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Effects of Surface Sterilizing Agents, Sucrose and Plant Growth Regulatory Hormone Concentration Levels on Micropropagation of *Bacopa monnieri* L.

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Authors' contributions

This work was carried out in collaboration among all authors. Author MKM designed the study, did the field work, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors PK and DS managed the analyses of the study. Authors RL managed the literature searches and were companion for field work. All authors read and approved the final manuscript.

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ABSTRACT

Background and Aims: *Bacopa monnieri* L. is used to treat sleep deprivation and anxiety. Microropagation is rapid and helps in exsitu preservation of this endangered plant.
Methods: Three surface sterilizing agents with different concentrations [ethanol (EtoH; 50 and 70%), mercuric chloride ($HgCl_2$; 0.1 and 0.5%) and sodium hypochloride (NaOCl; 0.1, 0.5 and 1%)] were used at different time intervals (1, 3, 5, 8 and 10 minutes) with and without hot water to obtain the good aseptic culture. Various concentrations of sucrose (30, 10 and 20 g/L) plus agar 7 g/L was used to observe the effect on root initiation and length in *Bacopa monnieri L.* The initiated explants

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(leaf, node and internode) were cultured on MS media and supplemented with different combination of 2, 4-D (2, 4-dichlorophenoxyacetic acid), KIN (Kinetin) and BAP (6-Benzyl amino purine), IAA (Indole-3-acetic acid), NAA (1-Naphthaleneacetic acid) and GA_3 (Gibberellin A_3) to induce callus, to initiate root and root length, to initiate auxillary bud induction and length and to multiple shoots after inoculation.

Results: It has been seen that 0.1% of $HgCl_2$ (Mercuric chloride) followed by warm water showed 100% aseptic culture conditions. MS media with sucrose (30 g/L) along with agar (7 g/L) showed multiple root initiation whereas MS media with 2,4-D (0 mg/L), KIN (2.0 mg/L) and BAP (2.0 mg/L); NAA (1.0 mg/L) + (KIN 1.0 mg/L) + BAP (1.0 mg/L) and BAP (2.0 mg/L) + KIN (1.0 mg/L) + GA_3 (0.5 mg/L) were found good for rapid callus growth, shoot length and multiple shoots respectively.

Conclusions: In conclusion, higher number of roots and increased root length was observed in MS Media + Sucrose (30 g/L) + Agar (7 g/L). It was also found that in the medium MS + 2,4-D (0 mg/L), KIN (2.0 mg/L) and BAP (2.0 mg/L) rapid callus growth was observed which turned pale yellow and of globular appearance. Highest shoot length was observed in MS + NAA (1.0 mg/L) + (KIN 1.0 mg/L) + BAP (1.0 mg/L) medium. Maximum number of multiple shoots was found in MS + BAP (2.0 mg/L) + KIN (1.0 mg/L) + GA3 (0.5 mg/L).

Keywords: Bacopa monnieri L; microropagation; surface sterilizing agents; sucrose; plant growth regulatory hormones.

1. INTRODUCTION

Bacopa monnieri L. (brahmi) is an old and vital "Medhyarasayana" tranquilize in the conventional arrangement of Indian prescription- the Ayurveda. In India, it develops in moist regions up to 1320 m. It forms a vital element of various Ayurvedic arrangements, for example, "Brahmighrit," "Brahmi-rasayana," "Sarasvatarisht," and "Brahmivati." The entire plant is utilized as a medication to treat epilepsy, mental pressure, and enhance knowledge and memory power, and nervousness [1]. Other than having hostile to inflammatory, pain relieving, and antipyretic properties, the plant is known to likewise have anticancer and cancer prevention agent properties [2]. The saponins - bacoside A and B have been demonstrated for nerving tonic properties [3].

Inadequate seed accessibility and issues related with seed engendering including short seed viability are the real imperatives of seed protection in the quality banks. In vitro clonal propagation, an essential for in vitro preservation by improved axillary branching was standardized. The normal natural surroundings of *Bacopa monnieri* is disintegrating day by day and the plant itself has turned out to be endangered because of numerous reasons. In vitro recovery holds enormous potential for the creation of fantastic plant based drug. Subsequently, there is the need to energize in vitro plant proliferation which is viewed as one of the essential procedures for ex-situ biodiversity preservation.

The best business utilization of tissue culture procedures has been in the generation of consistent with true sort plants at an exceptionally quick rate contrasted with ordinary techniques [4] and tissue cultured plants are accounted for to become quicker and develop sooner than their seed spread offspring [5]. Duplication of plants because of tissue culture can happen through improved development of axillary shoots and creation of extrinsic shoots either straight forwardly from the explant or through the middle of the phase of callus pursued by establishing of individual shoots and furthermore by physical cell embryogenesis [6,7].

