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IN VITRO MASS PROPAGATION OF *BACOPA MONNIERI* (LINN.) WETTST FROM NODAL EXPLANT - A MULTIPURPOSE MEDICINAL PLANT

T. Nagarajan[1*], S. Alagumanian[1], G. Jahirhussain[2], S. Subbaiya[1]

[1]PG and Research Department of Botany, H.H.The Rajah's College (Auto), Pudukkottai, Tamil Nadu, South India.

[2]P.G. and Research Department of Botany, Government Arts College (Autonomous), Karur - 639 005. Tamilnadu, India.

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***Correspondence for Author**
T. Nagarajan
PG and Research Department of Botany, H.H.The Rajah's College (Auto), Pudukkottai, Tamil Nadu, South India.

ABSTRACT

Bacopa monnieri is a highly valuable medicinal plant from the Scorphulariaceae family. It is used to derivate different pharmaceutical products, but it lost from their natural habitats due to indiscriminate collection for pay pharmaceutical demands. Therefore, the present study was carried out to determine the *in vitro* mass propagation of *Bacopa monnieri* (Linn.) Wettst. by using nodal explants. Nodal explants of *Bacopa monnieri* were cultured on MS basal medium supplemented with different concentrations of BAP and KIN ranging from 0.5-2.5 µM/L for multiple shoot induction. Two cytokinins tested, BAP was found to develop in shoot multiplication and higher number of shoots from the nodal explants when compared to KIN. Higher number of shoot was produced from all the concentrations of both BAP and KIN. The highest frequency (100%) of shoot induction and maximum number of shoot (**6.4±1.94**) was observed on 2.0 µM BAP with shoot length of **5.02±0.20** c.m. as well as in KIN the highest frequency (100%) of shoot induction and maximum number of shoot (**5.4±1.51**) was observed on 1.5 µM KIN with shoot length of **5.14±0.28** c.m. The isolated shoots were transferred to MS basal medium supplemented with different concentrations of IBA and NAA for root induction. The rooted plantlets were successfully transferred in soil through hardening and established in the field.

KEY WARDS: *In vitro*, Mass propagation**,** *Bacopa monnieri*, BAP, KIN.

INTRODUCTION

Plants have been an important source of medicine for thousands of medicine for years. According to an estimate by World Health Organization, herbal medicine is one of the most remarkable uses of plant based biodiversity. As many as 75 to 90% of the worlds rural people rely on herbal medicine for their primary health care on traditional remedies such as herbs for the tremendously growing world population, increasing anthropogenic activities, rapidly eroding natural ecosystem, etc the natural habitat for a great number of herbs and trees are dwindling.

Taxonomic classification of *Bacopa monnieri (Linn.)* Wettst

Kingdom: Plantae
Division: Tracheophyta
Class: Magnoliopsida
Order: Lamiales **(**Scrophulariales**)**
Family: Scrophulariaceae
Genus: *Bacopa*
Species: *Bacopa monnieri* (Linn.) Wettst

This plant is also known as "Brahmi" or "Nira-brahmi". The ancient ayurvedic texts recommend it to "rejuvenate the brain" and to improve the cognitive properties of the mind. The gurus of the religious schools from ancient India gave Brahmi to their disciples, so they could memorize hymns and vedic texts, and be more concentrated during meditation.

Bacopa monnieri, commonly known as water hyssop, is an herb often used in Ayurveda. Supplementing *Bacopa monnieri* has been shown to improve Cognition, by means of reducingAnxiety. It is also reliable for improving Memory formation. Though effects of this nature are usually studied in the elderly, *Bacopa monnieri* appears to affect young people as well, making it a useful Nootropic. Since *Bacopa monnieri* is also an Adaptogen, a relaxed person might experience a lack of motivation to work after supplementation. Theoretically, pairing *Bacopa monnieri* with a stimulant would ward off malaise, but this combination has not been tested.

Bacopa monnieri interacts with the dopamine and serotonergic systems, but its main mechanism concerns promoting neuron communication. It does this by enhancing the rate at which the nervous system can communicate by increasing the growth of nerve endings, also called dendrites. *Bacopa monnieri* is also an antioxidant. Taking *Bacopa monnieri* on an empty stomach may cause nausea, cramping, bloating, and diarrhea.

