ISSN: 2348-1900
Plant Science Today
http://www.plantsciencetoday.online

Research Article

In vitro clonal propagation, organogenesis and somatic embryogenesis in *Bacopa monnieri* (L.) Wettst

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Article history
Received: 24 June 2019
Accepted: 02 August 2019
Published: 01 October 2019

Guest Editor
Dr Nishikant Wase

Publisher
Horizon e-Publishing Group

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Abstract

Bacopa monnieri (L.) Wettst is a well-known medicinal herb in the Ayurveda. It is also used as laxative and curative for ulcers, inflammation, anaemia, scabies, leucoderma, asthma and epilepsy, enlargement of spleen, leprosy and others. *In vitro* propagation and regeneration through somatic embryogenesis of *B. monnieri* has played an important role in the production of healthy, disease-free plants with desirable traits. In *B. monnieri*, there are few reports which indicate rapid regeneration and somatic embryogenesis. For *in vitro* clonal propagation, the highest shoot formation was obtained when BAP 2 mg/ l used. The best response for rooting was obtained in IAA 1.0 mg/ l. The recorded survival rate of the plants was 70%. Plants were without any detectable phenotypic variations. Cytological study indicated that the chromosome number remain same (2n= 64) in *in vitro* and *in vivo* roots. A rapid, simple and efficient protocol for plantlet regeneration was achieved through embryogenic callus from leaf explants of *B. monnieri*. Callus induction and embryogenesis were significantly affected by presence/absence and type and concentration of growth regulators. Best organogenic callus induction was obtained in MS medium supplemented with BAP 5mg/ l. For induction of somatic embryogenesis, auxin (2, 4-D 1 mg/ l) was used in the culture medium subsequently in basal media for embryo maturation. Kn 0.2 mg/ l was the best for production of plantlet from embryo. Thus, this can be an easiest protocol for stable clonal propagation and plant regeneration through somatic embryogenesis in *B. monnieri*. The protocol used here for propagation and regeneration is much easier, low cost and reliable.

Keywords: *Bacopa monnieri*; *in vitro*; Organogenesis; Rooting; Shoot Bud Multiplication; Somatic Embryogenesis

Citation: Samanta D, Mallick B, Roy D. *In vitro* clonal propagation, organogenesis and somatic embryogenesis in *Bacopa monnieri* (L.) Wettst. Plant Science Today 2019;6(4):442-449. https://doi.org/10.14719/pst.2019.6.4.600

Indexing: Plant Science Today is covered by Scopus, Web of Science, BIOSIS Previews, ESCI, CAS, AGRIS, CABI, Google Scholar, etc. Full list at http://www.plantsciencetoday.online

Introduction

Bacopa monnieri (L.) Wettst. (Scrophulariaceae) is a well-known medicinal herb in the Ayurveda. The International Union for Conservation of Natural and National Resources has a long time ago listed *Bacopa monnieri* as a threatened species but at present *Bacopa monnieri* comes under Least Concern Category (1). The plant is commonly known as “Brahmi” and found as spreading herbs.

Multiple branches form a mat like structure if they are growing on hard surface. Small roots are produced from each node. White flowers can be seen in well grown plants during rainy season. It is also known as the Indian water hyssop. The plant is commonly found in wet, damp and marshy areas and is widely distributed throughout India, Nepal, Sri Lanka, China, Taiwan, Vietnam and is also found in Florida, Hawaii and other southern states of the USA where it can be grown in damp conditions by the pond or bog garden (2). This plant is used as a brain tonic, which is effective in maintaining vigour and intellect. It enhances the efficiency of transmission of nerve impulses by strengthening memory and cognition (3). It is also used as laxative and curative for ulcers, inflammation, anemia, scabies, leukoderma, asthma and epilepsy, enlargement of spleen, leprosy, eczema, rheumatism, insanity and snake bite, appetitive and cardiotonic (4). *B. monnieri* has been considered as the most important Indian medicinal plants evaluated on the basis of medicinal importance, commercial value and potential for further research and development (5). *Bacopa monnieri* has been used for centuries as a brain tonic, memory enhancer, revitalizer of sensory organs, anti-anxiety, cardio-tonic, diuretic; antidepressant and anticonvulsant agent was seen by some workers (6). In India and Pakistan, the plant is also used for all sorts of skin problems like eczema, psoriasis, abscess, ulcerations, leprosy, for chronic rheumatism as an ointment, asthma and hoarseness of the voice (7).