Surface sterilization is very important critical step in plant tissue culture. Mathur and Kumar 1998 and Shrivastava and Rajani 1999, employed various sterilization treatments to acquire aseptic conditions of tissue culture [8,9]. Multiple shoot formation in MS media with supplemented with growth regulators auxins and cytokins in diffefent combination, can affect the shoot. MS media supplemented with plant growth regulatory hormones BAP (6-Benzyl amino purine) 0.5 mg/L for *bacopa monnieri*, 1.0 mg/ L for *Paederia foetida* and *Centella asiatica* [10], benzyladenine and NAA (1-Naphthaleneacetic acid) for *Rauwolfia serpentine* [11], 6-benzyladenine (0.01-0.1 mg/l) for morphogenetic response, 6.8M thidiazuron for adventitious shoot buds induction [12], BAP (2 mg/l) and IBA (0.1 mg/l) for initial sprouting in *Centella asiatica* [13,14]. Shrivastava and Rajani 1999; Tiwari et al. 1998, 2000; Asha et al. 2013 have reported high morphogenic potential of *Bacopa monniera* in

plant tissue culture but reports on effect of different surface sterilization agents (EtOH: Ethyl alcohol; $HgCl_2$: Mercuric chloride; and NaOcl: Sodium hypochloride), sucrose concentration in MS media and plant growth regulatory hormones on root, callus, auxillary bud and multiple shoot induction and length are not explained properly [9, 15-17]. So, in this current study, the effects of different concentration of surface sterilization agents, sucrose concentration in MS media and plant growth regulatory hormones on micro propagation of root, callus, auxillary bud and multiple shoot induction and length in *Bacopa monnieri* L. have been explained.

2. METHODOLOGY

2.1 Source and Selection of Explants

The current study has been executed in the Department of Botany, Mizoram University (MZU), Aizawl (Mizoram). Fresh plantlets of *Bacopa monnieri* were acquired from plant nursery, Department of Botany, Mizoram University (MZU), Aizawl (Mizoram). Plant identification was done in the Department of Horticulture and aromatic medicinal plants (HAMP), MZU, Aizawl. After identification, they were maintained in pots in the greenhouse of MZU showing leaves and flowers (Fig. 1). The different parts (Internodes, nodes and leaves) were used as source of explants (disease free, young and healthy) as young cells are supposed to have retained their totipotency for *invitro* propagation.

2.2 Explants Preparation and Sterilization

Stem of *Bacopa monnieri* with leaves (20-25 cm in length) and nodes (8-12 in nuber) collected from greenhouse of MZU, HAMP and were carefully removed by using sterilized surgical blade and brought to the laboratory immediately and were washed under running tap water 10-20 min. to remove all the dust particles and microorganisms from the surface of *Bacopa monnieri* and followed by surfactant (Tween-20: 4-5 drops/ 100 ml water) and fungicide (0.2% Bavistin) to remove dust, microorganisms if any attached on *Bacopa monnieri* for 5 min. They were later treated with surface sterilants 0.1% mercuric chloride and 0.1% sodium hypochlorite solution for 5 minutes under aseptic condition in a Laminar air flow cabinet and followed by repeated rinsing (2-3 times) by using sterilized water for 5min. Leaves, nodes and internodes were separated and used for inoculation after surface sterilization. Experiments were done to examine the upshot of different surface sterilants [(EtoH: Ethanol; $HgCl_2$: Mercuric chloride; NaOCl: sodium hypochlorite) in different concentrations [EtoH (50 and 70%); $HgCl_2$ (0.1 and 0.5%); NaOCl (0.1, 0.5 and 1%)] at different time intervals (1, 3, 5, 8 and 10 minutes) without hot water (Fig. 2a) and with hot water (Fig. 2b) to obtain the good aseptic culture expressed as response (%).

Fig. 1. Photographic representation of leaves (Yellow colour arrow) and flowers (black colour arrow) of *Bacopa monnieri* (L.)

2.3 Nutrient Media and Its Sterilization

The basal culture media contains inorganic and organic salts, growth regulators and agar (all analytical grade chemicals purchased from Merck and Sigma, USA). The media used in this study was Murashige and Skoog (MS 1962) medium including growth hormone. Media was prepared and poured into sterilized conical flasks (500 mL) after adding all ingredients (macronutrients, micronutrients, amino acids, vitamins, carbon sources and growth regulators) in sterilized water and pH is adjusted to 5.8. The media containing high concentration of sucrose (30 g/L) supports the growth of many micro-organisms. So, media was autoclaved at 121°C at 15 p.s.i pressure for 15-20 min to prevent the micro-organisms growth. Various media was prepared by using different concentrations of sucrose (30, 20 and 10 g/L) (Table 1) and growth regulators (BAP: 6-Benzyl amino purine; 2,4-D: 2,4-dichlorophenoxyacetic acid; KIN: Kinetin; IAA: Indole-3-acetic acid; NAA:1-Naphtha-leneacetic acid; GA_3: Gibberellin A_3) for callus, root, auxillary bud and shoot induction (days) and length (cm) (Tables 2-5).

2.4 Culture Initiation and Condition

Sterilized explants (Internodes, nodes and leaves) were used in MS media containing test tubes and were then placed in an upright position in the test tube. After inoculation, test tubes were plugged and labelled under laminar flow to avoid cross contamination and were kept in tissue culture room at 25ºC ± 2ºC temperature (Temp.) and at 50%- 60% humidity with a day and night cycle (16 hours day light and 8 hours night) under the fluorescent light (3000lux). Sub culturing was done every three weeks in fresh media with same composition. Every day observation was done for callus, root, auxiliary buds and multiple shoot formation. Experiments were done to examine the upshot of different plant growth regulators with same media composition. All experiments were replicated 5 times and repeated 3 times and growth responses were observed every week.

