

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

37 **ABSTRACT**

38 **Background and Aims:** *Bacopa monnieri* L. is used to treat sleep deprivation and anxiety.
39 **Microropagation is rapid and helps in exsitu preservation of this endangered plant.**

40 **Methods:** **Three** surface sterilizing agents with different concentrations [ethanol (EtoH; 50 and 70%),
41 mercuric chloride (HgCl₂; 0.1 and 0.5%) and sodium hypochloride (NaOCl; 0.1, 0.5 and 1%)] were used at
42 different time intervals (1, 3, 5, 8 and 10 minutes) with and without hot water to obtain the good aseptic
43 culture. Various concentrations of sucrose (30, 10 and 20 g/L) plus agar 7 g/L was used to observe the
44 effect on root initiation and length in *Bacopa monnieri* L. The initiated explants (leaf, node and internode)
45 were cultured on MS media and supplemented with different combination of 2, 4-D (2, 4-
46 dichlorophenoxyacetic acid), KIN (Kinetin) and BAP (6-Benzyl amino purine), IAA (Indole-3-acetic acid),
47 NAA (1-Naphthaleneacetic acid) and GA₃ (Gibberellin A₃) to induce callus, to initiate root and root length,
48 to initiate auxillary bud induction and length and to multiple shoots after inoculation.

49 **Key Results:** It has been seen that 0.1 % of HgCl₂ (Mercuric chloride) followed by warm water showed
50 100% aseptic culture conditions. MS media with sucrose (30 g/L) along with agar (7 g/L) showed multiple
51 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
52 mg/L) + (KIN 1.0 mg/L) + BAP (1.0 mg/L) and BAP (2.0 mg/L) + KIN (1.0 mg/L) + GA₃ (0.5 mg/L) were
53 found good for rapid callus growth, shoot length and multiple shoots respectively.

54 **Conclusions:** In conclusion, higher number of roots and increased root length was observed in MS
55 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
56 (2.0 mg/L) and BAP (2.0 mg/L) rapid callus growth was observed which turned pale yellow and of
57 globular appearance. Highest shoot length was observed in MS + NAA (1.0 mg/L) + (KIN 1.0 mg/L) +
58 BAP (1.0 mg/L) medium. Maximum number of multiple shoots was found in MS + BAP (2.0 mg/L) + KIN
59 (1.0 mg/L) + GA₃ (0.5 mg/L).

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61

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

64

65 1. INTRODUCTION

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

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

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

88 Surface sterilization is very important critical step in plant tissue culture. Mathur and Kumar 1998 and
89 Shrivastava and Rajani 1999, employed various sterilization treatments to acquire aseptic conditions of
90 tissue culture [8, 9]. Multiple shoot formation in MS media with supplemented with growth regulators
91 auxins and cytokins in different combination, can affect the shoot. MS media supplemented with plant
92 growth regulatory hormones BAP (6-Benzyl amino purine) 0.5 mg/L for *bacopa monnieri*, 1.0 mg/ L for

93 *Paederia foetida* and *Centella asiatica* [10], benzyladenine and NAA (1-Naphthaleneacetic acid) for
94 *Rauwolfia serpentine* [11], 6-benzyladenine (0.01-0.1 mg/l) for morphogenetic response, 6.8M thidiazuron
95 for adventitious shoot buds induction [12], BAP (2mg/l) and IBA (0.1 mg/l) for initial sprouting in *Centella*
96 *asiatica* [13,14]. Shrivastava and Rajani 1999; Tiwari *et al.*, 1998, 2000; Asha *et al.*, 2013 have reported
97 high morphogenic potential of *Bacopa monniera* in plant tissue culture but reports on effect of different
98 surface sterilization agents (EtOH: Ethyl alcohol; HgCl₂: Mercuric chloride; and NaOCl: Sodium
99 hypochloride), sucrose concentration in MS media and plant growth regulatory hormones on root, callus,
100 auxillary bud and multiple shoot induction and length are not explained properly [9, 15-17]. So, in this
101 current study, the effects of different concentration of surface sterilization agents, sucrose concentration
102 in MS media and plant growth regulatory hormones on micro propagation of root, callus, auxillary bud and
103 multiple shoot induction and length in *Bacopa monnieri* L. have been explained.