The herb requirement is rising rapidly in view of the popularity of the *B. monnieri* based drugs (7). In view of wider market demand, there is need to conserve the wild stocks of *B. monnieri*. Due to progressively increasing demand, more than 90% of plant species used by industry are collected from the wild source of which 70% involves unorganized harvesting, leading to rapid depletion of the plant. It also needs immediate conservation and also to meet the growing demand of raw material of medicinal plants.

Tissue culture techniques may be used as alternative methods for propagation and the conservation of germplasm of this important and threatened medicinal plant in many countries. Micropropagation technique provides new possibilities for *in vitro* propagation and multiplication of plants and also recognized as an efficient tool for rapid clonal propagation (8). Till date a few reports are available on stable *in vitro* clonal propagation and somatic embryogenesis of *Bacopa monnieri*. Most of the work has been carried on plant regeneration by adventitious organogenesis from shoot tip, leaf and other parts of the plant. Different author worked on the micropropagation of *B. monnieri* to view the effect of auxins (IAA, NAA & 2,4-D) and cytokinins (BAP, Kn & TDZ) on shoot induction and IAA and TDZ on root induction (8). They also found induction of callus on Murashige and Skoog's medium (9) supplemented with NAA, 2,4-D and TDZ at various combinations and concentrations of plant growth regulators.

Work on somatic embryogenesis of *B. monnieri*, has not been done much (10). Earlier investigator cultured stem, leaf and flower bud explants *in vitro* for the regeneration of plantlets (11). A high frequency of embryoids (35 embryoids/ culture tube) after the transfer of nodular, leaf-derived callus onto MS medium supplemented with 2,4-D.

However, no reports are so far available on stability of the regenerates; in the study the stability of regenerated plants was verified at cytological level. The present investigation provides an important documentation about *in vitro* propagation and regeneration through somatic embryogenesis.

Materials and Methods

Materials

Healthy, young and disease free *Bacopa monnieri* plants were procured from the Medicinal Garden of the Botany Department of the Lady Brabourne College, Kolkata, West Bengal. The plants were identified by the experts of Calcutta University Herbarium (CUH), Kolkata (Fig. 1 a) and herbarium specimen was deposited at CUH, Kolkata (Acc No. 20041).

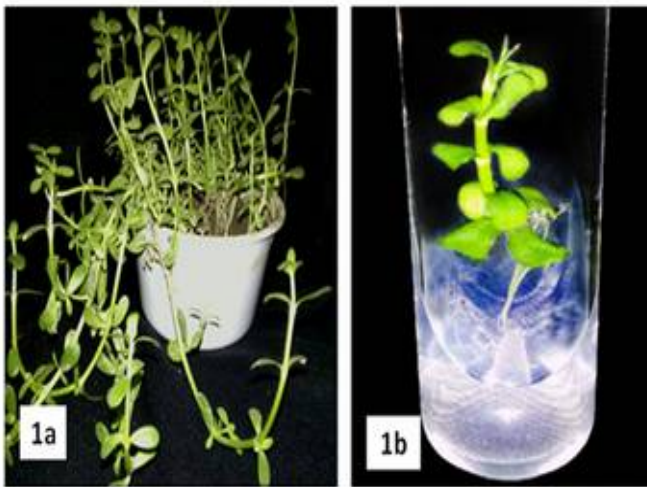

Fig. 1. a. *In vivo Bacopa* plant, **b.** *In vitro Bacopa* plant (ca ×1)

Establishment of culture of Bacopa monnieri- surface sterilization of explant

Explants (apical or axillary bud, internode and leaves) were washed under running tap water for about 25 minutes, then washed with the surfactant Tween 20 solution (2-6 drops in 100 ml solution) for 15 minutes to 20 minutes and finally rinsed with water till the surfactant was thoroughly removed. Further treatments were carried out inside a laminar air flow cabinet. The explants

(apical bud, axillary bud, node and internode) were surface sterilized with 0.1% (w/v) aqueous solution of Mercuric Chloride ($HgCl_2$) for about 10-12 minutes and then thoroughly washed with sterile double distilled water for about 10 minutes (12). Sterilized explants were cultured on MS medium supplemented with different concentrations of hormone.