2.5 Field Transfer for Hardening

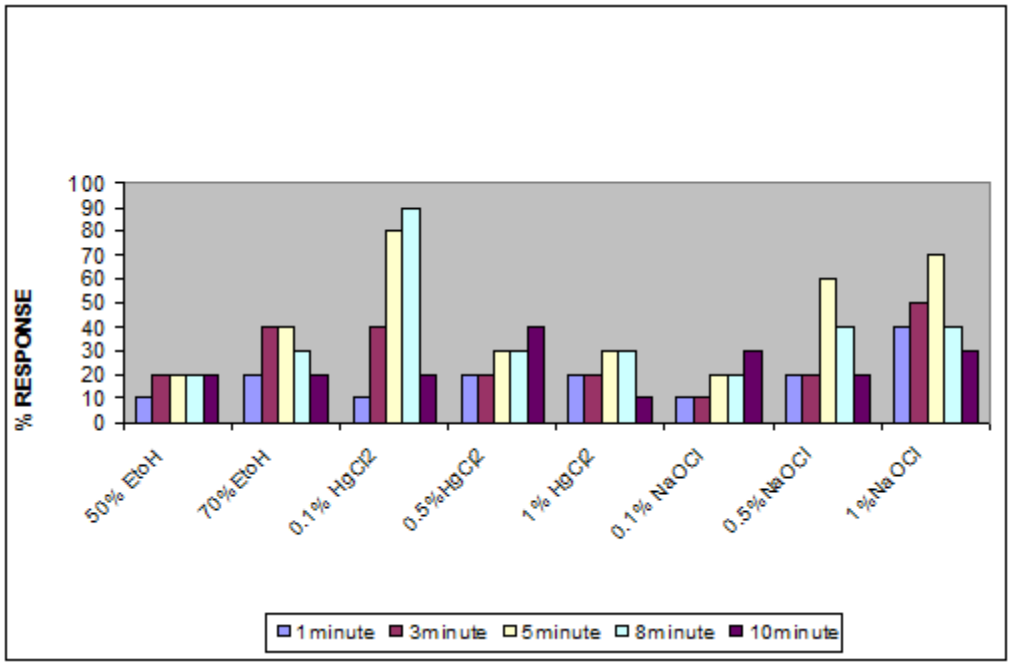
Rooted plantlets were washed and transferred to small plastic bags without any damage to the root systems from the plant tissue culture vessels. Temperature (25ºC ± 2ºC) and Humidity (70% - 80%) was maintained by spraying of cold water at an time interval of 3 to 4 hours using a hand sprayer with fine mist nozzle and also be maintained under shade in the green house. After 15- 20 days, plantlets were transplanted in the field.

2.6 Statistical Analysis

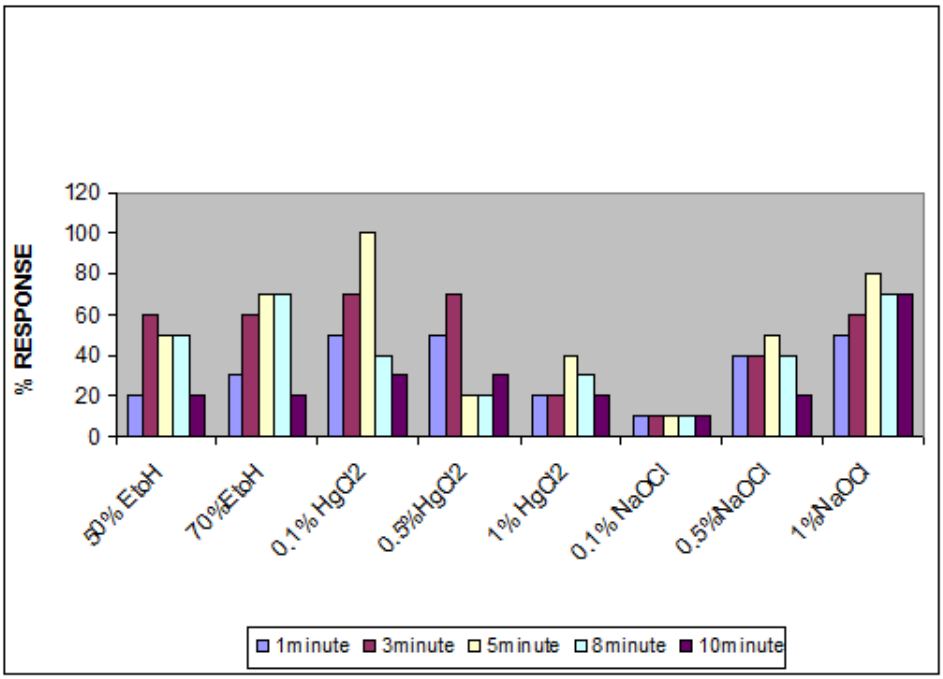
Simple statistical analysis was performed by using SPSS software (version 18.0). Data are represented as mean ± standard deviation.

3. RESULTS

Bacopa monnieri L. is a highly endangered plant, which has a wide range of remedy properties against many diseases. Hence, current study has carried out on standardization of *in vitro* culture technique of *Bacopa monnieri* L. via organogenesis for optimum culture conditions and mass multiplication of the plant. Different parts (Internodes, nodes and leaves) were used as explants but nodal explants were showed good response after 2-3 weeks of culture. These explants were sterilized by using different surface sterilizing agents (ethanol, mercuric chloride, sodium hypochlorite) with and without hot water and also inoculated with different concentrations of sucrose and combination of plant regulators (NAA, IAA, GA_3, KIN, BAP and 2,4-D) for optimum culture conditions and mass multi-plication of the plant.



