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An Enhanced *In-Vitro* Production of Saponins and Other Bioactives from *Bacopa monnieri* L. Penn.

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ABSTRACT

An important herb *Bacopa monnieri* commonly called Brahmi, is a well known source of neuroprotective saponins. The whole plant is commercially used in ayurvedic preparations as well as for extracting their bioactives at an industrial level to use them in herbal medicines and hence they are in great demand. Since they are mainly collecting from the wild, the plant is under high anthropogenic pressure. Moreover, the bioactive contents in the wild plants may vary due to environmental influence also. In this scenario, the current study was conducted to develop a biotechnological intervention to produce elite *Bacopa* plants with uniform bioactives though *in vitro* direct shoot regeneration of *Bacopa monnieri* from leaf explants using Murashige and skoog medium containing various growth hormones such as 6-benzyl aminopurine (BAP), Kinetin (KIN) and Thiodizuron (TDZ). Maximum shoot bud (162.33 ± 21.385) formation was observed in MS media supplemented with 0.2mg/L BAP and the shoot buds were developed into shoots in 4 weeks time. Rooting of these shoots was obtained in MS basal medium. Rooted plants were hardened successfully in *in vivo* conditions at 98.5% frequency. The *in vitro* shoots as well as field established plants that were raised through tissue culture were analyzed and compared with the mother plant for their phytochemical constituents with respect to phenols, flavonoids and saponins. Though flavonoids present in the *in vitro* shoots (277.5 ± 0.022 µg/g DW) showed a lesser value in compared with *in vivo* plants (397.5 ± 0.080 µg/g DW), the phenols and saponins present in *in vitro* plants (26.25 ± 0.084 µg/g DW and 92.6± 1.113 mg respectively) were comparable to *in vivo* plants (27.25± 0.056 µg/g and 93.1± 1.973 mg respectively). The *in vitro* culture system developed can be further scaled up to cater the need of this plant in future for industrial purpose.

Keywords: *Bacopa monnieri*, Shoot regeneration, growth regulators, phenols, flavanoids, saponins.

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INTRODUCTION

Bacopa monnieri L. (Family of scrophulariaceae), commonly known as brahmi in Indian system of medicine is a small, creeping and glabrous herb with purple flowers. The medicinal properties of *Bacopa monnieri* as memory and intelligence enhancer [1], anti-anxiety and anti-depressant agent and as an agent to treat poor cognition is well established. It is also used as a curative medicine for asthma, mental illness, and epilepsy [2, 3]. Anti-ulcerogenic activity [4], anti-cancer activity [5] and antioxidant properties [6] of this plant also endorsed the medicinal importance of this plant. The bioactives which are attributed to pharmacological activities of *Bacopa* are mainly classified into the groups of alkaloids, saponins and flavonoids. The major bioactive saponins are bacoside A and B [7,8]. The other Chemical constituents of the plant include bacoside A1, stigmasterol b- sitosterol, hersaponin and betulinic acid. According to [9] the main triterpenoid Saponins Bacoside A3 and *Bacopa*side II are responsible for the neuropharmacological effects of the plant. Bacosides acts on CNS and also improves the memory, speech and also corrects emotional aberrations and personality of an individual [10]. Based on clinical studies (Animal model and human volunteers), it has been proven that bacosides do not have any side effects [11] and may be recommended as a safe herbal drug to cure many disease. In plants, different environmental parameters such as temperature, rainfall, humidity tend to affects the production and types of secondary metabolites. Hence, growing plants in controlled environmental conditions using *in vitro* techniques can be an effective tool for secondary metabolite production. In the present study, we aimed at a rapid and enhanced regeneration of an elite *Bacopa monnieri* plant using leaf as a major explant and subsequent production of bioactive enriched shoot cultures and their establishment in pots for sustainable production of the phytochemicals without much variation in their content and quantity.

