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IN VITRO MICROPROPAGATION OF BACOPA MONNIERI L. USING NODAL EXPLANTS

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ABSTRACT

The present study investigated the impact of carbon source and light quality on the regeneration efficiency of nodal explants from *Bacopa monnieri* L. (Brahmi), a medicinal plant known for its therapeutic properties. Carbon sources, such as sucrose and glucose, are essential for providing energy and supporting metabolic processes in plant tissue cultures, which are crucial for the successful regeneration of shoots and roots. The study examined different carbon sources to determine their effect on the growth of nodal explants, aiming to identify the most effective one for enhancing regeneration efficiency. Similarly, light quality, which refers to the spectrum of light provided to the cultures, can significantly influence physiological processes such as photosynthesis, cell division, and photomorphogenesis.

Various light conditions, including different wavelengths and types of light (e.g., fluorescent or LED), were tested to evaluate their role in optimizing regeneration. The regeneration efficiency, which refers to the ability of the nodal explants to produce viable shoots or plantlets under these different treatments, was assessed based on parameters like shoot length, number of shoots, and overall plantlet health. While differences in regeneration efficiency were observed across various treatments, the findings are still considered preliminary. These results suggest that both carbon source and light quality influence the growth and development of *Bacopa monnieri* in vitro, but further experimentation involving sub-culturing and extended observation periods is necessary to draw definitive conclusions. Only with more detailed and sustained trials can the optimal conditions for enhancing regeneration efficiency be determined, which is essential for large-scale micropropagation of this important medicinal plant.

Keyword: Carbon source, regeneration, photosynthesis, preliminary, moisture content

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INTRODUCTION

Bacopa monnieri has been used as a revered medicine in the traditional Indian Ayurvedic system for centuries. According to Ayurveda, the human body is influenced by her three energy dimensions or

doshas viz. 'Vata', 'Pitta' and 'Kapha'. An imbalance of these components leads to the development of disease. An imbalanced "vata" leads to neurodegenerative diseases and nootropic herbs and drugs are used to improve memory, cognition, mood,

and other factors related to mental function. One such herb with nootropic activity is brahmi (*Bacopa monnieri*). It is also known as water heirloom hyssop, brahmi or jalamimba, and has been used in Ayurveda for centuries. It is classified as Medhyarasayana i.e. a drug for enhancing memory and intelligence (medhya). The name Brahmi comes from the Hindu pantheon word "Brahma, the creator of the universe". Since the brain is the center of creative activity, compounds that improve brain health are called Brahmi, which also means "bringing knowledge of the highest reality." Brahmi was originally found in India and is also grown in Sri Lanka, Nepal, Pakistan, Vietnam, South America, Hawaii, Florida etc.

Brahmi is a well-known Ayurvedic herb that is making a comeback as a way to treat memory problems. Its medicinal properties have been reported in both Indian and traditional Chinese literatures. Although many compounds have been isolated from brahmi, bacoside-A and bacoside-B are the major active ingredients. Many other secondary plant substances such as alkaloids, glycosides, flavonoids and saponins have been reported from it (Dutta and Basu, 1963; Chatterji et al., 1965; Basu et al., 1967). It helps to maintain neurotransmitter balance (Charles et al., 2011; Rauf et al., 2012). and has shown promise in the treatment of Alzheimer's disease, schizophrenia, Parkinson's disease (Jadiya et al., 2011), epilepsy (Reas et al., 2008) etc. besides its anti-stroke (Rehni et al., 2007), antidepressant (Shen et al., 2009), anxiolytic (Reas et al., 2008), antioxidant (Pandareesh M. and Anand, 2014), gastrointestinal and hepatoprotective (Siraj et al., 2012), endocrine (Kar et al., 2002), antimicrobial (Azad et al., 2012; Katoch et al., 2014), anti-inflammatory, analgesic (Jain et al., 1994) and relaxant properties (Rashid et al., 1990).