(a)



(b)

Fig. 2. Effect of various types of surface sterilizing agents (EtoH: Ethanol; $HgCl_2$: Mercuric chloride; NaOCl: sodium hypochlorite) in different concentrations [EtoH (50 and 70%); $HgCl_2$ (0.1 and 0.5%); NaOCl (0.1, 0.5 and 1%)] at different time intervals (1, 3, 5, 8 and 10 minutes) without hot water (a) and with hot water (b) of *Bacopa monnieri* (L.) explants to obtain aseptic culture, expressed as response (%)

3.1 Standardization of Explant Surface Sterilization

Three varieties (EtoH, $HgCl_2$ and NaOCl) of surface sterilizing agents were used in different concentrations EtoH (50% and 70%), $HgCl_2$ (0.1% and 0.5%) and NaOCl (0.1%, 0.5% and 1%) at different time intervals (1, 3, 5, 8 and 10 minutes) without hot water and with hot water to obtain the good aseptic culture because of collected plant explants from the outside of the laboratory infected with microorganisms. The explants responded (%), when treated with first surface sterilizing agent (EtoH), 70% of EtoH showed greater aseptic response in respective to time intervals than 50% of EtoH in the absence of hot water. Second agent ($HgCl_2$), 0.1% $HgCl_2$ showed greater sterilizing response within given time intervals than 0.5% and 1% $HgCl_2$. Third agent (NaOCl), 0.1% NaOCl showed 10% (1 min and 3 min), 20% (5 min and 8 min) and 30% (10 min) respectively followed by 0.5% and 1% NaOCl (20% at 1 min and 2 min, 60% at 5 min, 40% at 8 min, 20% at 10 min and 40% at 1 min, 50% at 3 min, 70% at 5 min, 40% at 8 min, 30% at 10 min. respectively (Figs. 2a and 2b). Based on observations, the explants treated with 0.1% mercuric chloride (HgCl2) for 8 minutes showed 90% of aseptic response followed by 70% of aseptic response when treated with 1% NaOCl at 5min respectively (Fig. 2a) used as a most effective sterilizing agents whereas when the explants treated with 0.1% mercuric chloride ($HgCl_2$) for 5 minutes was preceded by warm water treatment at 50ºC for 5 min gave 100% aseptic culture was obtained (Fig. 2b).

3.2 Effect of Different Combination of Sucrose along with MS Media on Root Initiation and Lenght in *Bacopa monnieri* L. Explants

Rooting in *Bacopa monnieri L. was* impartially unprompted and further no need to add any plant hormones, hence MS media with sucrose concentration 30 g/L (Fig. 3A), 10 g/L (Fig. 3B) and 20 g/L (Fig. 3C) plus agar 7 g/L was effected greatly on root initiation and length in *Bacopa monnieri L.* Root induction (6.02 ± 0.06) in days and root length (2.5 ± 0.15) in centimeter (cm)

was observed in MS Media + Sucrose 30 g/L + Agar 7 g/L followed by MS Media + Sucrose 10g/L + Agar 7 g/L (20.00 ± 0.05 and 0.6 ± 0.13), MS Media + Sucrose 20 g/L + Agar 7 g/L (15.03 ± 0.07 and 0.8 ± 0.02) and MS Media + Agar 7 g/L (0.00 ± 0.00 and 0.0 ± 0.00) respectively (Table 1). This study results showed that MS Media + Agar 7 g/L has no effect on root induction (0.00 ± 0.00) and root length (0.00 ± 0.00) (Fig. 3D and Table 1) as compared with other concentrations. MS Media + Sucrose 30 g/L + Agar 7 g/L was found to be a best media for root induction (days), number and root length (cm) (Fig. 3A and Table 1) as compared to others.

3.3 Effect of Different Combination of Plant Growth Regulatory Hormones along with MS Media on Callus Initiation in *Bacopa monnieri* L.

The initiated explants (leaf, node and internode) were cultured on MS media and supplemented with different combination of 2, 4-D (2, 4-dichlorophenoxyacetic acid), KIN (Kinetin) and BAP (6-Benzyl amino purine) to induce callus and enlargement after 12-14 days of inoculation (Table 2). However, callus formation (callus was globular white and was of pale yellow in colour) started after 20- 25 days at the ends of the explants. The explants cultured with the combination of plant growth hormones, 0 mg/L of 2,4-D, 2.0 mg/L of KIN and 2.0 mg/L of BAP (10.25 ± 0.17) (Fig. 4B and Table 2) followed by 1.0 mg/L of 2,4-D, 0.0 mg/L of KIN and 0.5 mg/L of BAP (11.45 ± 0.24) (Fig. 4A and Table 2) exhibited good and rapid growth of callus from leaves compared with other combination of 2,4-D, KIN and BAP (Table 2).

3.4 Effect of Different Combination of Plant Growth Regulatory Hormones along with MS Media on Root Initiation and Length in *Bacopa monnieri* L.