104

105 **2. METHODOLOGY**

106

107 **2.1 Source and selection of explants**

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

115 **2.2 Explants preparation and sterilization**

116 Stem of *Bacopa monnieri* with leaves (20-25 cm in length) and nodes (8-12 in nuber) collected from
117 greenhouse of MZU, HAMP and were carefully removed by using sterilized surgical blade and brought to
118 the laboratory immediately and were washed under running tap water 10-20 min. to remove all the dust
119 particles and microorganisms from the surface of *Bacopa monnieri* and followed by surfactant (Tween-20:
120 4-5 drops/ 100 ml water) and fungicide (0.2% Bavistin) to remove dust, microorganisms if any attached

121 on *Bacopa monnieri* for 5 min. They were later treated with surface sterilants 0.1% mercuric chloride and
122 0.1% sodium hypochlorite solution for 5 minutes under aseptic condition in a Laminar air flow cabinet and
123 followed by repeated rinsing (2-3 times) by using sterilized water for 5min. Leaves, nodes and internodes
124 were separated and used for inoculation after surface sterilization. Experiments were done to examine
125 the upshot of different surface sterilants [(EtoH: Ethanol; HgCl₂: Mercuric chloride; NaOCl: sodium
126 hypochlorite) in different concentrations [EtoH (50 and 70%); HgCl₂ (0.1 and 0.5%); NaOCl (0.1, 0.5 and
127 1%)] at different time intervals (1, 3, 5, 8 and 10 minutes) without hot water (Fig. 2a) and with hot water
128 (Fig. 2b) to obtain the good aseptic culture expressed as response (%).

129 **2.3 Nutrient media and its sterilization**

130 The basal culture media contains inorganic and organic salts, growth regulators and agar (all analytical
131 grade chemicals purchased from Merck and Sigma, USA). The media used in this study was Murashige
132 and Skoog (MS 1962) medium including growth hormone. Media was prepared and poured into sterilized
133 conical flasks (500mL) after adding all ingredients (macronutrients, micronutrients, amino acids, vitamins,
134 carbon sources and growth regulators) in sterilized water and pH is adjusted to 5.8. The media containing
135 high concentration of sucrose (30 g/L) supports the growth of many micro organisms. So, media was
136 autoclaved at 121°C at 15 p.s.i pressure for 15-20 min to prevent the microorganisms growth. Various
137 media was prepared by using different concentrations of sucrose (30, 20 and 10 g/L) (Table 1) and
138 growth regulators (BAP: 6-Benzyl amino purine; 2,4-D: 2,4-dichlorophenoxyacetic acid; KIN: Kinetin; IAA:
139 Indole-3-acetic acid; NAA:1-Naphthaleneacetic acid; GA₃: Gibberellin A₃) for callus, root, auxillary bud
140 and shoot induction (days) and length (cm) (Tables 2-5).

141 **2.4 Culture initiation and condition**

142 Sterilized explants (Internodes, nodes and leaves) were used in MS media containing test tubes and were
143 then placed in an upright position in the test tube. After inoculation, test tubes were plugged and labelled
144 under laminar flow to avoid cross contamination and were kept in tissue culture room at 25°C ± 2°C
145 temperature (Temp.) and at 50%- 60% humidity with a day and night cycle (16 hours day light and 8
146 hours night) under the fluorescent light (3000lux). Sub culturing was done every three weeks in fresh
147 media with same composition. Every day observation was done for callus, root, auxillary buds and
148 multiple shoot formation. Experiments were done to examine the upshot of different plant growth

149 regulators with same media composition. All experiments were replicated 5 times and repeated 3 times
150 and growth responses were observed every week.

