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Effect of different carbon sources on *in vitro* regeneration of Brahmi *Bacopa monnieri* (*L.*) An important memory vitalizer

Preeti Srivastava, Kavindra Nath Tiwari and Gaurava Srivastava

Abstract
Brahmi (*Bacopa monnieri*) is an important medhyarasayan plant and it is widely used in Indian traditional system of medicine for the improvement of memory/brain function. This medicinal herb is exploited from the wild population all over world so there is need to develop the *in vitro* protocol for the mass multiplication under various conditions and its conservation. The International Union for Conservation of Nature and Natural Resources (IUCN) has a long time ago listed *B. monnieri* as a threatened species.
MS medium containing 8.9 µM BA was found most suitable for induction of high frequency shoot buds from different explants e.g., leaf and node. We had evaluated different concentration range (0.5-10%) of different carbon sources (sucrose, mannitol and glucose) for enhancement of this regeneration event. It was observed that MS media modified with 5% sucrose was found most effective for shoot bud induction and shoot regeneration from nodal explants and it ranges 22.6 shoots/explants and for leaf explant 3% sucrose was found effective with 20.6 shoots/explant. These shoots were further elongated on MS basal media without growth regulator. For getting maximum rooting these elongated shoots were transferred on MS media containing 4.9 µM IBA. The plantlets after acclimatization transferred into field with 90% survival rate. Thus the present study could be utilized for better multiplication and conservation of this important threatened species.

Keywords: *Bacopa monnieri*; *in vitro* regeneration; carbon sources; growth regulator

Introduction
Plant cell, tissue or organ culture normally required the incorporation of carbon source to the culture medium [1-4]. Several tissue culture reports refer to the influence of the carbon on *in vitro* morphogenesis of different plant species. Among the many available carbon sources, sucrose has been major and most common one [5-8]. Sucrose is often assumed to be the best sugar in cell culture media because it is main transport sugar in most plant species. In some cases, sucrose is partially [9] or totally replaced by other carbon sources such as mannose, galactose, cellobiose, lactose and melibiose with different levels of success for supporting growth in culture [1, 10].
The sterilization method may also influence culture response due to alterations in pH, hydrolysis of sugars, and formation of toxic compounds during autoclaving [11]. When media containing sugars are autoclaved, the pH generally decreases. This acidification is most pronounced when monosaccharides were added [11-12], especially fructose [11], and when medium pH prior to autoclaving was between 5 and 8 [13]. During autoclaving of sucrose up to 25% can be hydrolyzed to fructose and glucose [11, 14], but the degree of hydrolysis depends on temperature during autoclaving, exposure time, volume autoclaved, initial pH, and other constituents of the medium.
High temperatures during autoclaving of culture media can result in formation of toxic compounds, such as 5-(hydroxymethyl)-2-furaldehyde (HMF). These compounds are primarily formed from fructose, whether added initially or produced during hydrolysis of sucrose [11, 15]. The toxicity of HMF can be eliminated by addition of activated charcoal [15-16], but activated charcoal also, non-specifically, adsorbs other compounds from the medium such as growth regulators [15, 17].

Bacopa monnieri (*L.*) Wettst (Scrophulariaceae) commonly known as Brahmi or Nirbrahmi is an ancient and renowned medicinal plant with legendary reputation as a memory vitalizer. In the traditional system of Indian medicines (Ayurveda), Brahmi is classified as medhya rasayana i.e., a drug which is supposed to minimize the effects of mental stress with improvement of intelligence and memory function and also effective in case of anxiety and necrosis [18-19]. Due to over-exploitation of large number of medicinal properties the plant is enlisted in endangered category [20].

This plant has wide variety of medicinal properties including anti-inflammatory, analgesic, antipyretic activities [21-22]. This traditional plant is being used for the treatment of epilepsy and insomnia, as a sedative and abolishing raw anxiety in India for 5,000 years [23]. Indian Materia Medica (Bhavaprakasha Nighantu 1,500 years AD) recommends this material as a means of improving memory and concentration) [24-25]. Vangalapati *et al*., [26] reported that commercially available preparations of *B. monnieri* improve brain function, concentration and memory in both young and older people. *B. monnieri* has several biological properties such as anti-inflammatory, analgesic, antipyretic, sedative, antiepileptic and antioxidant activity, immuno-modulatory, memory-enhancing property, anti-stress, adaptogenic activity, antianxiety, and anti-cancer [27]. Due to the presence of several pharmaceutically important compounds and multipurpose therapeutic uses of *B. monnieri*, these plants have been deleteriously harvested from the wild making them a threatened species [28-29].