Culture medium

Explants (apical bud, axillary bud, node and internode) were inoculated on MS medium supplemented with different hormone concentrations for shoot bud multiplication, rooting, callus induction and somatic embryogenesis. The pH of the medium was fine adjusted to 5.7-5.8 before autoclaving. Media contained 3% (w/v) sucrose and solidified with Gelrite 0.25% (w/v) (12). Molten medium was dispensed 20 ml (approximately) into each culture tube (25×150 mm) and plugged with non-absorbent cotton. The medium was autoclaved at 15 lbs/ sq inch pressure and 121°C for 15 minutes.

Culture conditions

All the cultures were maintained at 23±2°C temperature and 60-70% relative humidity, photoperiod of 16 hours day light and 8 hours dark having 3000 lux light intensity by cool white fluorescent tubes (Philips India Ltd.).

Shoot bud multiplication

Shoot multiplication of *Bacopa monnieri* is based on medium supplemented with cytokinin as the major PGR. For shoot bud multiplication, apical or axillary buds were used (13). Explants were cultured in MS media supplemented with BAP (1-4 mg/l) and Kn (1- 4 mg/l).

Root induction of microshoots

Shoot buds were transferred to MS medium containing different concentrations of IAA (0.25-2.00 mg/ l) (pH- 5.8) for root development.

Acclimatization

Rooted plantlets were removed from the culture medium followed by washing under running tap water to remove Gelrite®. Then, the plantlets were transferred to pot containing pre- soaked vermiculite and maintained inside a growth chamber set at 27^{0}C and 70-80% relative humidity. After 25 days they were transplanted to poly bags containing mixture of soil + sand + manure in 1: 1: 1 ratio and kept under shade house for a period of three weeks and subsequently established in the field conditions.

Induction of callus culture

For callus induction leaves, node and internodes were used. Explants were cultured in MS media supplemented with: 1-5 mg/ 1 2, 4-D and 1-5 mg/ l BAP.

Organogenesis

For organogenesis, calli were transferred to MS basal media without any hormone (pH 5.7).

Somatic Embryo induction

The embryo induction medium was supplemented with of 2, 4-D (1 mg/ l) for embryo induction followed by subculture in MS basal media for embryo maturation.

Plantlet formation from Somatic embryos

The plantlets formation medium was supplemented with Kn 0.2 mg/ l (pH 5.7).

SEM analysis

Samples were collected and washed twice with PBS, followed by fixing with 2.5% gluteraldehyde solution for 2 hr. The samples were dehydrated with sequential alcoholic treatment for 10 minute each and imagined using a scanning electron microscope.

Determination of chromosomal stability

Chromosome analysis involved somatic chromosome number determination from both *in vivo* and *in vitro* roots of *Bacopa* by propionic-orcein technique (14).

Data and statistical analysis

In all the cultures, visual observations were recorded such as nodal shoot proliferation, shoot number per explants, length of the regenerated shoots, root number per explants and average root length. Each experiment comprised of 10 replicates and each experiment was repeated thrice. Mean as well as standard error were calculated. The one-way analysis of variance (ANOVA) was carried out to detect the significance of differences among the treatment means. Dunn Kruskal-Wallis multiple comparison test was followed for analysis of different hormonal treatment in case of shoot bud multiplication. Here, *p*-values adjusted with the Benjamini-Hochberg method.

Results and Discussion

In vitro multiplication from apical and axillary bud

Bud breaking was found after 11 days of culture initiation. The highest percentage of multiple shoot induction was 80% on the medium augmented with 2.0 mg l^{-1} BAP (Fig. 1 b) followed by 70.00% on the medium containing1.0 mgl^{-1} BAP and 3.0 mgl^{-1} BAP but the number of shoot bud varied in these 2 hormones (Table 1). Lowest percentage of multiple shoots induction was found to be only 20.00% on the medium containing 1.0 mgl^{-1} Kn. The highest number of shoots per apical or axillary buds was obtained on the medium having 2.0 mg l^{-1} BAP followed by the medium fortified with 1.0 mgl^{-1} BAP. On the other hand, the minimum numbers of