151 **2.5 Field transfer for hardening**

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

157 **2.6 Statistical analysis**

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

160 **3. RESULTS**

161 **3. RESULTS**
162 *Bacopa monnieri* L. is a highly endangered plant, which has a wide range of remedy properties against
163 many diseases. Hence, current study has carried out on standardization of invitro culture technique of
164 *Bacopa monnieri* L. via organogenesis for optimum culture conditions and mass multiplication of the
165 plant. Different parts (Internodes, nodes and leaves) were used as explants but nodal explants were
166 showed good response after 2-3 weeks of culture. These explants were sterilized by using different
167 surface sterilizing agents (ethanol, mercuric chloride, sodium hypochlorite) with and without hot water and
168 also inoculated with different concentrations of sucrose and combination of plant regulatories (NAA, IAA,
169 GA₃, KIN, BAP and 2,4-D) for optimum culture conditions and mass multiplication of the plant.

170 **3.1 Standardization of explant surface sterilization**

171 Three varieties (EtoH, HgCl₂ and NaOCl) of surface sterilizing agents were used in different
172 concentrations EtoH (50% and 70%), HgCl₂ (0.1% and 0.5%) and NaOCl (0.1%, 0.5% and 1%) at
173 different time intervals (1, 3, 5, 8 and 10 minutes) without hot water and with hot water to obtain the good
174 aseptic culture because of collected plant explants from the outside of the laboratory infected with
175 microorganisms. The explants responded (%), when treated with first surface sterilizing agent (EtoH),
176 70% of EtoH showed greater aseptic response in respective to time intervals than 50% of EtoH in the
177 absence of hot water. Second agent (HgCl₂), 0.1% HgCl₂ showed greater sterilizing response with in

178 given time intervals than 0.5% and 1% HgCl₂. Third agent (NaOCl), 0.1% NaOCl showed 10% (1 min
179 and 3 min), 20% (5 min and 8 min) and 30% (10 min) respectively followed by 0.5% and 1% NaOCl
180 (20% at 1min and 2min, 60% at 5 min, 40% at 8 min, 20% at 10min and 40% at 1 min, 50% at 3 min, 70%
181 at 5 min, 40% at 8 min, 30% at 10 min. respectively (Figs. 2a and 2b). Based on observations, the
182 explants treated with 0.1% mercuric chloride (HgCl₂) for 8 minutes showed 90% of aseptic response
183 followed by 70% of aseptic response when treated with 1% NaOCl at 5min respectively (Fig. 2a) used as
184 a most effective sterilizing agents whereas when the explants treated with 0.1% mercuric chloride (HgCl₂)
185 for 5 minutes was preceded by warm water treatment at 50 °C for 5 min gave 100% aseptic culture was
186 obtained (Fig. 2b).

187 3.2 Effect of different combination of sucrose along with MS media on root initiation and length in 188 *Bacopa monnieri* L. explants

189 Rooting in *Bacopa monnieri* L. was impartially unprompted and further no need to add any plant
190 hormones, hence MS media with sucrose concentration 30 g/L (Fig. 3A), 10 g/L (Fig. 3B) and 20 g/L (Fig.
191 3C) plus agar 7 g/L was effected greatly on root initiation and length in *Bacopa monnieri* L. Root induction
192 (6.02 ± 0.06) in days and root length (2.5 ± 0.15) in centimeter (cm) was observed in MS Media +
193 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 ±
194 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
195 (0.00 ± 0.00 and 0.0 ± 0.00) respectively (Table 1). This study results showed that MS Media + Agar 7 g/L
196 has no effect on root induction (0.00 ± 0.00) and root length (0.00 ± 0.00) (Fig. 3D and Table 1) as
197 compared with other concentrations. MS Media + Sucrose 30 g/L + Agar 7 g/L was found to be a best
198 media for root induction (days), number and root length (cm) (Fig. 3A and Table 1) as compared to
199 others.

200 3.3 Effect of different combination of plant growth regulatory hormones along with MS media on 201 callus initiation in *Bacopa monnieri* L.

202 The initiated explants (leaf, node and internode) were cultured on MS media and supplemented with
203 different combination of 2, 4-D (2, 4-dichlorophenoxyacetic acid), KIN (Kinetin) and BAP (6-Benzyl amino
204 purine) to induce callus and enlargement after 12-14 days of inoculation (Table 2). However, callus
205 formation (callus was globular white and was of pale yellow in colour) started after 20- 25 days at the

206 ends of the explants. The explants cultured with the combination of plant growth hormones, 0 mg/L of 2,4-
207 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-
208 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
209 growth of callus from leaves compared with other combination of 2,4-D, KIN and BAP (Table 2).