MATERIALS AND METHODS

Tissue culture

Saplings of disease free *Bacopa monnieri* plant material was collected from Biotechnology centre, Bannerghatta road, Bangalore and reared in 12" pots near the laboratory premises. Uniform sized healthy leaves were collected was washed under running tap water with 1 to 2 drops of laboline for 15 minutes to clean the external dust/contaminants. This was followed by gentle wash in distilled water for 20 minutes for two cycles. Afterwards, the explants were sterilized with 0.1% Mercuric chloride for 7 minutes in a laminar air flow. Then the explants were removed from the solution and rinsed thoroughly for a minimum of five times with sterile distilled water. These explants were cut into pieces (0.3 – 0.5 cm) longitudinally, and inoculated in MS media containing 1.5% gelzan, 3% sucrose and varying concentrations and combinations of plant growth regulators (BAP 0.1to 1.0mg/L, Kinetin 0.1 to 1.0mg/L, TDZ 0.01 to 0.2mg/L individually or in combinations) [12]. Cultures were incubated at 25±2ºc under 50-60% relative humidity and at a 12hr photoperiod. A photon flux intensity of 50-60μEM^{-2} s^{-1} at the level of the cultures was provided by cool white fluorescent tubes (Philips, Mumbai). *In vitro* developed healthy shoots were transferred to MS basal media as well as the media supplemented with varying concentrations of auxins (IAA 0.1to 2.0mg/L and IBA 0.1to 2.0mg/L) for root induction. Three weeks after root induction, the *in vitro* rooted plants were washed under running tap water to remove the traces of medium and transferred to polybag containing sand and soil in the ratio of 1:1. The plants were kept in a mist house for 4 weeks for their acclimatization. Then the healthy plantlets were successfully transferred to the pots.

Phytochemical analysis

Mother plants as well as *in vitro* derived shoots were collected, shade dried and powdered by using a morter and pestle. A known amount of the sample was refluxed for 6hrs with ethanol in a ratio of 1:10 (wt/v).The extract was filtered and made dry by evaporating the solvent in a rotor evaporator. The residue was weighed and purified or resuspended at a suitable concentration with different solvents and used for the estimation of different phytochemical constituents.

Estimation of total phenols

The total phenol in the plants were estimated by folin- ciocalteau method [13] gallic acid was used as a standard at a concentration of 10 to 50 µg/ml and test solution (plant extract) 0.1g (dw)/ ml were made upto 3ml with distilled water. 0.5 ml of folins phenol reagent was added to all tubes and incubated for 3min at

room temperature. And 2ml of 20% sodium carbonate was added and kept in boiling water bath for 1 min followed by measuring the absorbance of these samples at 650nm using UV-Vis spectrophotometer.

Estimation of total Flavonoids

Aluminium chloride method is used to estimate the amount of flavonoids in the plant by following the method developed by [14]. In this procedure, working standard concentration was kept from 50 to 250 µg/ml and test solution (plant extract) 0.1g/ ml were made upto 2.5ml with distilled water. 75µl of 5% $NaNO_2$ was added and kept 5mins for incubation at room temperature. Then, 150µl 10% Aluminium chloride added to the tubes. After 6min, 0.5ml 1N sodium hydroxide was added and absorbance was measured at 510nm using UV-Visible spectrophotometer.

Extraction of Saponins

The total saponins were extracted from the whole plant of *Bacopa monnieri* by using a standard protocol with appropriate modifications [15]. One gram of powdered plant material was taken in a test tube with 20 ml of 20% aqueous ethanol, and incubated it in water bath at 55˚c for 4 hrs with continuous stirring. The mixture was filtered and re- extracted with 20ml of 20% aqueous ethanol. The re-extracted filtrate was combined s in a 250 ml separating funnel. Then, 20 ml of petroleum ether was added and shaken vigorously, aqueous phase was collected. The aqueous phase was taken in a separating funnel and 60 ml of n-butanol was added. By vigorously shaking, 10ml of 5% aqueous NaCl was added. To remove traces of solvent, upper layer was collected and washed repeatedly with aqueous NaCl. The upper layer was collected and heated in a water bath, followed by drying in an oven at 40˚C.