The initiated explants (leaf, node and internode) were cultured on MS media and supplemented with different combination of IAA

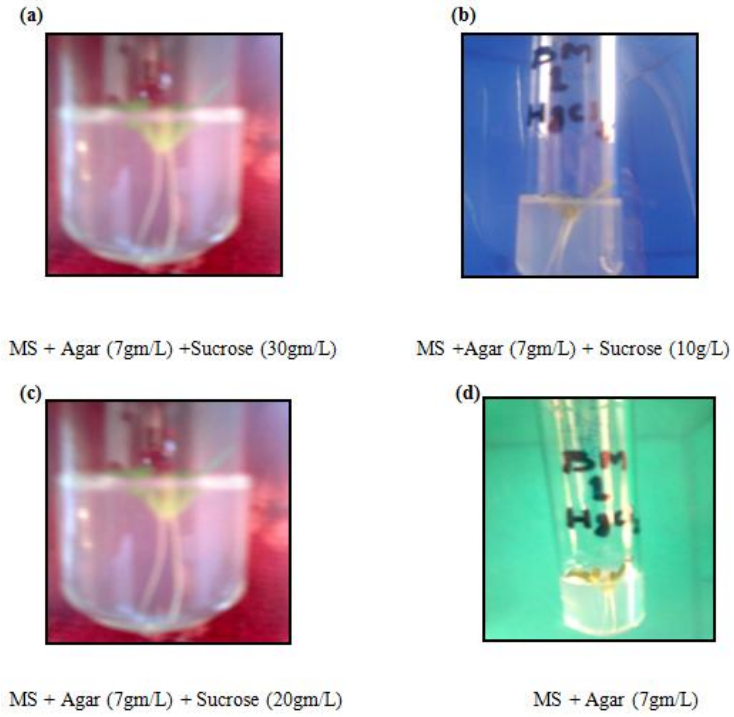


Fig. 3. Effect of different concentrations (a): MS Media + Sucrose 30 (g/L) + Agar 7 (g/L); (b): MS Media + Sucrose (10g/L) + Agar 7 (g/L); (c): MS Media + Sucrose 20 (g/L) + Agar 7 (g/L); (d): MS Media + Agar 7 (g/L) of sucrose (g/L) along with MS (Murashige and Skoog) media and agar (7g/L) on root initiation and root length of *Bacopa monnieri* L. Black colour arrow symbol represents root induction and root length of *Bacopa monnieri* L. after five days of inoculation

(Indole-3-acetic acid), KIN (Kinetin) and BAP (6-Benzyl amino purine) to initiate root and root length (Table 3). However, root initiation was started after 5-6 days of inoculation. After 10 days, 1-2 cm length of single and multiple roots were formed. The explants cultured with the combination of plant growth hormones, 0 mg/L of IAA, 0.0 mg/L of KIN and 0.5 mg/L of BAP showed good and rapid growth of root (6.85 ± 0.97) and root length (1.17 ± 0.11) (Fig. 5B and Table 3) followed by 1.0 mg/L of IAA, 1.0 mg/L of KIN and 0.0 mg/L of BAP, exhibited root induction (8.08 ± 1.09) and root length (0.87 ± 0.06) (Fig. 5A and Table 3) compared with other combination of IAA, KIN and BAP (Figs. 5**C** and 5D and Table 3).

3.5 Effect of Different Combination of Plant Growth Regulatory Hormones along with MS Media on Auxiliary Bud Induction and Length in *Bacopa monnieri* L.

MS media supplemented with different combination of NAA (1-Naphthaleneacetic acid), KIN (Kinetin) and BAP (6-Benzyl amino purine) to initiate auxiliary bud induction and length (Table 4). After inoculation, bud initiation was started from 9-10 days, which multiply into shoot buds and leaves after 21-25 days. However, for auxiliary bud induction (18.09 ± 1.45) and length (10.50 ± 0.32) was observed in the MS media containing combination of NAA (1 mg/L), KIN (1 mg/L) and BAP (1 mg/L) (Fig. 6B and Table 4) followed by 0.0 mg/L of NAA, 1.0 mg/L of KIN and 0.5 mg/L of BAP, exhibited auxiliary bud induction (10.12 ± 1.08) and bud length (8.50 ± 0.16) (Fig. 6A and Table 4) compared with other combination of NAA, KIN and BAP (Table 4).

3.6 Effect of Different Combination of Plant Growth Regulatory Hormones along with MS Media on Multiple Shoot Induction and Number in *Bacopa monnieri* L.

The cultured explants were showed multiple shoots within two weeks of inoculation in MS media with BAP (6-Benzyl amino purine), KIN (Kinetin) and GA_3 (Gibberellin A_3). After successive sub culturing, MS media with combination of BAP (2.0mg/L), KIN (1.0 mg/L) and GA_3 (0.5 mg/L) was given best response of multiple shoots induction (8.25 ± 0.57) and

Table 1. Upshot of various concentrations of sucrose (g/L) along with MS media on root initiation (days) and root length (cm) of *Bacopa monnieri* L.

Various concentrations of sucrose (g/L) with MS media	Root initiation (days)	Root length (cm)
MS Media + Sucrose 30 (g/L) + Agar 7 (g/L)	6.02 ± 0.06	2.5 ± 0.15
MS Media + Sucrose (10 g/L) + Agar 7 (g/L)	20.00 ± 0.05	0.6 ± 0.13
MS Media + Sucrose 20 (g/L) + Agar 7 (g/L)	15.03 ± 0.07	0.8 ± 0.02
MS Media + Agar 7 (g/L)	0.00 ± 0.00	0.0 ± 0.00

Data was expressed as mean ± standard deviation (N = 30 explants/concentration). MS: Murashige and Skoog

Table 2. Effect of various combinations of plant growth regulatory hormones on *in vitro* callus induction (days) of *Bacopa monnieri* L.