shoots per explant were obtained in the medium supplemented with 1.0 mgl^{-1} Kn. So, BAP 2 mg/ l was the best hormone for *Bacopa* in respect to percentage of shoot bud formation and number of bud formation per explants (15). Statistical analysis showed that the growth regulators differ significantly among themselves with respect to type and concentration for *in vitro* multiplication (Table 1). Micropropagation of *Bacopa monnieri* indicated that it is feasible for rapid propagation, faster introduction of new cultivars with desirable traits and for rapid multiplication of disease-free, healthy propagation material. The efficacy of shoot multiplication is influenced by several factors, such as genotype, media composition, *in vitro* environmental factors, PGR etc. Shoot multiplication depends on the initiation and activity of meristems which are hormonally controlled mainly by cytokinin (16).

Table 1. Effects of different concentrations of BAP and Kn for shoot bud multiplication (after 20 days of culture)

Growth regulators (mg/l)	% of shoot formation	No of shoot developed/ apical or axillary bud*	Shoot length** (mm)
BAP 1.0	70	9-13	12±0.45
BAP 2.0	80	12-17	20±0.13
BAP 3.0	70	7-12	16±0.19
BAP 4.0	60	4-9	15±0.87
Kn 1.0	20	2-3	10±0.51
Kn 2.0	30	7-9	12±0.45
Kn 3.0	30	4-5	14±0.13
Kn 4.0	30	4-5	14±0.12

*Explant= apical and axillary bud.
**Data represents Mean ± SE of 10 replicates.

Table 4. Response of callus for induction of somatic embryo (after 21 days of culture)

Growth regulators (mg/l)	No. of embryo found/ culture tube
2,4-D 0.25	21
2,4-D 0.50	25
2,4-D 1.00	35
2,4-D 1.50	30
2,4-D 2.00	28
IAA 0.25	07
IAA 0.50	10
IAA 1.00	11
IAA 1.50	15
IAA 2.00	15

Note: To test if the growth regulators for shoot (different concentrations of BAP, Kn) differ significantly among themselves H0: The growth regulators for shoot formation do not differ significantly among themselves, H1: The growth regulators differ significantly among themselves. Kruskal- Wallis rank sum test was carried out and the p – value was < 0.05, therefore we rejected H0 and concluded that the growth regulators differed significantly among themselves. Dunn (1964) Kruskal-Wallis multiple comparison (p-values adjusted with the Benjamini-Hochberg method) was conducted. Post hoc test showed that the following pairs of treatments BAP1, BAP4; BAP2,BAP4; BAP1,Kn1; BAP2,Kn1; BAP2,Kn3; BAP1,Kn4; BAP2,Kn4 differed significantly as the p- values for the respective pairs were <0.05.

Rooting

Successful rooting of *in vitro* shoots prior to their establishment in soil is a prerequisite for any propagation method. Rooting of shoots is the most critical step also in the production of complete plants and their subsequent survival. Auxin added exogenously to *in vitro* generated shoots promotes root formation. Individual elongated healthy shoots were isolated and transferred to rooting media and rooting was highest (90%) on full-strength MS medium containing 1 mg/ l IAA (Fig. 1 b). Statistical analysis showed that the different IAA concentrations used here, differed significantly among themselves (Table 2).

Table 5. Regeneration of plantlets from somatic embryo

Day	No. of roots developed*	No. of shoots developed*
7	3 ± 1.56	1.9 ± 1.10
14	4.2 ± 2.30	3 ± 1.56
21	6.3 ± 2.06	5.9 ± 2.08
28	9.2 ± 2.35	7.8 ± 1.81

*Data represents Mean ± SE of 10 replicates

Table 6. Measurement of SEM photoghaph

Type of cell	Length (µm)	Width (µm)
Stomata *in vivo* leaf	17.68	6.89
Stomata *in vitro* leaf	23.77	8.18
Organogenic callus	2884.23	2636.53
Embryogenic callus	1552.69	2018.25
Globular stage	13.58	13.71
Heart shaped stage	4.09	4.47
Torpedo stage	303.76	294.69
Bipolar stage	39.24	31.01

Table 2. Response of different concentrations of IAA for root formation (after 30 days of culture)

Growth regulators (mg/l)	% of root formation	No of root formation/ shoot	Root length* (cm)
IAA 0.25	80	7-13	1.45±0.07
IAA 0.50	80	9-16	1.95±0.08
IAA 1.00	90	13-20	2.50±0.06
IAA 1. 50	90	7-11	2.14±0.05
IAA 2.00	80	5-11	2.10±0.05

*Data represents Mean ± SE of 10 replicates.