RESULTS AND DISCUSSION

***In vitro* culture of leaves**

The explants subjected to culture initiation were free from colored phenolic oxidates but colorless exudates released from the cut basal ends into the medium were not turned brown. Current study was to raise the multiple shoots from small slices of leaf explants. Irrespective of the leaf segments, the explants inoculated in MS medium containing hormones (BAP 0.1 to 1.0 mg/L, KIN 0.1 to 1.0 mg/L, TDZ 0.05 to 0.2 mg/L), all responded with shoot bud initiation on the 5^{th} day of inoculation (Fig 1b) and fully formed shoots were formed in three weeks of culture in the same media (Fig 1c&1d)) supplemented with various cytokinins. However, there was no shoots bud formation from any of the leaf segments inoculated in basal medium (Fig.1a). Adventitious shoot formation is a reliable technique for clonal propagation as it prevents somaclonal variations in the cultures. The type of tissue or explant used for clonal multiplication also influences the chances of genetic variation. Because of the non- uniform nature of callus tissue [16].

Many other research studies on other medicinal plant species have shown the use of cytokinin alone or in combination with other in different concentrations. For example for *Paederia foetida* and *Centella asiatica* multiple shoots were obtained in MS medium supplemented with BAP 1.0 mg /L [17], and in *Rauwolfia serpentina* on MS medium supplemented with BAP and NAA [18]. The large number of shoot buds were formed as a function of cytokinin activity and among the three cytokinins tested, BAP supplemented at an optimal concentration of 0.2mg/l was the best to induce formation of up to 162.33 ± 21.385 callus free healthy shoot buds (Table 1) from a single explants at 100% frequency. Enhanced shoot formation in the sub-cultured leaves was consistent with the improved acclimatization and participation of the available meristems in shoot multiplication reported in other species as well [19]. Relatively fewer shoots were obtained in other concentrations and at higher concentrations callusing was prominent and the formation of shoot buds was less. Callus induction stimulated by all the cytokinins at higher concentrations also induced differentiation of a few condensed shoots scattered over a massive greenish callus. Each leaf segments with healthy green shoot buds were sub-cultured in basal MS media and also with cytokinin supplementation were more caulogenic with substantial increase in harvestable shoots per explants than the primary leaf segments employed for culture initiation. [20] inferred from that the size of the leaf and the position of explants in media plays an important role for the regeneration of adventitious buds. Our results didn't show any variation in the frequency of regeneration with respect to the size of leaves or how they placed in the media. The shoots excised individually or as clumps (3.5-4.5 cm) transferred to basal medium devoid of hormone rooted at an

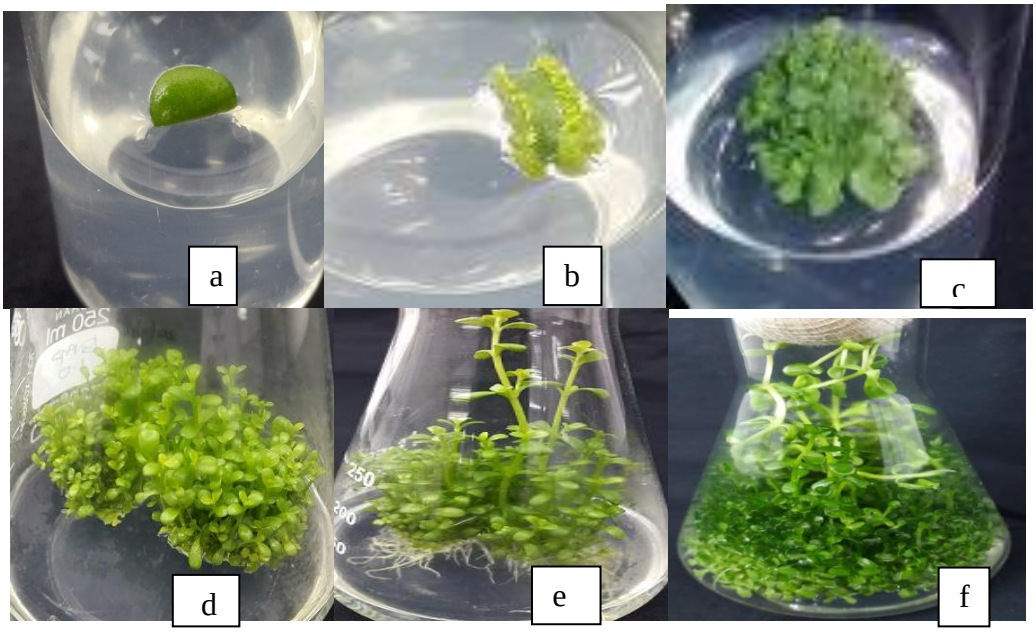
equal efficiency (100%) with the formation roots after 15-20 days (Fig. 1e). Though supplementation of the medium with auxins (IAA, IBA) at 0.1-1.0 mg/l increased the number of roots per explants, specific requirement of any auxins were not essential for rooting and subsequent establishment of the shoots in soil. During root formation also, the shoots showed continuous growth with respect to the length as well as in number (Fig.1f).