Bacopa monnieri is a non-aromatic herb. The leaves are succulent, oblanceolate and opposite. The flowers are small, radial, white, and have 4-5 petals. It also grows in brackish water. Propagation is by cuttings. It commonly grows in the wetlands of India, Sri

Lanka, China, Pakistan, Taiwan, Vietnam, tropical and southern Africa, Madagascar, Australia, the Caribbean, and Central and South America. Due to its high medicinal efficacy, it is in great demand in the market. Therefore, large-scale propagation by micropropagation is a requisite. The present study was undertaken to study the influence of carbon sources and light quality on response of in vitro nodal cultures in brahmi.

MATERIALS AND METHODS

Materials: Nodal explants acquired from in vitro multiplication stage cultures of brahmi were used for the experiment. The study was conducted at the Plant Tissue Culture Laboratory in the Division of Horticulture and Forestry, ICAR-Central Island Agriculture Research Institute (CIARI), Port Blair, Andaman and Nicobar Islands.

Equipment's: Hot plate cum magnetic stirrer, pH meter, autoclave, laminar air flow cabinet, Hot air oven, air purifier, bead sterilizer, analytical balance, Lux meter.

Glassware: The glassware used in the study were glass rod, beaker (1,000 ml), measuring cylinders of different capacities (25ml, 100ml, 250ml, 500ml and 1000ml), funnel, test tubes (150 mm × 25 mm), culture bottles.

Chemicals: 6- Benzyl aminopurine (BAP), various constituents of Murashige and Skoog's Medium, Cleri Gel, sucrose, glucose, fructose, isopropyl alcohol, 0.1 N NaOH, 0.1 N HCL etc.

Miscellaneous: Deionized water, Forceps, scalpel, spatula.

Preparation of various stock solutions

Composition of various stock solutions used to prepare the MS medium is as follows.

Macronutrients

Stock Solution MS-A (Macro-nutrients)	Quantity (for 500 ml)
NH ₄ NO ₃	8.25g
KNO ₃	9.5g
CaCl ₂ .2H ₂ O	2.2g
MgSO ₄ .7H ₂ O	1.85g
KH ₂ PO ₄	0.85g

Stock solution was prepared by dissolving the constituents and making up the volume to 500 ml.

Micronutrients

Stock Solution MS-B (Micronutrients)	Quantity (for 100ml)
MnSO ₄ .4H ₂ O	1115 mg
ZnSO ₄ .4H ₂ O	430 mg
H ₃ BO ₃ .4H ₂ O	310 mg
KI	41.5 mg
Na ₂ MoOH.2H ₂ O	1.25 mg
CuSO ₄ .5H ₂ O	1.25 mg
COCl ₂ .6H ₂ O	1.25 mg

Stock solution was prepared by dissolving the constituents and making up the volume to 500ml.

MS-C Stock solution

Stock Solution MS-C (Chelating agents)	Quantity (for 100ml)
Na ₂ EDTA.H ₂ O	373 mg
FeSO ₄	278 mg

This stock solution was prepared by dissolving the constituents and making up the volume to 100 ml and it should be checked visually for precipitation of ingredients.

MS-D Stock solution

Stock solution (Vitamins)	Quantity (for 100ml)
Glycine	20 mg
Nicotine acid	5 mg
Pyridoxine HCl	5 mg
Thiamine HCl	1 mg
Meso-inositol	100 mg

This stock solution was prepared by dissolving the constituents and making up the volume to 100 ml. The stock solutions were stored in refrigerator till further use.

To prepare the growth regulator, 10 mg of 6-Benzylaminopurine (BAP) was accurately weighed using an analytical balance. The BAP was then dissolved by adding 0.1 N NaOH. After ensuring complete dissolution, the final volume of the solution was adjusted to 20 ml using deionized water.

Preparation of growth regulators

To prepare the growth regulator, 10 mg of 6-Benzylaminopurine (BAP) was accurately weighed using an analytical balance. The BAP was then dissolved by adding 0.1 N NaOH. After ensuring complete dissolution, the final volume of the solution was adjusted to 20 ml using deionized water.