In a recent study, *B. monnieri* was placed second in a priority list of the most important Indian medicinal plants evaluated on the basis of medicinal importance, commercial value and potential for further research and development [30-31]. *Bacopa* contains several alkaloids such as nicotine, Brahmin, herpeetine, saponins, such as hersaponin, bacoside A, B, C & D; several other chemicals like stigmastanol, β-sitosterol & stigmasterol [32-33]. An alcoholic extract of *Bacopa* showed anticancer activity against Walker carcinosarcoma 256 (intramuscular) in rat [34] and significantly inhibited sarcoma 180 cell culture growth, possibly by affecting DNA replication [35]. It also improved the performance of rats in several learning tests which measured the acquisition, consolidation and retention of the learnt responses [36].

B. monnieri has been recognized as the major priority species on the basis of their medicinal importance, commercial value & potential for further research & development [31, 37]. The establishment of contamination free nodal cultures (85%) of *B. monnieri* required the addition of an antibiotic trimethoprim (TMP; 50 mg/l) & fungicide bavistin (BVN, 50 mg/l) in GR-free MS medium [38], and this combination were synergistically enhance the micropropagation [39].

The objective of this study was to determine the effect of different type and concentration of sugar on *in vitro* regeneration using different explant type of *B. monnieri*. By optimizing the best sugar type and concentration for tissue culture media to achieve maximum proliferation from different explants of *B. monnieri*.

Material and methods

Plant material

Plants of *B. monniera* were collected from the 'Ayurvedic garden' of Banaras Hindu University. Terminal shoots bearing 4 – 5 nodes were cut off from the plants growing in the field, washed under running tap water for 10 min, and soaked in 1% cetrimide solution (Hi Media, Bombay, India) for 10 min. Surface sterilization was done as follows: a quick dip in 70% ethanol, a 6-min treatment with 0.1% $HgCl_2$ solution and, finally, six washes with sterilized distilled water. Segments containing a single node of the surface-sterilized shoots were placed vertically on (MS) basal medium to which 150 mg/l bavastin (BASF, Bombay, India) and 50 mg/l trimethoprim (Hi Media, Bombay, India) were added before autoclaving. The disinfected cultures were transferred to MS basal medium after 14 days. The shoots produced from the axillary buds of the nodal explants were used as the source of single node (1 – 1.5 cm), internode (about 1 cm) and leaf (proximal 1/4th portion trimmed) explants for subsequent experiments. All the explants were placed horizontally on the medium, and the leaves were placed with their dorsal side upward.

Culture conditions

Single disinfected nodal segments and leaves were cultured on MS basal medium (1962) supplemented with 3% (w/v) sucrose (Himedia, India) and 0.8% (w/v) agar for culture initiation and these served as explant sources for subsequent experiments. The pH of the medium (supplemented with respective growth regulators) was adjusted to 5.8 with 1 N NaOH or 1 N HCl before addition of 0.8% (w/v) agar (Himedia, India). In all the experiments, the chemicals used were of analytical grade (Himedia and Sigma). The medium was dispensed into culture vessels (Borosil, India) and autoclaved at 105 kPa (121ºC) for 15 min. The surface-disinfected explants were implanted vertically on the culture medium [test tubes (150x25mm) containing 15 ml medium] and plugged tightly with non-absorbent cotton. All the cultures were incubated at 25±2 ºC under 16 h photoperiod of 45 – 50 µmol m^{-2} s^{-1} irradiance provided by cool white fluorescent tubes (Philips, India) and with 55 – 60% relative humidity (RH). All subsequent subcultures were performed at four weeks intervals.