The rooted plants were weaned from the flasks and washed under running tap water to remove the adhered media and agar (Fig.1g). The plants were transplanted in disposable cups with potting mixture containing top soil and sand (1:1) and maintained under regular irrigation in 75% shade showed 100% establishment (Fig.1h&i). Hardening protocol was assessed by calculating its survival percentage. Similar studies at *in vitro* conditions were carried using many plants such as *Morus indica L.* [21], *Withania somnifera Dunal*. [22], *Musa cuminata Colla* [23] and *Terminalia arjuna Roxb* [24].

Table 1: Effect of cytokinins on *in vitro* regeneration from leaf of *Bacopa monnieri*

PGR's (mg/l)			Mean number of	
BAP	**Kinetin**	**TDZ**	**Shoot buds**	**Frequency of bud formation**
Basal	Basal	Basal	0	0
0.1	-	-	124.21 ± 18.062	95
0.2	**-**	**-**	**162.33 ± 21.385**	**100**
0.5	-	-	138 ± 23.259	80
1.0	-	-	119.67 ± 13.05	70
-	0.1	-	102±22.17	60
-	0.2	-	115.33± 12.503	60
-	0.5	-	128.33 ± 16.502	90
-	1.0	-	119.67 ± 32.393	65
-	-	0.05	139.33 ± 15.947	95
-	-	0.1	124.33 ± 31.21	85
-	-	0.2	131.67 ± 12.342	90

This data represents the mean number of 20 replicates repeated twice, recorded after 4weeks of culture values followed by the same letter in the superscript.



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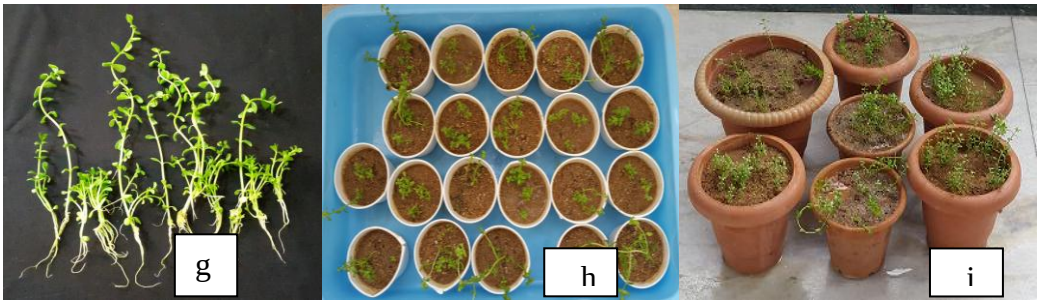

Figure 1: a-Leaf explant in basal medium b&c- shoot initiation from leaf tissue d- *in vitro* shoot multiplication e- *In vitro* rooting of shoots f- completely grown plants g-planlets h&i- plants established in soil.