Fig.1. Morphological features of *Bacopa monnieri* (Linn.) Wettst

The literal meaning of the term Mandukaparna is a plant having leaves resembling the shape of frogs. And it also means, the stolons grow in the manner of jumping frog. Centella asiatica is the accepted source of this herb.[1] It is a stoloniferous creeping herb, rooting at nodes. The whole plant is reported to be a nervine and cardiotonic, astringent and diuretic.[2] Charaka includes this under Vayasthapana varga,[3] the group of drugs that are capable of maintaining the youthful vigour and strength. There is, however, some confusion with regard to the drugs Mandukaparni and Brahmi. This may be due to the lack of description of the two drugs in the texts, attribution of similar properties to them and also application of the same synonyms.[1]

Drug standardization or quality assurance forms an essential step before its therapeutic utility.[3] The word standardization should encompass the entire field of study from the birth of a plant to its clinical application. Most of the pharmacopoeias suggest macro-microscopic characterization and chemical profiling of botanical material turn out to be a pilot step in drug standardization.[4]

In several industrialized societies, plant – derived prescription drugs constitute an element in the maintenance of health. Medicinal plants are an integral component of research developments in the pharmaceutical industry. Such research focuses on the isolation and direct use of active medicinal constituents, or on the development of semi-synthetic drugs, or still again on the active screening of natural products to yield synthetic pharmacologically-active compounds.

India has great diversity of medicinal plants. These medicinal plants are used in Siddha, Ayurvedic and Unani medicines and a number of pharmaceutical products. *Bacopa monnieri* (L.), commonly known as ‘Brahmi’ is a member of the family scrophulariaceae, is most placed in the priority list of Indian medicinal plants.[5] It is commonly found on the banks of rivers and lakes. It has been used for centuries in folklore and traditional system of medicine. It has a great market demand due to its high medicinal values. It is prescribed for a variety of therapeutic indications including antipyretic, anti-inflammatory, analgesic, epilepsy, insanity, anticancer, antioxidant activities and memory enhancement.[6],[7] It contains different types of saponins such as bacosides A, B, C and D which are the active triterpenoid principles and known as “memory chemicals”.[8]

Micropropagation

Micropropagation is referred to as the true-to-type clonal propagation of any selected genotype under the in vitro condition by plant tissue culture technique. It is often associated with mass production of plants at a competitive price. This is one of the many plant tissue culture techniques wherein plants can be produced either through organogenesis or somatic embryogenesis at large scale. Practically any part of a plant can be induced to regenerate into complete plant under in vitro condition. They can be multiplied and rooted under in vitro or ex vitro condition or can be made into artificial seeds for automated sowing of these propagules under natural conditions.

These new aseptic propagation methods are reliable and present a new tool in the plant propagation industry for those with the inclination to use them. Grains with increased yield, trees with better form and faster growth, plants with disease resistance, uniform crops able to be harvested at an optimum time, plants with known characteristics that are able to be better marketed are the advantages accrue as the advance of technology goes on.

Micropropagation provides a fast and dependable method for production of large quantity of uniform plants in a short time throughout the year. This technique ensures round the year propagation with high multiplication rate that could be utilized in scaling up the production at commercial level. Hitherto plants impossible to propagate are now being done with ease.

MATERIALS AND METHODS

Collection of explants

The field grown *Bacopa monnieri* (Linn.) Wettst (Scrophulariaceae) was selected for the source of explants in the present Study. Nodal regions with axillary bud of two weeks older plants were used as explants source for micropropagation.

Culture Medium

The nutrient medium consists of inorganic salts, carbon source and organic supplements. In addition, vitamins and growth regulators are also added to the medium. In the present study, the basal medium consists of the mineral salts and organic nutrients of Murashige and Skoog (MS) salts[9] with B5 vitamins[10] are used. For convenience, throughout this chapter, MS medium with MS salts plus B_5 vitamins is being referred as MS medium. The basal medium is supplemented with various concentrations and combinations of different growth regulators.

Growth regulators

The prwsent study the hormone concentration was used in µM (micromole). These growth regulators were used as supplement to the basal medium individually as well as in different combinations.

Cytokinins : **BAP** (6-benzylaminopurine)
KIN (6-furfurylaminopurine)

Auxins : **IBA (Indole butyric acid)**
NAA (Naphthalene acetic acid)

The present study was performed in the basal medium with MS salts, B5 vitamins, 3% sucrose and 0.8% agar.

The basal medium was variously supplemented with factorial combinations of different growth regulators ranging from 2 – 10 µM BAP or KIN alone for shoot multiplication or in combination of both the cytokinins. After adding all the supplements (various concentrations of different hormones) to the basal medium, the pH of the medium was adjusted to 5.8. The molten medium was dispensed in culture tubes or culture bottles or conical flasks and was capped with cotton plugs.