Procedure for preparation of culture media:

A 1-liter capacity beaker was taken, rinsed thoroughly with deionized water, and then filled with approximately 500 ml of deionized water. The beaker was placed on a hot plate-cum-magnetic stirrer, and a magnetic bar was added to the beaker. The magnetic stirrer was turned on, and 100 ml of MS-A solution was added to the beaker. After stirring for 2 minutes, 10 ml each of MS-B, MS-D, and MS-C solutions were added sequentially. Following this, 1 mg of BAP was introduced into the solution. To provide the necessary carbon source, 30 g of sucrose, glucose, or fructose (depending on the treatment) was added, and the volume of the solution was adjusted to 1000 ml with deionized water. The pH of the solution was then checked using a pH meter, and it was adjusted to 5.73 as needed. The solution was boiled, and 5.5 g of Clerigar was slowly added while continuously stirring. After the preparation was complete, the media was poured into culture bottles, each receiving 30 ml of the solution. The bottles were then autoclaved at 15 psi and 121°C for 15 minutes. Finally, the culture bottles were transferred to an

aseptic culture storage section and observed for 2 days to check for any signs of contamination.



Figure 1: Inoculation of nodal explants

Inoculation of explants of *B. monnieri*

The culture bottles were transferred to the culture inoculation room, where the air conditioner and air purifier were activated to maintain a controlled environment. The laminar airflow system was turned on, followed by the activation of the blower and light. The laminar airflow bench was cleaned with 70% isopropanol to ensure aseptic conditions. Sterilized tools, including forceps, scalpels, paper, culture media bottles, and cotton, were placed inside the laminar airflow cabinet. The UV lamp was switched on for 10 minutes to sterilize the chamber effectively. Bottles containing multistage cultures were wiped with 70% isopropanol before being transferred into the laminar airflow cabinet. Nodal explants were carefully excised from the mother plants using sterilized forceps and scalpels. The selected nodal explants were then inoculated onto the prepared culture media under sterile conditions.

Incubation:

After inoculation, lid was closed tightly and bottles are then sealed with cling film. Cultured bottles were, then, transferred to culture incubation room at $24 \pm 2^\circ\text{C}$ and 18 h photoperiod conditions.

Transfer of cultures from white LED light to Blue + Red LED light to study its effect on shoot regeneration in in vitro cultures of Brahmi Equipment:

Lux meter: Model lx-1108, Lutron company, made in Taiwan.

Blue + Red LED

Mean light intensity under this condition was 1,276.66 lux.

White LED

Mean light intensity under this condition was 4,260 lux.

Biochemical Analysis

The *Bacopa monnieri* herbage used in this study was collected from the Horticultural Plants Propagation Unit of the Division of Horticulture and Forestry at ICAR-CIARI, Port Blair. For the estimation of chlorophyll and carotenoids, the acetone method was employed. The necessary equipment included an analytical weighing balance, biospectrometer, and centrifuge. Pre-chilled 80% acetone was used as the reagent. Miscellaneous items required for the procedure included a mortar and pestle, spatula, micro-pipette (ranging from 100 μL to 1000 μL), micro-pipette tips (100 μL to 1000 μL), and centrifuge tubes.

Procedure

Leaf samples were accurately weighed to known values. The leaf surfaces were gently cleaned and wiped with cotton soaked in deionized water to remove any impurities. The cleaned leaf samples were then homogenized in a mortar and pestle with an appropriate volume of pre-chilled 80% acetone. The final volume was adjusted to 20 ml by adding additional 80% acetone into a centrifuge tube. The samples were centrifuged at 5000 rpm for 10 minutes at 4°C . After centrifugation, the supernatant was carefully collected into a separate set of centrifuge tubes.

