Standardization of a Rapid Micropropagation Protocol for Medicinal Plant, *Bacopa monnieri* (L.) Wettst.

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Abstract :

Bacopa monnieri (L.) Wettst. is a valued herb since ancient times owing to its various therapeutical properties. Studies show that bacosides A, B, C and D, also known as "memory chemicals" are the most important components of *Bacopa monnieri*, thereby designating it as the second most important medicinal plant of India. To maintain the constant supply of raw materials and reduce the pressure on natural population, development of an efficient, reliable and rapid *in vitro* micropropagation protocol is essential. The present study was carried out to standardize a rapid micropropagation protocol for *Bacopa monnieri* by using different growth regulators. Furthermore, anatomical studies of propagules were also performed. A comparative analysis of the pharmacological features and ana- tomical markers of *Bacopa* plants from different sources showed similarities.

Key words : *Bacopa monnieri*, medicinal plant, bacosides, micropropagation, phytoconstituents.

Introduction

Bacopa monnieri (L.) Wettst. is a small, non-aromatic, creeping succulent and water loving herb of Plantaginaceae, grows in ponds, tidals, and wetlands in tropical and semitropical areas. It is mostly distributed in marshy areas of India, Nepal, Sri Lanka, China, Pakistan, Taiwan and Vietnam. In traditional Indian system of medicine, the *Ayurveda*, where it is known as "Brahmi," after Brahmâ, the creator God (Hindu) of the Universe and has been extensively used for the treatment of anxiety, and in improving intellect and memory for several centuries.

Scientific studies showed that *Bacopa monnieri* possessed many pharmacological effects included central nervous effects (memory enhancement, antidepressant, anxiolytic, anticonvulsant and antiparkinsonian), antioxidant, gastrointestinal, endocrine, antimicrobial, anti-inflammatory, analgesic, cardiovascular and smooth muscle relaxant effects. The effective alkaloids are brahmine, nicotinine, herpestine, bacosides A[3-(á-L-arabinopyranosyl)-O-â-D-glucopyranoside-10,20-dihydroxy-16-keto-dammar-24-ene], triterpenoid saponins , saponins A, B and C, betulinic acid , D-mannitol , stigmastanol , â-sitosterol , stigmasterol and pseudojujubogenin glycoside (Behera et al., 2016). The alkaloids are produced from two independent biosynthetic pathways; Mevalonate (MVA) pathway occurring in the cytoplasm and the methylerythritol 4-phosphate (MEP) pathway in the plastid for triterpenoid saponins. Triterpenoid saponins are secondary metabolites of isopentenyl pyrophosphate (IPP) oligomers and represent the largest group of phytochemicals (Bhakuni et al., 1969; Bishayee et al. 2011; Rajan 2015; Gupta 2017, Moquammel Haque et al., 2017). Prevoius studies report *Bacopa monnieri* (L.) Wettst. as an efficient plant material for micropropagation (Tiwari et al., 2000; Behera et al., 2016).

The present study is aimed to standardize a rapid micropropagation protocol and to perform comparative pharmacological analysis of the *in vitro* micropropagation of *Bacopa monnieri* with those available in the markets.

Materials and methods

Selection and sterilization of explants

The plants are collected from the Rishra market, Hooghly. Axillary meristems and adventitious buds were selected as explants. They were cleaned under running tap water and washed with 2.5% liquid detergent solution for 2 minutes followed by 2.5% (w/v) Tween 20 for 15 minutes. Then, the explants were surface disinfected with 0.1% HgCl2 for 8 minutes and rinsed with sterile distilled water several times to remove all traces of HgCl2 and then explants were transferred to sterile petridish containing sterile filter paper to soak the surface water of the explants.

Transfer of explants in media

Two types of growth regulators were used- Kinetin and Naphthalene Acetic Acid (NAA). The axillary meristems and adventitious buds were aseptically implanted on MS medium (Murashige and Skoog, 1962) supplemented with specific concentrations of hormones [Kinetin (0.5-4.5 mg/L) and NAA (0.5-2.5 mg/L)] in three biological and three technical replicates for each. The cultures were incubated and observed.

Anatomical study of propagules

The micropropagated propagule was used for anatomical studies. It was thoroughly washed with water to remove the media and then blotted dry. The fine sections were mounted in glycerine and observed.

Preliminary phytochemical analysis of *Bacopa monnieri* extracts

Collection and Authentication

Bacopa monnieri plants were collected from two different markets of Hooghly district: Rishra (B1) and Chandannagar (B2) and authenticated by herbarium sheets. Micropropagated plantlets (B3) were also taken.