Sterilization of Culture Medium and Glassware's

The culture medium containing high concentration of sucrose supports the growth of several microorganisms. These microbes generally grow much faster than the explants and finally spoil the culture. So it is very essential to maintain a complete aseptic environment inside the culture tube. Therefore, the culture medium, glassware's, forceps and scalpels was sterilized by autoclaving at 1.06 kg cm^{-2} and 121°C for 15 min. As well as the same procedure to follow the sterilization of. During this period much care was taken to avoid denaturation of growth regulators and vitamins that were incorporated into the medium. The culture tubes left free until agar in the medium become solidified. Then the tubes were transferred to inoculation chamber for inoculation.

Sterilization of explant

The explants were taken from the field grown mature plants. The explants consisting of the nodal regions with axillary bud were surface sterilized by rinsing in running tap water for 30 minutes. Then they were washed in an agitated solution of liquid detergent (Teepol) for 5 minutes and followed by distilled water for 2-3 times for removing the traces of liquid

detergent. After thorough washing, the materials were taken in to the Laminar Flow Chamber where they were disinfected with 70% alcohol for 30-60 seconds followed by 0.1% mercuric chloride for 3-5 minutes. Finally, the materials were thoroughly rinsed with sterile distilled water for 4-5 times to remove the traces of mercuric chloride.

Inoculation Procedure

Before starting inoculation all the requirements such as culture tubes, containing media, spirit lamp, sterile water, glassware and explants, were placed in the laminar air flow chamber. The platform surface of the chamber was swapped with 70% alcohol. After swapping the chamber with 70% alcohol, the UV light was switched on for 30 minutes. After 30 minutes, the UV light was switched off and the white fluorescent light was switched on. Before inoculation, hands were rinsed with absolute alcohol. The instruments were sterilized by dipping in absolute alcohol followed by flaming and cooling.

The inoculation was carried out in the vicinity of flame. The surface sterilized explants were aseptically transferred to the respective culture media in the Laminar Flow Chamber. The explants were taken out from beaker and at the same time the cotton plug of the culture tube was slightly opened in front of the spirit lamp flame, the explant was put it the medium and immediately covered with cotton plug. The explants with nodal regions were inserted in the medium vertically. Cultures were transferred to fresh media with the same hormone concentration at 4 week intervals.

Culture Conditions

The cultures were maintained in a culture room at 25±2°C under 16 hr photoperiod with a light intensity of 30-40 µM m^{-2} s^{-1} supplied by cool white fluorescent tubes. These growth conditions were referred to as standard culture conditions for *in vitro* studies.

Culture Maintenance

The nodal explants regions, were initially cultured on MS solid medium in test tubes. After 4 weeks, the initiated shoot multiples were subcultured on MS basal medium fortified with the same growth regulator concentrations and combinations or whichever is the best for further multiplication. To facilitate higher number of shoot formation, the explants were also subcultured on conical flasks and/or culture bottles which can provide more space and more medium for growth and multiplication.

Rooting

In vitro raised shoots of 2 cm and above were excised from the culture tube or culture bottle and subcultured into MS medium fortified with 3% sucrose (w/v) and 0.8% agar (w/v). The medium was further supplemented with different concentrations (2.0-10.0 µM) of IAA, IBA or NAA. The root number and length were measured in each culture medium.

Hardening and Acclimatization

Plantlets with well-developed roots were dislodged from the culture medium and roots were washed gently under running tap water to remove the adhering medium. Plantlets were transferred to plastic cups (10 cm diameter) containing autoclaved garden soil, farmyard manure and sand (2:1:1). Each plantlet was irrigated with distilled water every 2 days for 2 weeks followed by tap water for one week. The potted plantlets were initially maintained under culture room conditions (3 weeks) and later transferred to normal laboratory conditions (2 weeks).

The potted plantlets were initially covered with porous polyethylene sheets to maintain high humidity and were maintained inside the culture room. The relative humidity was reduced gradually. After 30 days the plantlets were transplanted to the field under shade for 3 weeks and then transplanted to the soil for further growth and development.