The absorbance of the supernatant was recorded at wavelengths of 663, 646, and 470 nm, corresponding to the absorption maxima for chlorophyll a, chlorophyll b, and total carotenoids, respectively. Chlorophyll a, chlorophyll b, and total carotenoid concentrations were then calculated using the following equations:

- 1) Chlorophyll a ($\mu\text{g/ml}$) = $(12.21 \times A663) - (2.81 \times A646)$
- 2) Chlorophyll b ($\mu\text{g/ml}$) = $(20.13 \times A646) - (5.03 \times A663)$
- 3) Total carotenoids ($\mu\text{g/ml}$) = $(1000 \times A470 - 3.27 \times \text{Chl a} - 104 \times \text{Chl b}) / 198$
- 4) The resulting values were then converted to $\mu\text{g/g}$ using the appropriate conversion factor.

Estimation of moisture content (%)

The materials required for this procedure included the herbage of *Bacopa monnieri* (Brahmi), an analytical weighing balance, a hot air oven, petri plates, a spatula, and a micro pipette with tips (100 μL to 1000 μL). To begin, the empty petri plates were

weighed using an analytical weighing balance. A known amount of sample was placed in the petri plates, with two replications, and the plates were then placed in a hot air oven set at 105°C for 3 hours to dry. After drying, the weight of the sample was recorded. The moisture content of the sample was then calculated using the formula:

$$\text{Moisture content (\%)} = \frac{w_2 - w_3}{w_2 - w_1} \times 100$$

Where **w₁** is the weight of the empty petri plate, **w₂** is the weight of the empty petri plate with the sample, and **w₃** is the weight of the empty petri plate with the dried sample.

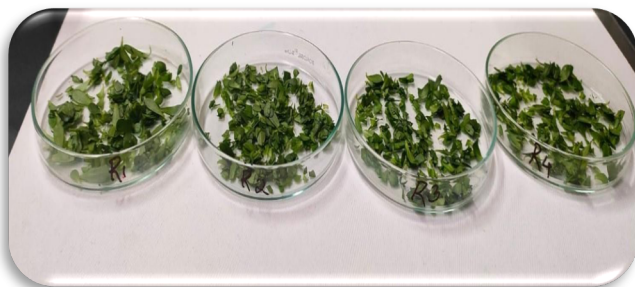


Figure 2: Sample used for moisture content determination

The ash content was determined using an industrial muffle furnace, an analytical weighing balance, and miscellaneous equipment such as forceps, heat- and temperature-resistant gloves, crucibles, and a spatula. Initially, empty crucibles were prepared, and the samples that had previously been used for moisture content determination were transferred into the empty crucibles and weighed again. The crucibles with the samples were then placed in the muffle furnace, where they were heated at 550°C for 3 hours. After this, the crucibles were allowed to cool overnight. Once cooled, the crucibles containing the dried samples were weighed, and the values were recorded.

The ash content (%) was then calculated using the following formula:

Ash content (%):

$$\text{Ash content (\%)} = \frac{\text{Crucible+sample initial wt (g)} - \text{Crucible+sample final wt (g)}}{\text{Original wt. of fresh sample (g)}} \times 100$$

Where **w₁** is the weight of the empty crucible, **w₂** is the weight of the crucible and sample before heating, and **w₃** is the weight of the crucible and sample after heating.



Figure 3: The samples, after ashing, were carefully removed from the crucibles and weighed.

Determination of vitamin C content using titrimetric method:

The instruments required for this procedure included an analytical weighing balance, bio spectrometer, and centrifuge. The glassware used consisted of a 100 ml measuring cylinder, 150 mm $\times$ 25 mm test tubes, a 50 ml conical flask, burette stand, funnel, beaker, and a micro pipette with tips (100 μL -1000 μL). Additional materials included mortar and pestle, centrifuge tubes, spatula, and filter paper. The reagents used were 4% oxalic acid, 2,6-Dichlorophenolindophenol dye, stock standard (1,000 ppm), and working standard (100 ppm) solutions of ascorbic acid.

To begin the procedure, the leaf samples were accurately weighed to a known value. The pre-weighed leaf samples were then crushed in a mortar and pestle with an appropriate volume of 4% oxalic acid. The extracted sample was collected in a centrifuge tube, and the volume was adjusted to 30 ml. The samples were centrifuged at 5000 rpm for 5 minutes at 4°C in a cooling centrifuge, after which the supernatant was filtered using filter paper.