Estimation of total phenols, flavonoids and saponin content in the *in vitro* multiplied shoots and their mother plant

The capacity for producing and accumulating many valuable chemical compounds similar to the parent plant in nature by plant cell, tissue, and organ cultures has been highlighted from the inception of *in vitro* technology. The strong and growing demand in today's market for natural, products has refocussed attention on *in vitro* plant materials as a potential source for bioactives, and has paved a new way for research exploring secondary metabolite expression *in vitro*. The deliberate stimulation of defined chemical products under highly controlled micro-environmental regimes also provides an excellent way for indepth investigation of biochemical and metabolic pathways. Phenolic compounds mainly act as free radical terminators and bioactivities to inhibit lipoxygenase and to chelate metals [25]. The analysis showed that the total phenol content present in the *in vivo* plant was 27.25 ± 0.0084 µg/g DW was comparably equal to the *in vitro* plant in the range of 26.25 ± 0.0056 µg/g DW. Biotechnological approaches, specifically plant tissue culture plays a vital role in search for alternatives to production of desirable medicinal compounds from plants. Since it was observed, that production of secondary metabolites is generally higher in differentiated plant tissues, there were attempts to cultivate whole plant organs, i.e. shoots or roots under *in vitro* conditions with the aim to produce medicinally important compounds [26].

Flavonoids are a group of compounds having numerous biological activities including inhibition of cell growth, inhibition of protein kinase activity, inhibition of apoptosis, inhibition of MMP secretion, inhibition of tumor cell invasion, and inhibition of adhesion and spreading of cells; flavonoids also have anti-angiogenic properties. It is reported that the intake of foods containing flavonoids reduces even the risk of cancer. Flavonoids are also tried to produce by using different biotechnological approaches, such as callus cultures, cell suspension cultures and/or organ cultures [27]. The flavonoid content present in the *in vivo* plant was 397.5±0.080 µg/g DW more than the *in vitro* plant 277.5± 0.022 µg/g DW. The presence of high flavonoid content in the plant will have greatest antioxidant property can be explore to produce new drugs [28].

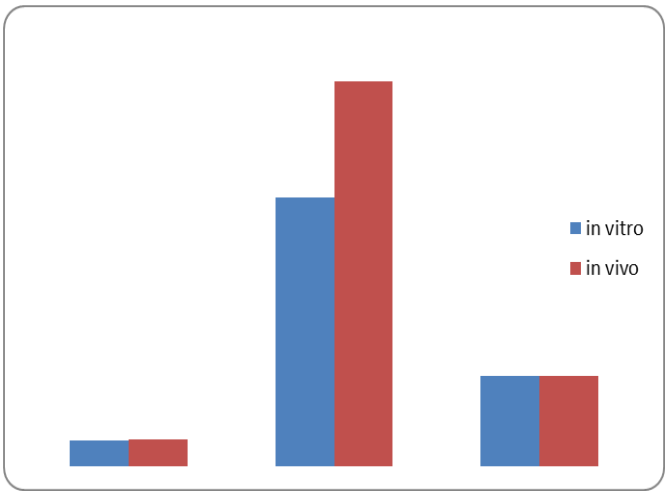

Figure 2: Total phytochemical content of *in vitro* and *in vivo* plant of *Bacopa monnieri*.

The saponin was extracted based on [15] protocol. In *in vitro* grown shoots of *Bacopa*, saponins content was estimated to be 92.6 ± 1.1135 mg/gm (DW) which was almost equal to and comparable with the same in the mother plant (93.1 ± 1.9731 mg/gm (DW). Fig.2 describes a comparative analysis of phenols, flavonoids and saponin present in the *in vivo* and *in vitro Bacopa* plant. Since the shoot cultures produced *in vitro* is endowed to produce a comparable amount of the bioactives with the *in vivo* plants, we can expect this methodology of producing bioactive shoot cultures to contribute to the future studies in this plant species for large scale production of certain bioactives. Especially accumulation of saponins in the shoot cultures can be exploited for its large scale production in a possible array of memory enhancing agents in future.

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