Sl no.	Hormone concentration (mg/L)			Callus induction (days)
	2,4-D	KIN	BAP	
1.	0.5	0.0	0.0	25.08 ± 1.54
2.	1.0	0.5	0.0	28.27 ± 1.61
3.	0.5	1.0	0.5	34.12 ± 1.89
4.	1.0	0.0	0.5	11.45 ± 0.24
5.	2.0	0.5	1.0	13.12 ± 0.29
6.	0.0	2.0	2.0	10.25 ± 0.17

Data was represented as mean ± standard deviation (N = 30 explants/combination). 2,4-D: 2,4-dichlorophenoxyacetic acid; KIN: Kinetin; BAP: 6-Benzyl amino purine

Table 3. Effect of various combinations of plant growth regulatory hormones on *invitro* root initiation (days) and root length (cm) of *Bacopa monnieri* L.

Sl no.	Hormone concentration (mg/L)			Root induction (days)	Root length (cm)
	IAA	KIN	BAP		
1.	1.00	1.00	0.00	8.08 ± 1.09	0.87 ± 0.06
2.	0.00	0.00	0.50	6.85 ± 0.97	1.17 ± 0.11
3.	1.00	1.00	1.00	13.25 ± 3.57	0.64 ± 0.04
4.	0.00	1.00	1.00	12.09 ± 2.85	0.78 ± 0.22

Data was showed as mean ± standard deviation (N = 30 explants/combination). IAA: Indole-3-acetic acid; KIN: Kinetin; BAP: 6-Benzyl amino purine

Table 4. Effect of different combinations of plant growth regulatory hormones on *invitro* auxillary bud induction (days) and auxillary bud length (cm) of *Bacopa monnieri* L.

Sl no.	Hormone concentration (mg/L)			Auxillary bud Initiation (days)	Auxillary bud length (cm)
	NAA	KIN	BAP		
1.	1.00	0.50	0.00	7.15 ± 0.25	1.36 ± 0.03
2.	0.00	1.00	0.50	10.12 ± 1.08	8.50 ± 0.16
3.	1.00	1.00	1.00	18.09 ± 1.45	10.50 ± 0.32
4.	1.00	1.00	2.00	12.21 ± 1.12	6.90 ± 0.14
5.	2.00	1.00	1.00	11.05 ± 1.11	5.60 ± 0.11
6.	1.00	2.00	0.00	15.14 ± 1.75	4.80 ± 0.21
7.	3.00	1.00	1.00	16.23 ± 1.82	7.20 ± 0.19

Data was represented as mean ± standard deviation (N = 30 explants/combination). NAA: 1-Naphthaleneacetic acid; KIN: Kinetin; BAP: 6-Benzyl amino purine

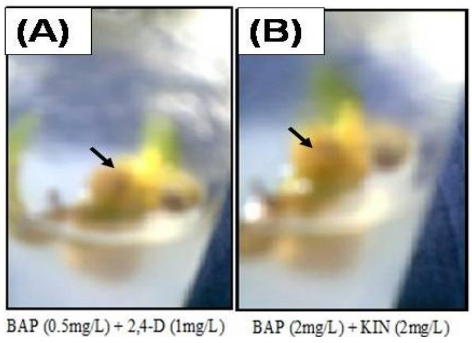


Fig. 4. Effect of different combinations of plant growth regulatory hormones with different concentrations (a): BAP: 6-Benzyl amino purine (0.5 mg/L) plus 2,4-D: 2,4-dichlorophenoxyacetic acid (1 mg/L); (b): BAP: 6-Benzyl amino purine (2.0 mg/L) plus KIN: Kinetin (2 mg/L) on callus initiation of *Bacopa monnieri* L. Black colour arrow indicates callus induction of *Bacopa monnieri* L. after 25 days of culture

number (4.09 ± 0.09) (Fig. 7**A** and Table 5) followed by 1.0 mg/L of BAP, 1.0 mg/L of KIN and 0.0 mg/L of GA_3, exhibited multiple shoots induction (10.45 ± 1.42) and number (3.60 ± 0.24) (Fig. 7**A** and Table 5) compared with other combinations of BAP (6-Benzyl amino purine), KIN (Kinetin) and GA_3 (Gibberellin A_3) (Table 5).

Plantlets were transplanted to polyethylene bags and kept at green house conditions for few weeks, later shifted to field transfer for hardening. The developed protocols can be very useful in conservation and propagation of *Bacopa monnieri.*

4. DISCUSSION

Plant tissue culture is a biotechnological tool and depends on various factors such as type of explants, surface sterilizing agents, explants age, concentration and combination of plant hormones for taking care of standardization of protocol and the issues of propagation of multipurpose and endangered therapeutic plants in India [18]. Three (EtoH, HgCl2 and NaOCl) surface disinfecting agents were utilized in various concentrations EtoH (half and 70%), HgCl2 (0.1% and 0.5%) and NaOCl (0.1%, 0.5% and 1%) at various time interims (1, 3, 5, 8 and 10 minutes) without hot water and with hot water to get the great aseptic culture on account of gathered plant explants from the outside of the research center tainted with microorganisms. Various parameters (Type of explant, age, treatment time) led to death of explants [19,20] hence ethyl alcohol and sodium hypochloride were not suitable and efficient sterilants in case of *Bacopa monnieri* L.