For the titration of the standard ascorbic acid solution, 1 ml of the stock standard was diluted with 9 ml of 4% oxalic acid in a test tube. Next, 5 ml of the

The ascorbic acid content in the sample was calculated using the appropriate formula.

working standard ascorbic acid solution was placed into a conical flask, and 10 ml of 4% oxalic acid was added. This solution was titrated against a dye solution, prepared by dissolving 42 mg of NaHCO₃ and 52 mg of DCPIP, then making up the volume to 200 ml with deionized water. The titration continued until the appearance of a pink color, and the titre value was recorded.

The sample solution was then titrated in the same manner using the dye solution, and the titre value was noted.

$$\text{Ascorbic acid } \left(\frac{\text{mg}}{100 \text{ g}} \right) = \frac{0.5 \text{ mg} \times \text{titre value of sample (ml)} \times \text{volume made up (ml)}}{\text{titre value of working standard (ml)} \times 5 \text{ ml} \times \text{wt of sample (g)}} \times 100$$

RESULT

Table 1: Effect of carbon source and lights on shoot regeneration (number of shoots/explants) in *in vitro* cultures of Brahmi

Treatments	Day 12	Day 15	Day 17	Day 20	Day 22	Day 24
T ₁	5.533 a	6.469 a	6.485 a	6.774 a	7.387 abc	7.854 a
T ₂	5.069 a	6.280 a	6.480 a	6.898 a	7.407 abc	7.723 a
T ₃	4.551 a	6.129 a	5.743 a	5.983 a	6.635 c	7.066 a
T ₄	5.409 a	6.520 a	6.862 a	7.016 a	8.331 a	8.500 a
T ₅	5.611 a	6.327 a	6.716 a	7.018 a	7.305 bc	7.906 a
T ₆	5.359 a	6.005 a	6.332 a	6.787 a	7.951 ab	8.167 a

T₁ sucrose B+R LED, T₂ sucrose white LED, T₃ glucose B+R LED, T₄ Glucose white LED, T₅ Fructose white LED, T₆ Fructose B+R

There was no significant effect of treatments on number of shoots regenerated per explant Except on day 22.

Table 2: Effect of carbon source and lights on Root induction (%) in *in vitro* cultures of brahmi

Treatments	Day 12	Day 15	Day 17	Day 20	Day 22	Day 24
T ₁	0.740 a	32.548 a	26.352 a	28.653 a	29.951 a	28.948 a
T ₂	0.740 a	8.136 b	16.926 a	19.708 a	18.317 ab	18.317 a
T ₃	0.740 a	14.549 b	14.549 a	16.850 ab	24.050 a	24.050 a
T ₄	0.740 a	13.546 b	19.447 a	19.447 a	32.548	28.497 a
T ₅	0.740 a	0.740 b	0.740 b	3.041 b	7.644 a	7.644 a
T ₆	0.740 a	12.692 b	16.926 a	16.926 ab	16.926 ab	16.926 a

T₁ sucrose B+R LED, T₂ sucrose white LED, T₃ glucose B+R LED, T₄ Glucose white LED, T₅ Fructose white LED, T₆ Fructose B+R

Treatments were found to be non-significant on day 12 and day 24, while significant differences amongst the treatments were observed on other days for root induction.

Table 3: Effect of carbon source and lights on callus induction (%) in *vitro* cultures of brahmi

Treatments	Day 12	Day 15	Day 17	Day 20	Day 22	Day 24
T ₁	13.546 c	13.546 c	45.649 a	49.249 a	58.750 b	68.252 a
T ₂	46.075 a	46.075 a	56.255 a	67.826 a	65.360 ab	66.76 a
T ₃	24.050 bc	24.050 bc	48.246 a	65.950 a	65.950 ab	81.057 a
T ₄	34.850 ab	34.850 ab	47.655 a	68.252 a	83.359 a	81.862 a
T ₅	24.050 bc	24.050 bc	38.449 ab	63.648 a	71.556 ab	72.855 a
T ₆	18.317 bc	18.317 bc	19.708 a	46.073 a	35.895 c	39.752 b

T₁ sucrose B+R LED, T₂ sucrose white LED, T₃ glucose B+R LED, T₄ Glucose white LED, T₅ Fructose white LED, T₆ Fructose B+R

Treatments were found to differ significantly at 1% and 5% level of significance for callus induction.