Effect of Basal media

The MS media (1962) was evaluated for their effects on *in vitro* growth and development of *B. monnieri*. All the basal media contained 3% (w/v) sucrose and solidified with 0.8% (w/v) and 8.9 µM BA. Different concentrations of cytokinins, including 0.44-22.2 µM of Benzyladenine (BA), 0.46-23.2 µM of Kinetin (Kin) and 0.45-22.7 µM of Thidiazuron (TDZ) had been tested and 8.9 µM BA was found most effective.

Effect of Carbon sources

Nodal segments and leaves were cultured on MS medium supplemented with different type of sugar (sucrose, glucose and mannose) and different concentrations range (0.5-10%) including 3% (w/v) sucrose, 0.8% (w/v) agar and 8.9 µM BA as a standard.

Acclimatization and transfer of plantlets to soil

Plantlets with well-developed roots were removed from the culture medium and after washing the roots gently under running tap water, plantlets were transferred to plastic pots (10 cm diameter) containing sterilized soilrite (Kel Perlite, Vishwasnagar, Karnataka) and garden soil (2:1). All were irrigated with 1/8 MS basal salt solution devoid of sucrose and inositol every 4 days for two weeks. The potted plantlets were covered with porous polyethylene sheets for maintaining high humidity and were maintained under the culture room conditions. The relative humidity was reduced gradually and after 30 days the plantlets were transplanted to a botanical

evaluation garden and kept under shade in a net house for further growth and development. The morphology, growth characteristics and floral features were examined.

Statistical analysis

The effect of different treatments on multiple shoot bud induction was compared to detect the significance of differences among the treatment means using a three-factor nested design (ANOVA) and Duncan multiple range test (DMRT) at a 5% probability level according to Gomez and Gomez [40]. The experiment had two replicates; each replicate consisted of 20 culture tubes. The frequency and number of adventitious shoot buds were recorded after 4 and 7 weeks. Results of subculture experiment was expressed in terms of mean values ± standard error with two replicate, each containing 10 explants. In the rooting experiment treatment means were compared by randomized block design (RBD) and DMRT having a total of 40 explants in two replicates.

Result and Discussion

The response of *in vitro* culture to different carbon source added to the medium frequently tested during medium optimization. Sugars have two functions in the culture media as carbon source and as osmotic regulator. Cell growth depends on carbon compound utilization for the formation of major cell components and as an energy source. The percentage of leaf explants with callus was affected by different carbon source, where as to some extent sucrose affected the callus regeneration from nodal explant too (Table1).

Carbon sources and shoot regeneration

This study evaluated the effect of different carbon type and concentration on *in vitro* regeneration of *B. monnieri* when incorporated into the medium. Although carbohydrates are of prime importance for *in vitro* organogenesis [41], carbon metabolism *in vitro* is still not clearly understood [42]. It is well established that carbohydrate requirements depend upon the stage of culture and may show differences according to the species [43]. Nodal as well as leaf explants were cultured on MS medium supplemented with 0.5 – 10% (w/v) of different sugar i.e.: sucrose, mannitol and glucose. These media were supplemented with 8.9 µM BA and 0.8% (w/v) agar. Among the three carbon sources, sucrose was found best for both the explants over mannitol or glucose for shoot regeneration (Table 1). Similar results were obtained in micropropagation of Cork oak where sucrose was proved to be the best choice as carbon source [44], *Kaempferia* [45]; *Cocos nucifera* L. [46]; *Mentha piperita* [47], *Stevia rebaudiana* [48]; *Metroxylon sagu Rottb* [49] and *Nauclea diderrichii* [50]. This is due to its efficient uptake across the plasma membrane [51]. However, glucose was most effective for shoot proliferation in Prunus [52], beech culture [53], *Dendrobium* [54] and Strawberry Cv. "Elsanta" [3]. The shoot multiplication was greater in the presence of sorbitol than sucrose [55-56]. Similarly, fructose (4%) was reported as best sugar for *in vitro* regeneration of *Solanum viarum* [57] and *Solanum nigrum* (Linn.) (Sridhar and Naidu, 2011) [58]. Sucrose and glucose gave a similar rate of proliferation in sour cherry [51]. However, sucrose and glucose induced highest frequency of organogenesis in *Bixa orrellana* [59]. Various authors have reported that different carbon sources such as glucose, fructose, mannitol and sorbitol are significantly important for *in vitro* regeneration of *Asparagus* [60], Cucumber [61]. Satisfactory shoot proliferation from nodal explants was obtained on sucrose (5 %, w/v) whereas glucose and mannitol resulted in less number of shoots and shoot lengths also varied. In case of sucrose there is an increasing order of shoot regeneration with increasing the concentration from 0.5 % to 5 %, but at 10 % only few buds were visible similarly in case of Strawberry Cv. "Elsanta" [3].