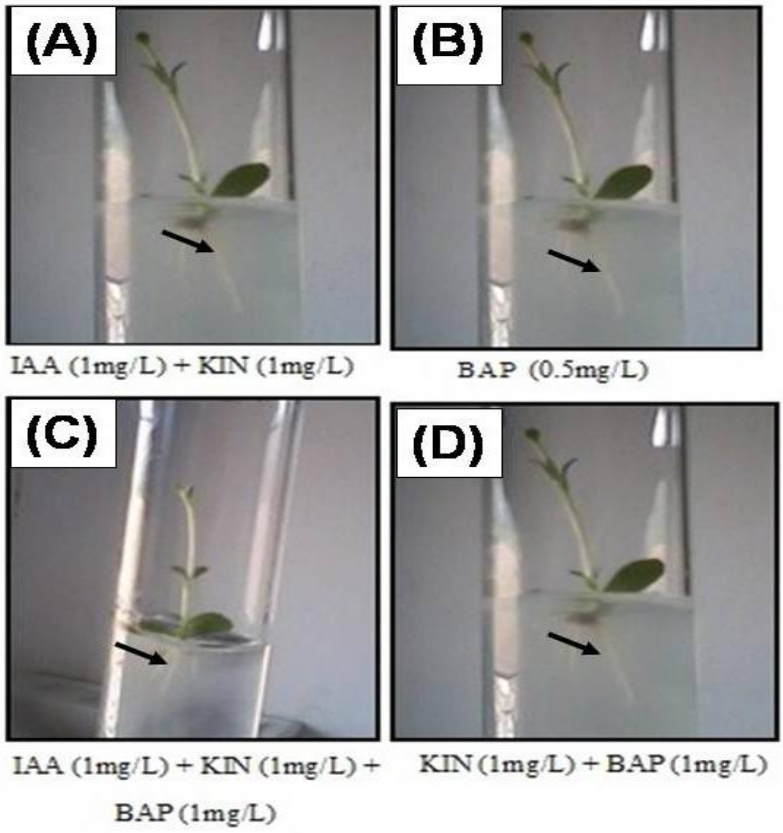


Fig. 5. Effect of different combinations of plant growth regulatory hormones with different concentrations (a): IAA: Indole-3-acetic acid (1.0 mg/L) plus KIN: Kinetin (1.0 mg/L); (b): BAP: 6-Benzyl amino purine (0.5 mg/L); (c): IAA: Indole-3-acetic acid (1.0 mg/L) plus KIN: Kinetin (1.0 mg/L) plus BAP: 6-Benzyl amino purine (0.5 mg/L); (d): KIN: Kinetin (1.0 mg/L) plus BAP: 6-Benzyl amino purine (0.5 mg/L) on root initiation and root length of *Bacopa monnieri* L. Black colour arrow indicates root induction and root length of *Bacopa monnieri* L

Table 5. Effect of various combinations of plant growth regulatory hormones on *in vitro* multiple shoots initiation (days) and number of shoots of *Bacopa monnieri* L.

Sl no.	Hormones (mg/L)			Shoot initiation (days)	Number of shoots
	BAP	KIN	GA_3		
1.	1.00	1.00	0.00	10.45 ± 1.42	3.60 ± 0.24
2.	2.00	1.00	0.50	8.25 ± 0.57	4.09 ± 0.09
3.	2.00	2.00	0.00	16.21 ± 1.85	2.27 ± 0.02
4.	5.00	0.00	0.00	13.02 ± 1.52	2.74 ± 0.03
5.	2.00	1.00	0.00	14.23 ± 1.68	4.09 ± 0.08
6.	1.00	1.00	0.00	12.08 ± 1.12	2.42 ± 0.03
7.	1.00	1.00	0.50	11.05 ± 1.08	2.06 ± 0.02

Data was showed as mean ± standard deviation (N = 30 explants/combination). BAP: 6-Benzyl amino purine; KIN: Kinetin; G

Establishing in *Bacopa monnieri* L. was fairly unprompted and advance no compelling reason to include any plant hormones, thus MS media with various sucrose concentrations (30 g/L,10 g/L and 20 g/L) in addition to agar 7 g/L was affected extraordinarily on root commencement and length in Bacopa monnieri L. Root enlistment (6.02 ± 0.06) in days and root length (2.5 ± 0.15) in centimeter (cm) was seen in MS Media + Sucrose 30 g/L + Agar 7 g/L. Our results were

similar to that of previous studies [21, 22], the impact of sucrose was broke down as regenerative potential and development of recovered shoots amid culture period. The most surprising rate of recovery was seen in the medium enhanced with 2% sucrose with most surprising number and length of recovered shoots.

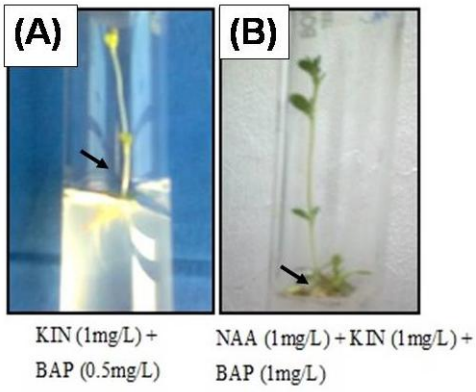


Fig. 6. Effect of different combinations of plant growth regulatory hormones with different concentrations (a): KIN: Kinetin (1.0 mg/L) plus BAP: 6-Benzyl amino purine (0.5 mg/L); (b): NAA:1-Naphthaleneacetic acid (1.0mg/L) plus KIN: Kinetin (1.0 mg/L) plus BAP: 6-Benzyl amino purine (1.0 mg/L) on axillary bud induction and length of *Bacopa monnieri* L. Black colour arrow indicates axillary bud induction and length of *Bacopa monnieri* L. after 15 days of inoculation