Phytochemical analysis by standard methods

The dried and powdered leaves were extracted with Methanol and Distilled water. The extracts were subjected to preliminary qualitative tests for identification of various phytochemicals (Patil et al., 2014; Jerusha et al., 2016).

Powder microscopy

The finely grounded powder is used for powder microscopy. 50% Safranine is used to infuse color.

Results

Effect of different hormones on the explants

Initially axillary meristems and adventitious buds adventitious buds were selected as explants and were placed in MS media supplemented with different concentration of the hormones Kinetin and NAA. Most responsive growth was achieved by using axillary meristem as explants supplemented with different concentration of kinetin **(Fig.1A-H).** There is a considerable increment in the number of roots (as observed in the data collected within an interval of 8 days). The number of shoots also increases but on a much slower rate. After 24 days, it is observed that Kinetin at concentration of 0.5 and 1.5 mg/L showed the maximum number of roots. Whereas at 3.5 mg/L concentration of kinetin shows the maximum number of shoots**(Fig.1I-J) (Table.1)**.

Initiation of propagules

Some propagule like structures were found to be initiated after 24 days in the MS medium supplemented with 1.5 mg/L and 3.5 mg/L kinetin. Emergence of shootlets were observed from propaguleson MS medium supplemented with 1.5mg/L kinetin within the 32nd day. Micropropagules grew and became enlarged. They were again subcultured on the 42nd day **(Fig.2A-D)**.

Cauolorhizogenesis of propagules was observed on MS medium supplemented with 3.5 mg/L kinetin. Then, they were transferred to jam bottle on the 50th day **(Fig.3A-G).**

Prominent roots were observed on the enlarged micropropagules when transferred to plastic glasses on the 60th day. Emergence of plantlets were observed after a few days **(Fig.4A-D)**.

Table.1. Total number of shoot initials after 45 days.

Concentration of hormone (mg/L)	Replica set	Explant No.	No. of shoot initials	Average no. of shoot initials
MS + K (3.5 mg/L)	1	1	8	13.67±6.66
		2	12	
		3	21	
	2	1	10	11.67±6.66
		2	6	
		3	19	
	3	1	11	13.33±5.86
		2	9	
		3	20	

Anatomical study of the propagules

Anatomical structures are very important for studying biological specimens for the purpose of classification, pathological changes and the physiological significance of certain cell organelles or structures in relation to the habitat (Anju et al., 2017; Coopoosamy and Naidoo, 2011). The 24 day propagules were selected for anatomical study. **Fig.5A** showed the crosssection of propagule showing several crevices under simple microscope (x5X). **Fig.5B-E** showed several regions like shoot apical meristem consisting of cells containing chloroplasts. This may be the sites of emergence of individual micropropagules. Different zones of green cells were observed indicating differentiation **(Fig.5D)**. Also, glandular trichome **(Fig.5C)**, vessels with scalariform thickening **(Fig.5F)** were observed. **Fig.6.A-C** showed clear

zones indicating lysis of cells. Profound green mesophyll cells indicating differentiation **(Fig.6D)**. The anatomical study speaks about the ongoing differentiation of cells, the glandular trichome indicating the emergence of epidermal layer, mesophyll cells consisting of the cortical region and vessels indicating the emergence of stelar region. The lysigenous cavities may indicate the zone of separations of individual micropropagules.

Phytochemical characterization

Aim for the phytochemical analysis is the characterization of an active principle responsible for some toxic or beneficial effect shown by a crude plant extract when tested against a living system. Quantification of the different phytoconstituents present in the plants is of equal impor- tance (Harborne, 2007) and can be used for identification of distince characters (Anju et al., 2017). After extraction, methanolic ex- tract was found to be yellowish green and aqueous extract was found to be dark brown in color. All phytochemical analysis was performed with these extracts.From the present study, it is evident that the carbohydrates, flavonoids, terpenoids, alkaloids and quinones are present is higher in methanolic extract than aqueous extract. Sa- ponins and phenols are present in high amount in aqueous extract.