Note: To test if the growth regulators for root (different concentrations of IAA) differ significantly among themselves . H0: The growth regulators for root formation do not differ significantly among themselves, H1: The growth regulators differ significantly among themselves. One way ANOVA was conducted and the p-value was <0.05 therefore we rejected H0 and concluded that the growth regulators (different concen-tration of IAA) differed significantly among them- selves. After Post-hoc test (Tukey multiple comparisons of means) it was concluded that Growth regulator pairs IAA 1.0, IAA 0.25; IAA 1.50,IAA0.50; IAA2.00,IAA0.05; IAA 1.50,IAA1.0; IAA2.0,IAA1.0 differed significantly (the respective p-values were <0.05).

Acclimatization

The transfer of plantlets from *in vitro* to *ex vitro* conditions is a very important step in the

structural and physiological adaptation of plants; this is the beginning of the autotrophic life of plants. Micropropagated plants require their successful acclimatization and subsequent transfer to the field. The survival rate of the plants in field conditions was recorded as 70%.

Table 3. Frequency of organogenic callus induction (after 35 days of culture)

Medium	Frequency	Callus morphology
2,4-D 1 mg/l	86.66	Thick compact yellow callus
2,4-D 2 mg/l	33.33	Yellowish-white callus
BAP 5mg/l	66.66	Thick green callus

Induction of callus

Very little callus growth was seen in internode and node explants as compared to leaf explant. The calli were green to light yellowish in color with soft structures. After 10 days of culture the first swelling response for callus was observed in 2,4-D (1 mg/ l). In 2,4-D (2 mg/ l), the response for callus induction was seen after 17 days and in BAP (5 mg/ l) the response was seen after 50 days of culture (Fig. 2 a). Higher concentration of 2,4-D (>2 mg/ l) caused blackening of callus and when we used <5 mg/ l BAP, no callus formation was achieved. Callusing started at the cut ends of the explants after 10-17 days of culture in case of MS Basal media with 2,4-D 1mg/ l and 2,4-D 2 mg/ l. After 60 days of culture callus induction and vigorous growth were obtained in case of MS Basal media supplemented with BAP 5 mg/ l. Hence, it can be concluded that BAP 5mg/ l needs more time to initiate callus induction. The frequency of callus formation was highest when 1mg/ l 2,4-D was used (86.66%) (Table 3). Influence of organic supplements on production of shoot and callus biomass was found in other plant like *Centella asiatica* (17).

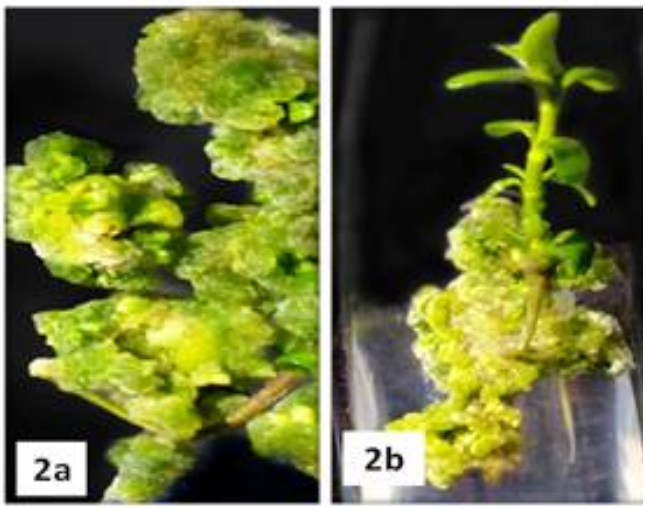

Fig. 2. a. Organogenic callus, **b**. Plantlet regeneration from organogenic callus (ca ×2)

Organogenesis

Plantlets formation was observed after 15 days of culture when intermodal and leaf callus were transferred to MS basal media (Fig. 2 b). As hormone free media was responsible for both shoot and root formation thus this protocol for regeneration from callus was much more cost effective. This protocol for regeneration from callus was much more cost effective.