The explants were cultured on MS media with different combination of 2, 4-D (2, 4-dichlorophenoxyacetic acid), KIN (Kinetin) and BAP (6-Benzyl amino purine) to induce callus and enlargement after 12-14 days of inoculation. However, callus formation (callus was globular white and was of pale yellow in color) started after 20- 25 days at the ends of the explants. Based on results, callus induction requires the presence of auxins or cytokinins or both in the media based on the source of explants. Previous studies suggested that by addition of 1μL BAP and 2, 4-D, initiated a thin layer of granular callus after 4 weeks of culture. However, all the explants turned brown to black at the base after 6 weeks of culture [9]. Atefeh et al. also reported, 2, 4-D (2.5 mg/L) and KIN (0.5 mg/L) in MS medium was best media for callus induction in cumin (*Cuminum cyminum*) [23]. Highest percentage of callus induction was acquired with media, supplemented with 2.4-D and KIN in Juniperus excels L. [24] and Kelussia odoratissimia Mozaff [25].

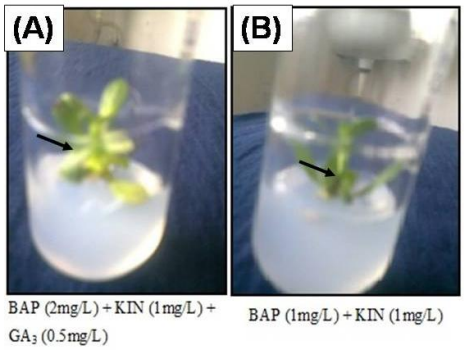


Fig. 7. Effect of different combinations of plant growth regulatory hormones with different concentrations (a): BAP: 6-Benzyl amino purine (2.0 mg/L) plus KIN: Kinetin (1.0 mg/L) plus GA_3: Gibberellin A_3 (0.5 mg/L); (b): BAP: 6-Benzyl amino purine (1.0 mg/L) plus KIN: Kinetin (1.0 mg/L) on multiple shoot induction and number, of *Bacopa monnieri* L. Black colour arrow indicates multiple shoot induction of *Bacopa monnieri* L

The started explants were cultured on MS media and enhanced with various mix of IAA (Indole-3-acidic acid), KIN (Kinetin) and BAP (6-Benzyl amino purine) to start root and root length (Table 3). Be that as it may, root commencement was begun following 5-6 days of inoculation. Following 10 days, 1-2 cm length of single and various roots were formed. The explants cultured with the plant development hormones, 0 mg/L of IAA, 0.0 mg/L of KIN and 0.5 mg/L of BAP indicated great and fast development of root (6.85 ± 0.97) and root length (1.17 ± 0.11). Based on results, MS media with any additional growth regulator showed 100% root formation. MS media with 2 mg/L of indole-3-butyric acid gave 87% of root growth [10]. Singh et al. 1999 and Rani et al. 2000 also reported that root growth in MS media with BAP (0.5 mg/L) within 6 days of culture and 90% of growth rate in 2.46μm IBA [26,27] .

MS media enhanced with various combinations of NAA (1-Naphthaleneacetic acid), KIN (Kinetin) and BAP (6-Benzyl amino purine) to start auxiliary bud induction and length. After inoculation, bud commencement was begun from 9-10 days, which increase into shoot buds and leaves following 21-25 days. Be that as it may, for auxiliary bud induction (18.09 ± 1.45) and length (10.50 ± 0.32) was seen in the MS media

containing combination of NAA (1 mg/L), KIN (1 mg/L) and BAP (1 mg/L). Previous studies on medicinal plants (Paederia foetida, Centella asiatica and Rauwolfia serpentine) have shown effects of hormones (alone and in combination) on auxillary bud induction and bud length [9, 11, 26]. MS media supplemented with BAP (1.0 mg /L) in Paederia foetida and Centella asiatica [26] and benzyladenine and NAA in Rauwolfia serpentine [11] showed optimum auxillary bud proliferation, growth and length.

The cultured explants were demonstrated numerous shoots inside about fourteen days of inoculation in MS media with BAP (6-Benzyl amino purine), KIN (Kinetin) and GA3 (Gibberellin A3). After progressive sub culturing, MS media with combination of BAP (2.0mg/L), KIN (1.0 mg/L) and GA3 (0.5 mg/L) was given best numerous shoots induction (8.25 ± 0.57) and number (4.09 ± 0.09). Our results suggested that MS media is very effective for shoot multiplication. These results were associated with previous studies [1,28-30].

5. CONCLUSION

In conclusion, higher number of roots and increased root length was observed in MS Media + Sucrose (30 g/L) + Agar (7 g/L). It was also found that in the medium MS + 2,4-D (0 mg/L), KIN (2.0 mg/L) and BAP (2.0 mg/L) rapid callus growth was observed which turned pale yellow and of globular appearance. Highest shoot length was observed in MS + NAA (1.0 mg/L) + (KIN 1.0 mg/L) + BAP (1.0 mg/L) medium. Maximum number of multiple shoots was found in MS + BAP (2.0 mg/L) + KIN (1.0 mg/L) + GA3 (0.5 mg/L).

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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