Table 4: Effect of carbon source and lights on shoot induction (%) in *vitro* cultures of Brahmi

Treatments	Day 12	Day 14	Day 17	Day 20	Day 22	Day 24
T ₁	57.747 a	68.292 a	68.292 a	74.153 a	76.454 a	78.756 a
T ₂	56.571 a	67.869 a	67.869 a	70.608 a	74.465 a	79.397 a
T ₃	55.446 a	71.851 a	71.851 a	72.855 a	82.356 a	84.657 a
T ₄	62.055 a	72.855 a	72.855 a	74.153 a	84.657 a	84.328 a
T ₅	61.503 a	70.607 a	70.607 a	80.471 a	81.863 a	81.863 a
T ₆	63.353 a	71.851 a	71.851 a	71.851 a	74.153 a	74.153 a

T₁ sucrose B+R LED, T₂ sucrose white LED, T₃ glucose B+R LED, T₄ Glucose white LED, T₅ Fructose white LED, T₆ Fructose B+R

Treatments were not found to be significantly different during all the days of observation.

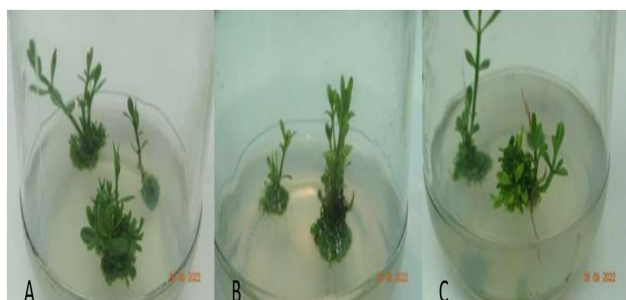


Figure 4: *In vitro* response of nodal cultures of Brahmi in media supplemented with Sucrose (A), Fructose (B) and Glucose (C) and maintained under white LED light condition

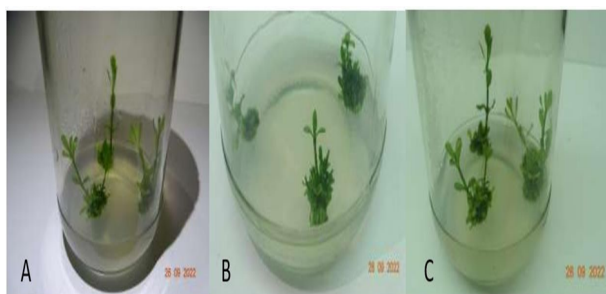


Figure 5: *In vitro* response of nodal cultures of Brahmi in media supplemented with Sucrose (A), Fructose (B), and Glucose (C) and maintained under B+R LED light condition.

Biochemical analysis of *Bacopa monnieri*

Parameters	Values
Chlorophyll a (µg/g)	372.57 ± 7.761
Chlorophyll b (µg/g)	122.31 ± 2.929
Total Chlorophyll Content (µg/g)	494.88 ± 4.832
Chlorophyll Ratio (a:b)	3.05 ± 0.135
Total Carotenoids (µg/g)	72.85 ± 9.778
Vitamin C (mg/100 g)	7.7975 ± 1.5545
Ash content (%)	1.188 ± 0.0685
Moisture content (%)	93.045 ± 0.286

CONCLUSION

The present study provides preliminary insights into the influence of carbon source and light quality on the regeneration efficiency of nodal cultures of *Bacopa monnieri* (Brahmi). Although variations in the responses to different treatments were observed, the

findings are not yet conclusive. Further sub-culturing and more detailed observations are necessary to draw definitive conclusions regarding the optimal conditions for regeneration.

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