Extract from all the three sources (B1, B2, B3) showed similar results with high amount of different phytochemicals. Thus, it can be concluded that the phytochemical content in the micropropagated plantlet remain uncompromised as the amount of phytochemical is similar with that of the plants from the two different markets**(Fig.7).**

Powder microscopy

Whole plant powder showed presence of simple, round to oval shaped starch grains, prismatic calcium oxalate crystals, xylem vessels with spiral thickenings and septate fibres. Stem powder is light brown in colour and bitter in

taste. The various diagnostic characteristics of stem pow- der observed are septate fibres, pitted xylem tracheids, simple, round and oval starch grains and parenchymatous cells with crystals. Leaf powder is green in colour and bitter in taste. The various diagnostic characteristics of leaf pow- der are prismatic calcium oxalate crystals in mesophyll tis- sue, macro scelereid and parenchymatous cells. Propagules showed presence of simple, round to oval shaped starch grains, prismatic calcium oxalate crystals, pitted xylem trac- heids and parenchymatous cells **(Fig.8A-C).** The propagules showed similar pattern of anatomical markers as obtained from the different sources **(Table.2)**.

The present study standardize a rapid protocol for an *in vitro* propagation of *Bacopa monneiri* (L.) Wettst. The time taken to get plantlets in soil is about 60 days. Most responsive growth was achieved by using axillary meristem as explants supplemented with kinetin (1.5mg/L and 3.5 mg/L). Emergence of shootlets from propagules were observed on MS medium supplemented with 1.5mg/L kinetin within the 32nd day. Whereas, cauolorhizogenesis of propagules was observed on MS medium supplemented with 3.5 mg/L kinetin.

The anatomical study of the propagules revealed the presence of apical meristem like region consisting of cells containing chloroplasts. Glandular trichomes, vessels with scalariform thickening, mesophyll cells were also observed. Lysis of cells are clearly seen which may indicate the zone of separations of individual micropropagules.

The results of the qualitative biochemical assay showed the presence of active phytochemicals for example saponins, terpenoids, alkaloids, phenols, quinines, etc. that may lead to the future production of herbal medicines. Powder microscopy helped us to identify the anatomical markers. The anatomical markers for stem are presence of pitted xylem tracheids, simple, round and oval starch grains, parenchymatous cells with crystals and for leaf are presence of prismatic calcium oxalate crystals, simple and round starch grains and parenchymatous cells. All the above mentioned anatomical markers were also observed in whole plant powder and micropropagated propagules of *Bacopa monnieri* (L.) Wettst.

Table.2. Characterization of anatomical markers B1 designated as Bacopa monneiri collected from Rishra market and B3 designated as micropropagated Bacopa monneiri propagules. +++ = Present in high amount; ++= Present in moderate amount; += Present in small amount; -= Absent

Sl. No.	Character	Plant source(B1)			Plant source(B3)
		Whole plant	Leaf	Stem	Propagules
1	Crystal	++	+++	+++	+++
2	Septate fibre	+	-	+	+++
3	Parenchymatous cells	+++	+++	+++	+++
4	Macro sclereid	-	++	-	+
5	Pitted tracheid	-	-	+++	++
6	Starch grains	-	-	+++	++

Conclusion

Bacopa monnieri (L.) Wettst. is a popular medicinal plant, which is being used for various medicinal properties. Here we have successfully developed an efficient *in vitro* propagation protocol for *B. monnieri* for 60 days. The method used was cost effective and required very little amount of chemicals and simple equipments like hot air oven, autoclave and laminar air flow and a tissue culture room. The regenerated plants will be useful for constant supply of uniform raw materials for commercial secondary metabolite extraction. This will reduce the pressure on natural population of this valuable medicinal plant species and thus be indirectly useful for conservation of this plant species. Qualitative biochemical assay can be used to further explore the medicinal properties of the herb. These also serve as biochemical markers. It has several beneficial properties that can be further exploited to develop new effective pharmaceuticals. Microscopic identification of medicinal plant is essential to distinguish any adulterants or other similar species in the crude herbal mixtures. Microscopic pharmacological characteristics of different plant parts of *Bacopa monnieri* may be used as anatomical markers to identify the plant and its medicinally important plant parts.