Experimental Design, Data Collection and Statistical Analysis

The design of all the experiments was a complete randomized block and each experiment consisted of five explants per flask and five replicate culture flasks per plant growth regulator treatment. The parameters recorded were frequency (number of cultures responding in terms of multiple shoot proliferation and root development), number of shoots per explant, shoot length, number of roots per shoot, root length and survival rate (%). All of the experiments were repeated five times. The analysis of variance (ANOVA) appropriate for the design was carried out to detect the significance of differences among the treatment means were compared using Duncan's Multiple Range Test (DMRT) at a 5% level of significance.[16]

RESULTS AND DISCUSSION

Nodal explants of *Bacopa monnieri (*Linn.) Wettst. were cultured on MS basal medium supplemented with different concentrations of BAP and KIN of both these cytokinins ranging from **0.5 - 2.5** µM/L for shoot multiplication. Multiple shoots were initiated within 12 days of inoculation. Maximum number of shoots was observed in 20-30 days. The data in respect

of shoot induction frequency, number of shoots and length of shoots on different concentrations of each hormone on nodal explants were presented in **Table 1 and Fig.2**.

Higher number of shoots was produced from all the concentrations of both BAP and KIN. The highest frequency (**100%**) of shoot induction and maximum number of shoot (**6.4±1.94**) was observed on 2.0 µM BAP with shoot length of **5.02±0.20 c.m**. as well as in KIN **1.5** µM/L to produce high frequency of (100%) maximum number of shoots (**5.4±1.51**) in the shoot length of **5.14±0.28 c.m.** the 30 days of shoot multiplication and proliferation the shoots are isolated. The isolated shoots were transferred to MS basal medium supplemented with different concentrations of IBA and NAA ranging from **0.5 - 2.5** µM/L for root induction.

In micropropagation technique, shoots are directly induced from the nodal explant with axillary buds where meristematic tissue is present. This technique is primarily used to produce pathogen free plantlets. Nowadays, it is widely used to get a mass propagation within a short period. Since the meristematic region is the very active site, the axillary buds are readily proliferated. The efficiency of shoot multiplication depends on plant growth regulators and types of explants.[11],[12],[13]

Table 1. Effect of different concentrations of cytokinins on shoot induction from the nodal explant of *Bacopa monnieri (Linn.)* Wettst.

BAP	KIN	Percentage of response (%)	Number of shoots	Shoots length
0.5 µM	--	90	4.8±1.30	3.64±0.36
1.0 µM	--	95	5.2±0.83	4.36±0.38
1.5 µM	--	80	4.6±1.14	4.52±0.51
2.0 µM	--	100	6.4±1.94	5.02±0.20
2.5 µM	--	95	5.6±1.81	4.54±0.27
--	0.5 µM	95	5.2±1.92	3.84±0.27
--	1.0 µM	80	5.2±1.48	4.22±0.25
--	1.5 µM	100	5.4±1.51	5.14±0.28
--	2.0 µM	95	4.8±1.30	4.76±0.33
--	2.5 µM	85	4.2±0.83	4.66±0.36

Mean ±standard deviation of 5 replicates per treatment in three repeated experiments

In many plants, multiple shoots were obtained from the shoot tips or axillary buds by administering BAP or KIN.[14],[15],[16],[17],[18],[19] [26],[27],[28],[29],[30] In the present study nodal explants with axillary bud were taken as explants source. The nodal explants showed active site of positive morphogenetic response and readily developed multiple shoots. The propagation rate and morphogenetic response significantly varied to a greater extent according to the explant type. Shoot tips have always been preferred for *in vitro* studies

because they can be handled easily and restore their regeneration potential over other explants. Some earlier findings showed that more number of shoots were produced from the nodal explants.[18],[20],[21],[22]

