as observed in the present study paves a way for enhanced qualitative supply under controlled conditions. An increase in the active principle content will be made possible by cell suspension culture as reported by various workers. Propagation of *B. monniera* through seeds is slow due to short viability and frequent seedling death. Its vegetative propagation is slow due to poor performance of propagules (Volluri *et al.*, 2011). Therefore, this method will overcome the inadequacy and extinction of the raw material from the natural sources. Future studies can be targeted on standardization of large scale production of *Bacopa* saponins in bioreactors to meet the increased demand in the area of neuropharmacology.

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