Somatic Embryo induction and maturation

The success of micropropagation through somatic embryogenesis relies on somatic embryo induction and maturation and conversion of embryos to plantlets. Somatic embryo development was observed in the callus growing in the 2,4-D 1mg/ l followed by their respective transfer in basal media. The different developmental changes were seen (Fig. 3 a-e). After 7 days of induction about 10-15/ culture tube globular (embryo) structure were observed per culture tube (Table 4). All the stages of somatic embryo development were obtained simultaneously. Somatic embryos were observed on the third subculture itself (*i.e.*, between 80 and 90 days from the inoculation of explant) subsequent transfer facilitated development of large numbers of somatic embryos. There is other report which also inferred that withdrawal of auxin produced embryos faster than transferred to another media (18). Globular stage was characterized by small, globose or spherical; globular structure; pale yellow in colour (Fig. 3 a) followed by heart shaped stage which was a three lobed structure with a central depression; pale yellow in colour (Fig. 3 b). In torpedo stage the structure resembles an elongated heart; large and pale yellow in colour (Fig. 3 c) and further elongation of the torpedo shape stage (Fig. 3 d) formed bipolar structure with defined root and shoot meristems that gave rise to a complete plant within a short span of time (Fig. 3 e).

Somatic Embryo to Plantlet formation

Shoot and root developments started after 7 days of culture and proper plantlets started developing after 21 days of culture in MS media supplemented with Kn 0.2 mg/ l. Individual embryos were loosely arranged with the explants and when the embryos were transferred to the media containing Kn 0.2mg/ l, shoots were developed after 15 days followed by rooting (Table 5). So, satisfactory embryo germination was not observed until cytokinin was added. There are also records of using cytokinin for plantlet regeneration from embryogenic calli (Fig. 3 e). The stress regulated genes are induced during embryo maturation and germination and thus certain changes may occur. It is reported that growth regulators significantly influenced the frequency of somatic embryogenesis and plant regeneration (19).