In present study sucrose was found optimum for both nodal as well as leaf explants regeneration. For nodal and leaf explants, 5% and 3% sucrose supplemented media were found optimum respectively (Fig 1 a., b. and 2 a., b.), where as in case of mannitol for nodal explants 5% concentration was optimum but shooting frequency was less than sucrose and for leaf 2% was optimum but shooting frequency was very low as well as very less difference among shooting of 1 and 2%. For glucose supplemented media, both for nodal and leaf explants 2% was found optimum but shooting frequency was very low as compare to both sucrose and mannitol (Table 1). A similar response was observed in Prunus [52]. Rasheed and Yaseeen, [62] reported 3% sucrose as optimum for growth and multiplication of *Asparagus densiflorus*. Alkhateeb [63] reported that above 6% sugar concentration rooting efficiency get reduced in date palm.

Carbohydrates are also critically important for embryo formation and somatic embryogenesis. Slesak and Przywara [64] reported that fructose was not given any embryogenesis whereas glucose was given very less embryo formation but maltose was found optimum for embryogenesis in *Brassica napus*. MS medium augmented with 3% fructose was the best carbohydrate for the production of multiple shoots followed by sucrose, maltose and glucose from nodal explants of *Sphaeranthus indicus* [65].

Zavattieri *et al*., [66] reported that there was no difference in rooting frequency on different media supplemented with different sugars. In case of shoot cultures of the apple Scion cultivar Macspur no difference was achieved in shoot multiplication between sucrose, mannitol and glucose, whereas for shoot elongation on manitol followed by glucose was found optimum. Whereas, glucose and mannitol promoted root induction slowly even at thirty days of culture. However, sucrose readily promoted light green silky as well as lengthy roots. All the roots turned white after forty-five days of culture. It was difficult to isolate shoots with roots from each carbon source containing shoot multiplication medium (MS + 8.9 µM BA) because of damage to the roots which were difficult to harden. All the further experiments were conducted on sucrose.

Caulogenesis

The sugar type and sugar concentration were affect the callus formation *in vitro*. In case of sucrose with increasing the concentration from 0.5 to 5% caulogenesis also increased and there was no callus formation in 10 %, where as in case of mannitol and glucose the order found was random. Michel *et al*., [67] reported among 6 different sugar evaluated 4% glucose was found optimum for caulogenesis in *Gossypium hirsutum* L.

Rooting and establishment in field

The excised shoots showed 100% rooting within two week of subculture in all the treatments. For rooting of the shoots normal 3% sucrose supplemented full MS medium was found optimum similar to *Eclipta alba* [68] (Fig 3 a., b.). Rooting from the basal end of the shoots occurred on medium without growth regulators, but better results were obtained with the addition of auxins at 4.9–5.7 μM. The root number and length

was maximum in 4.9 μM IBA supplemented medium [69] with a 1.6- and 1.3-fold increase compared to the control. MS medium supplemented with IBA (4.9 µM) was more effective for root induction than IAA and NAA (Fig.2) [70]. However, IAA and NAA formed slender roots in both medium.

Less amount callus formation occurred in all the types of auxins in full strength MS media. IBA favored rooting in other medicinal plants also [71-72]. IBA was more effective for root induction than IAA or NAA. Similar responses were observed in different plant species. [73-75].

Hardening of regenerated plants

The rooted plantlets established well upon transfer to pots containing sterilized soilrite. Plants after hardening for 3 weeks were transferred to Ayurvedic garden of Banaras Hindu University with almost 100% survival. 100% plantlet survival was seen after hardening on garden soil and soilrite mix (1:2) for three weeks (Fig.4). There was no detectable variation among the acclimatized plants with respect to morphological growth characteristics and floral features. All the micropropagated plants were free from external defects.