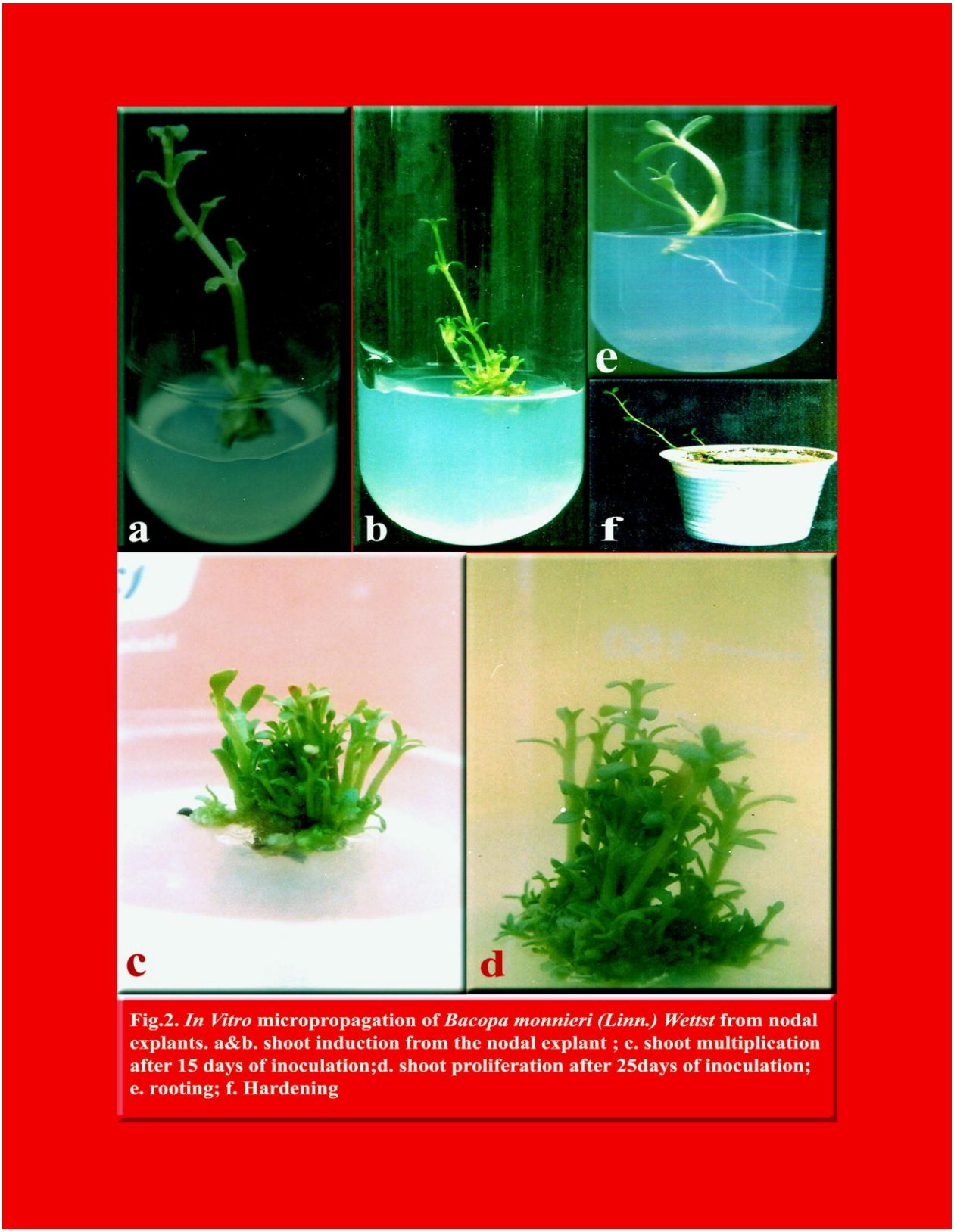

Fig.2. *In Vitro* micropropagation of *Bacopa monnieri (Linn.) Wettst* from nodal explants. a&b. shoot induction from the nodal explant ; c. shoot multiplication after 15 days of inoculation;d. shoot proliferation after 25days of inoculation; e. rooting; f. Hardening

Fig.2. *In Vitro* mass propagation of *Bacopa* monnieri (Linn.) Wettst from nodal explants

Shoot responses from node explants were tried in different concentrations; in this study it was found that BAP 2.0 mg/l with MS medium showed good response to shoot induction from node explants.[23] similar reported the BAP with MS medium, in this concentration the node explants of *S. trilobatum* showed better multiple shoot within 20 days after inoculation. BAP and MS medium individually and in combination induced a higher frequency of adventitious shoots from single explants of *S. xanthocarpum*,.[24], [25]

CONCLUSION

Micropropagation was carried out from the nodal explants with axillary buds of *Bacopa monnieri* (Linn.) Wettst. Nodal explants with axillary buds were grown on MS basal medium supplemented with different concentrations BAP or KIN. Of the two cytokinins BAP was found to actively induce more number of shoots from nodal explants when compared to KIN. The MS basal medium supplemented with 2.0 µM/L BAP showed the maximum number of **6.4±1.94** shoots per nodal explant with shoot length of **5.02±0.20 c.m.** and 1.5 µM/L KIN produced the maximum number of **5.4±1.51** shoots per node with shoot length of **5.14±0.28 c.m.**

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