Acknowledgement

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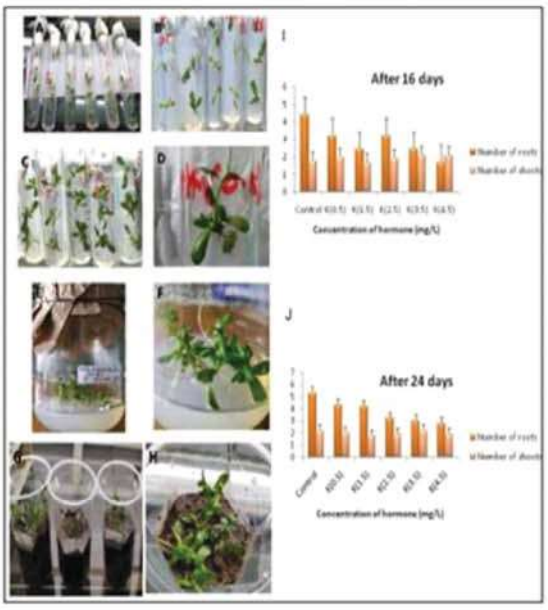

Fig.1. Micropropgation of *Bacopa monnieri* with axillary meristem [MS+K(0.5-4.5 mg/L)] on (A) 0 day, (B)After 8 days, (C) After 16 days , (D) After 24 days , (E-F) After 32 days (G-H) hardening in soil (40-60 days).

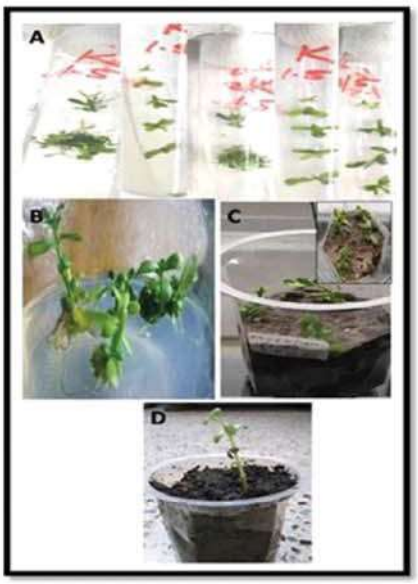

Fig.2. Micropropagation of *Bacopa monnieri*[MS+K(1.5mg/L)] on (A) 24th day; (B) Prominent visualization of propagules showing emergence of shootlets on 32nd day when transferred to jam bottle; (C-D) Some propagules were transferred to plastic glasses for hardening.

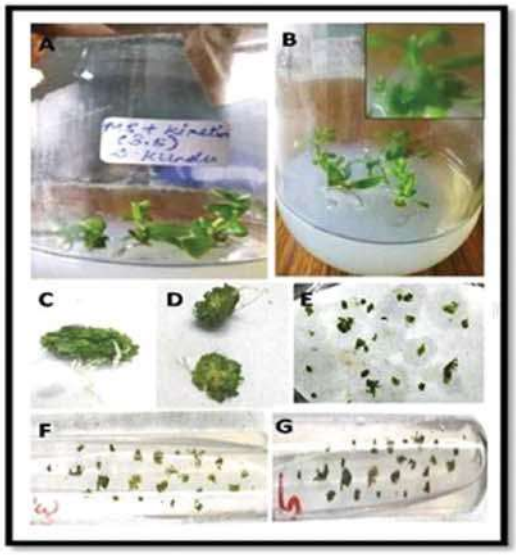

Fig.3. Micropropagated plantlets of *Bacopa monnieri* in MS+K(3.5mg/l) (A-B) showing initiation of propagules on 24th Day ; (C-E) Individual micropropagules were observed (32nd Day) and separated (F) Transfer of micropropagules to MS+K(3.5mg/l) (G) Enlarged micropropagules as observed on 37th day.

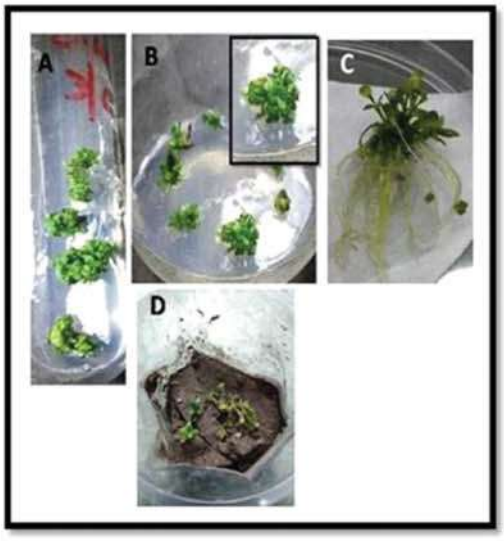

Fig.4. Enlarged micropropagules showing shoot and root emergence on (A) 42nd Day propagule isolated; (B-C): Shoots transferred to jam bottle on 50th Day ; (D) finally transferred to soil on 60th Day.