Conclusion

Present study revealed that the *in vitro* plantlet production system was achieved on MS medium + 8.9 μM BA with 5% sucrose for nodal explants and 3% sucrose for leaf explants. The highest number of roots was achieved on full strength MS medium containing 4.9 µM IBA and it was very suitable for hardening. In conclusion it may be stated that the protocol presented in this study yields efficient shoot and root regeneration for nodes and leaves due to effect of variation of sugar type and concentration. These results will encourage large scale micropropagation of this important medicinal plant. The protocol reported here could also be used for conservation of this medicinal herb.

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Table 1: Effect of different sugar type and concentration on nodal and leaf explants of *Bacopa monniera*

Explant type	Type of Sugar	Concentration of Sugar (%)	Callogenesis	Organogenesis		
				No. of buds ± S.E.	No. of shoots ± S.E.	Length of shoots (cm) ± S.E.
Node	Sucrose	0.5	+	6.0 ± 0.58^{d}	4.5 ± 0.29^{c}	0.8±0.1^{e}
		1.0	++	13.9 ± 0.47^{c}	11 ± 0.29^{bc}	1.8±0.26^{d}
		2.0	+++	21 ± 0.58^{b}	14.4 ± 0.30^{b}	2.4±0.34^{c}
		3.0	++++	35.4 ± 0.31^{ab}	18.7 ± 0.24^{ab}	3.9±0.11^{b}
		5.0	++++	36.5 ± 0.87^{a}	22.6 ± 0.31^{a}	5.5±0.23^{a}
		10.0	--	1.1 ± 0.33^{e}	-	-
Leaf		0.5	+	9.5 ± 0.29^{d}	1.125 ± 0.43^{cd}	0.3±0.12^{cde}
		1.0	++	11.5 ± 0.29^{cd}	3.5 ± 0.29^{c}	0.5±0.22^{cd}
		2.0	+++	14.5 ± 0.29^{c}	11.6 ± 0.31^{bc}	0.66±0.14^{c}
		3.0	++++	23.5 ± 0.87^{b}	20.6 ± 0.35^{a}	5.0±0.27^{a}
		5.0	++++	27.3 ± 1.45^{a}	14 ± 0.15^{b}	3.6±0.25^{b}
		10.0	--	-	-	-
Node	Mannitol	0.5	+	5.45 ± 0.29^{c}	4.5 ± 0.29^{c}	0.8±0.1^{e}
		1.0	++	19 ± 0.58^{bc}	11 ± 0.29^{bc}	1.8±0.26^{d}
		2.0	++	24.6 ± 0.20^{ab}	14.4 ± 0.30^{b}	2.4±0.34^{c}
		3.0	+	21.43 ± 0.26^{b}	15.7 ± 0.24^{ab}	3.9±0.11^{b}
		5.0	+	26.5 ± 0.87^{a}	17.6 ± 0.31^{a}	5.5±0.23^{a}
		10.0	--	-	-	-
Leaf		0.5	++	4.9 ± 0.19^{c}	0.87 ± 0.09^{cd}	0.3±0.12^{d}
		1.0	++	11 ± 0.58^{b}	4.6 ± 0.29^{ab}	0.5±0.22^{cd}
		2.0	+	28.9 ± 0.58^{a}	4.77 ± 0.19^{a}	0.66±0.14^{c}
		3.0	++	4.57 ± 0.29^{cd}	0.89 ± 0.06^{c}	5.0±0.27^{a}
		5.0	+	10.9 ± 0.49^{bc}	1.5 ± 0.29^{b}	3.6±0.25^{b}
		10.0	--	-	-	-
Node	Glucose	0.5	+	4.3 ± 0.25^{d}	0.77 ± 0.15^{d}	0.5±0.12^{e}
		1.0	+	5.6 ± 0.32^{cd}	2.7 ± 0.29^{cd}	1.4±0.22^{d}
		2.0	+	20.5 ± 0.29^{a}	6.5 ± 0.29^{b}	4.2±0.31^{a}
		3.0	+	15.6 ± 0.29^{b}	13.7 ± 0.25^{a}	3.1±0.17^{b}
		5.0	+	9.6 ± 0.26^{c}	3.7 ± 0.28^{c}	2.5±0.13^{c}
		10.0	--	1.3 ± 0.33^{e}	-	-
Leaf		0.5	+	1.4 ± 0.29^{e}	0.5±0.14^{d}	0.37±0.16^{d}
		1.0	+	12.3 ± 1.5^{c}	3.7 ± 0.24^{c}	1.2±0.18^{c}
		2.0	+	21 ± 0.58^{a}	5.7 ± 0.25^{a}	2.1±0.28^{b}
		3.0	++	16.5 ± 0.87^{b}	4.6 ± 0.29^{b}	5.0±0.24^{a}
		5.0	+++	7.4 ± 0.29^{d}	3.6 ± 0.32^{cd}	1.9±0.21^{bc}
		10.0	--	1 ± 0^{ef}	-	-