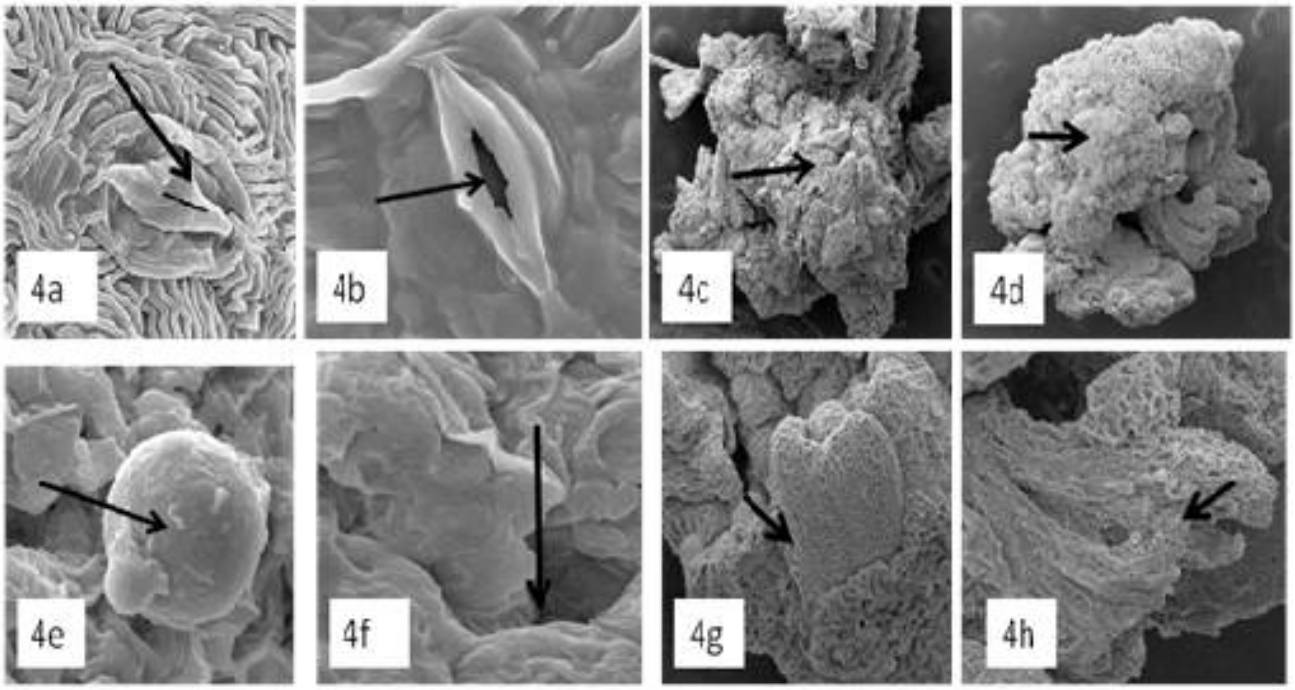

Fig. 4 a-h. Photograph from SEM analysis **a**. stomata *in vivo* leaf, **b**. stomata of *in vitro* leaf, **c**. organogenic callus, **d**. embryogenic callus, **e**. globular stage of somatic embryogenesis, **f**. heart shaped stage of somatic embryogenesis, **g**. Torpedo stage of somatic embryogenesis, **h**. bipolar stage of somatic embryogenesis (ca ×92)

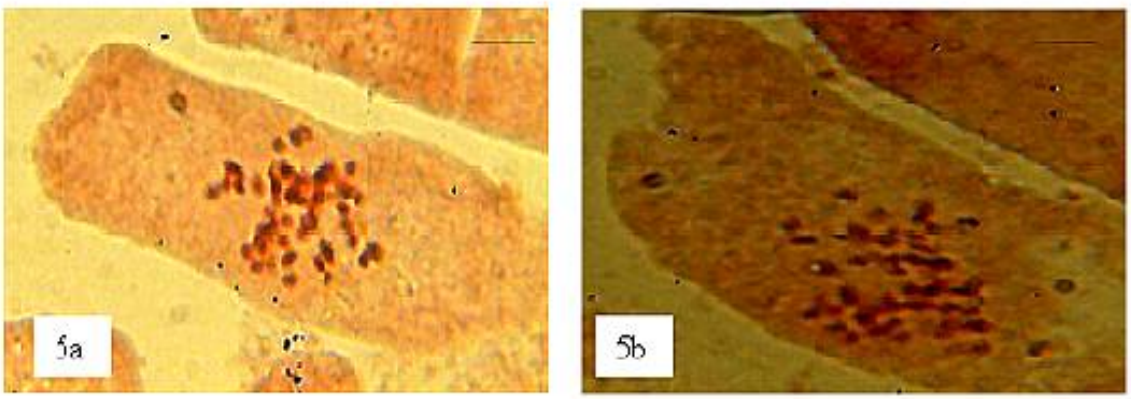

Fig. 5 a-b. Photographs of somatic metaphase plate **a**. *In vivo* plant, **b**. *In vitro* plant (ca ×1790, bar= 5 µm)

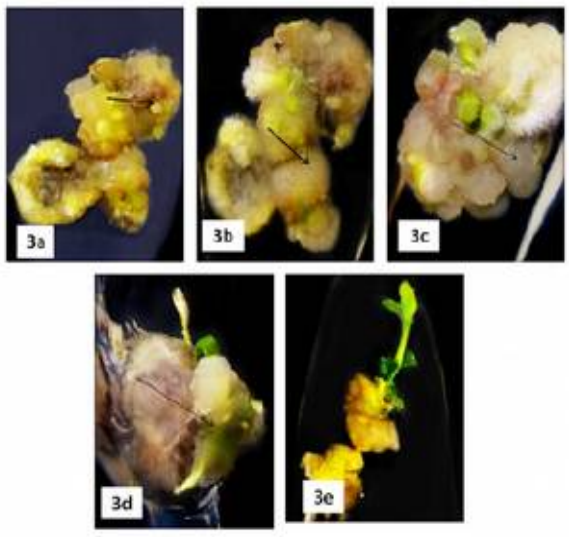

Fig. 3 a-e. Stages of somatic embryogenesis **a**. globular, **b**. heart shaped, **c**. torpedo, **d**. bipolar, **e**. plantlet formation (ca ×1.5)