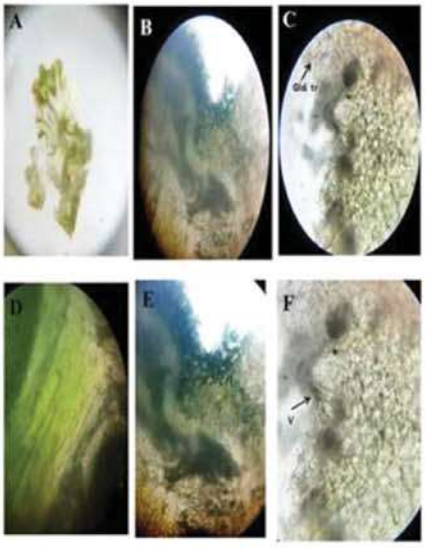

Fig.5.Cross section of propagules show- A: Propagule under simple microscope(x5X); B&E: Several shoot apical meristem like regions with cells containing chloroplast; C: Glandular trichome ; D: Different zones of green cells; F: Vessels with scalariform thickening. Keywords: Gld tr= Glandular trichome, v= Vessel.

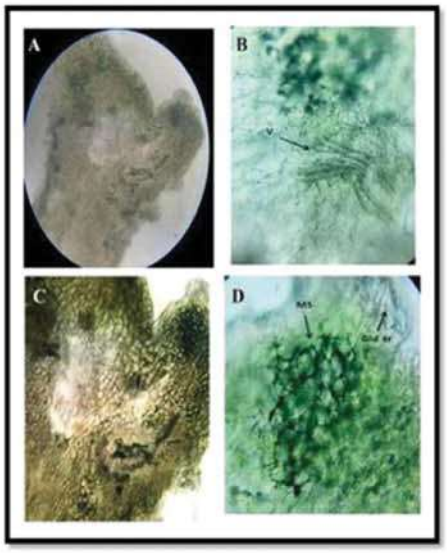

Fig.6. Cross section of propagules showed A&C: Lysis of cells; B: Vessels with scalariform thickening; D: Presence of green mesophyll cells and glandular trichomes. Keywords: v = vessel, Ms = Mesophyll cell, Gld tr = Glandular trichome.

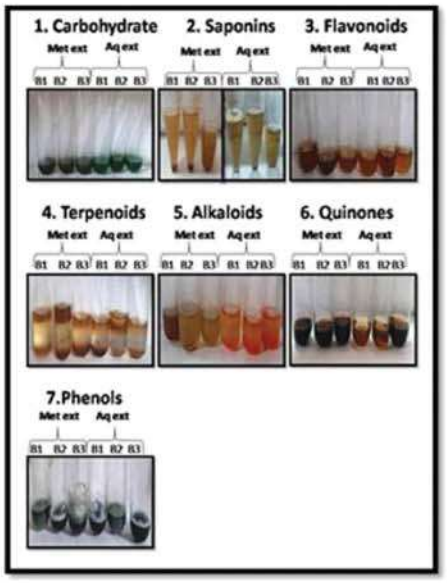

Fig.7.Phytochemical screening (Qualitative Analysis) of *Bacopa monnieri*. Met: Methanolic; Aq: Aqueous extracts; B1: Rishra market; B2: Chandannagar market; B3: Micropropagated.

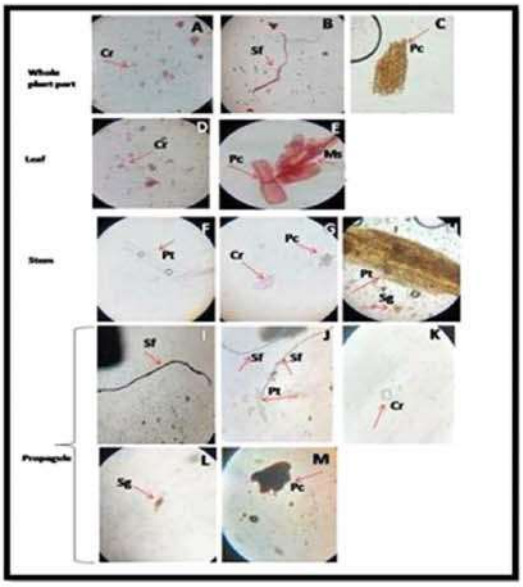

Fig.8. Powder characteristics of *Bacopa monnieri* (L.) Wettst. A-C: Whole plant; D-E: Leaf; F-H: Stem; I- M: Propagule. Keywords: Cr = Crystal, Sf = Septate Fibre, Pc = Parenchymatous Cells, Ms = Macro Sclereid, Pt = Pitted tracheid, Sg = Starch Grains.

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