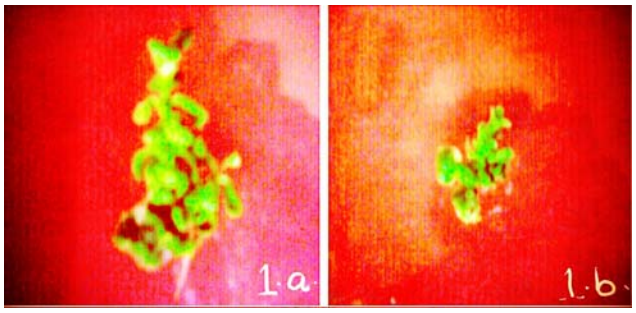

Fig 1: 1. a. Induction from leaf of *B. monniera* on medium MS + 8.9 µM BA + 3% sucrose and **1.b.** from node at 5% sucrose

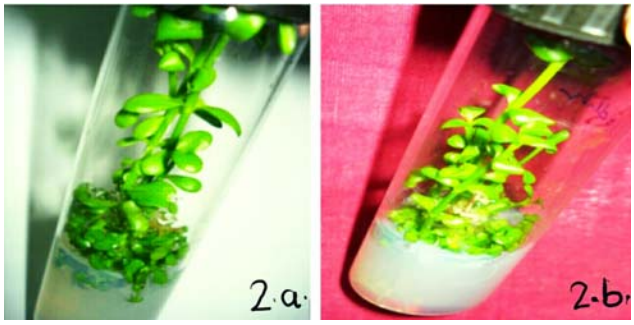

Fig 2: 2.a. Elongation of shoots regenerated from nodal explant at 5% sucrose and **2.b.** elongation of shoots regenerated from leaf at 3% sucrose

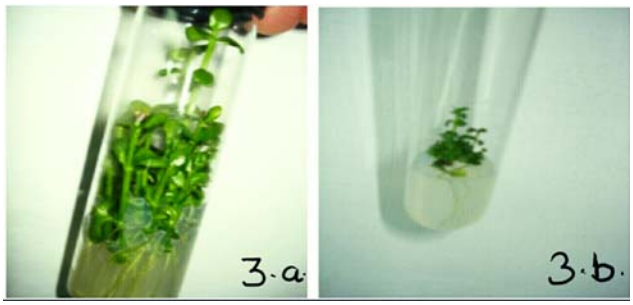

Fig 3: 3.a. rooting of induced shoots regenerated from nodal explants, **3.b.** rooting of induced shoots regenerated from leaf explant

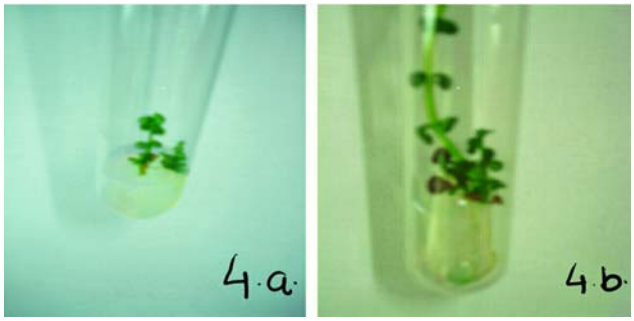

Fig 4: 4.a. Induction on 2% mannitol from leaf and **4.b.** 3% glucose from node

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