SEM analysis

The size of stomata was compared using Scanning Electron Microscope (SEM) and the measurements were represented in (Table 6). Size of stomata did not very much in *in vivo* and *in vitro* plant. Different measurements of organogenic callus, embryogenic callus, different stages of somatic embryo development were represented in Table 6 (Fig. 4 a-h). SEM analysis was helpful for accurate measurement and exact structure determination which were very difficult from normal photographs. The exact structure of different stages of somatic embryogenesis can be visualized using SEM. The structure stomata and differences in pore size *in vitro* and *in vivo* leaf can be measured accurately with the help of SEM.

Determination of chromosomal stability

The stability was determined through chromosome analysis. Somatic chromosome

number remains the same in *in vivo* (2n= 64 chromosomes) and *in vitro* plantlets. So, at chromosome level stability was maintained (Fig. 5 a-b).

Conclusion

This research was undertaken to establish an efficient protocol for mass propagation of *Bacopa* from apical and axillary buds and conservation in natural habitats. Results showed that micropropagation, callus induction, somatic embryo induction and maturation were influenced by type and concentration of hormone. Murashige and Skoog (MS) basal medium containing 2 mg/ l BAP was the best for shoot proliferation and the use of both apical and axillary bud was beneficial for clonal propagation. Elongated shoots were successfully rooted in MS basal supplemented with IAA 1.00 mg/ l. Somatic chromosome number remains the same in *in vitro* (2n= 64 chromosomes) and *in vivo* roots which revealed the chromosomal stability of regenerates. Regenerates after proper acclimatization were transferred to an *ex vitro* environment with 70% survival rate and no phenotypic changes was detected in comparison to *in vivo* plants of *Bacopa*. From the above study, it was concluded that leaves were best explant for callus induction of *Bacopa*. The effect of source and plant growth regulators on callus induction of *Bacopa* was explored here. We generally found that auxin is suitable for callus induction but in this study the highest amount of organogenic callus developed in MS medium supplemented with BAP 5 mg/ l, so this can be a new report for callus induction using cytokinin. A plant regeneration system was established *in vitro* from embryogenic callus. Somatic embryogenesis was influenced by the presence and absence of auxin hormone for embryo induction and maturation. Somatic embryo induction was best influenced in the presence of 2,4-D 1 mg/ l, but for subsequent embryo maturation hormone free MS Basal media was the beneficial. So in *Bacopa* tissue culture, morphogenetic response depends on plant growth regulators. Thus, our result on in *vitro* culture and somatic embryogenesis showed the considerable importance for large scale propagation. These protocols described in the present study were reproducible and can be used in future for further developments of the crop and may be applicable for other economically plants as well. The present study on callus induction and regeneration of *Bacopa monnieri* may help conservation of the species and possibly will lead to the production of secondary metabolites and extraction of active compounds from callus sources.

Author's contribution

DS has done Somatic Embryogenesis, SEM analysis, chromosome analysis, data analysis and prepared the content of the manuscript. BM has done collection of literature, identification and characterization of the plant. DR collected plants and subsequently established the culture followed by shoot bud multiplication, root induction, organogenic callus induction and acclimatization. DR also provided inputs and supervised the work.

Acknowledgements

This work was financially supported by the DBT and PG Department of Botany, lady Brabourne College. For SEM analysis authors also gratefully acknowledge the instrumental facilities provided by technician of Saltlake Campus, University of Calcutta. The authors like to thank Dr. Saswati Laha, Department of Botany, Bethune College to give immense support to this work including useful suggestions given by her in the field of collection of material and organogenesis part. We are grateful to Dr. Debabrata Maity, Department of Botany, University of Calcutta for plant identification and suggestions. We would sincerely thank Dr. Snigdha Pain, Consultant, Sankhya Analytical Research Pvt. Ltd., for statistical analysis and Dr. Sandip More, Visiting Scientist, GSI, for his co-operation in SEM analysis. We are also thankful to Dr. Suchita Sinha, Head, Department of Botany, Lady Brabourne College for her constant encouragement.

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