210 **3.4 Effect of different combination of plant growth regulatory hormones along with MS media on** 211 **root initiation and length in *Bacopa monnieri* L.**

212 The initiated explants (leaf, node and internode) were cultured on MS media and supplemented with
213 different combination of IAA (Indole-3-acetic acid), KIN (Kinetin) and BAP (6-Benzyl amino purine) to
214 initiate root and root length (Table 3). However, root initiation was started after 5-6 days of inoculation.
215 After 10 days, 1-2 cm length of single and multiple roots were formed. The explants cultured with the
216 combination of plant growth hormones, 0 mg/L of IAA, 0.0 mg/L of KIN and 0.5 mg/L of BAP showed
217 good and rapid growth of root (6.85 ± 0.97) and root length (1.17 ± 0.11) (Fig. 5B and Table 3) followed
218 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
219 length (0.87 ± 0.06) (Fig. 5A and Table 3) compared with other combination of IAA, KIN and BAP (Figs.
220 5C and 5D and Table 3).

221 **3.5 Effect of different combination of plant growth regulatory hormones along with MS media on** 222 **auxillary bud induction and length in *Bacopa monnieri* L.**

223 MS media supplemented with different combination of NAA (1-Naphthaleneacetic acid), KIN (Kinetin) and
224 BAP (6-Benzyl amino purine) to initiate auxillary bud induction and length (Table 4). After inoculation, bud
225 initiation was started from 9-10 days, which multiply into shoot buds and leaves after 21-25 days.
226 However, for auxillary bud induction (18.09 ± 1.45) and length (10.50 ± 0.32) was observed in the MS
227 media containing combination of NAA (1 mg/L), KIN (1 mg/L) and BAP (1 mg/L) (Fig. 6B and Table 4)
228 followed by 0.0 mg/L of NAA, 1.0 mg/L of KIN and 0.5 mg/L of BAP, exhibited auxillary bud induction
229 (10.12 ± 1.08) and bud length (8.50 ± 0.16) (Fig. 6A and Table 4) compared with other combination of
230 NAA, KIN and BAP (Table 4).

231

232

233 **3.6 Effect of different combination of plant growth regulatory hormones along with MS media on**
234 **multiple shoot induction and number in *Bacopa monnieri* L.**

235 The cultured explants were showed multiple shoots within two weeks of inoculation in MS media with
236 BAP (6-Benzyl amino purine), KIN (Kinetin) and GA₃ (Gibberellin A₃). After successive sub culturing, MS
237 media with combination of BAP (2.0mg/L), KIN (1.0 mg/L) and GA₃ (0.5 mg/L) was given best response of
238 multiple shoots induction (8.25 ± 0.57) and number (4.09 ± 0.09) (Fig. 7A and Table 5) followed by 1.0
239 mg/L of BAP, 1.0 mg/L of KIN and 0.0 mg/L of GA₃, exhibited multiple shoots induction (10.45 ± 1.42) and
240 number (3.60 ± 0.24) (Fig. 7A and Table 5) compared with other combinations of BAP (6-Benzyl amino
241 purine), KIN (Kinetin) and GA₃ (Gibberellin A₃) (Table 5).

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

245

246 **4. DISCUSSION**

247 Plant tissue culture is a biotechnological tool and depends on various factors such as type of explants,
248 surface sterilizing agents, explants age, concentration and combination of plant hormones for taking care
249 of standardization of protocol and the issues of propagation of multipurpose and endangered therapeutic
250 plants in India [18]. Three (EtoH, HgCl₂ and NaOCl) surface disinfecting agents were utilized in various
251 concentrations EtoH (half and 70%), HgCl₂ (0.1% and 0.5%) and NaOCl (0.1%, 0.5% and 1%) at various
252 time interims (1, 3, 5, 8 and 10 minutes) without hot water and with hot water to get the great aseptic
253 culture on account of gathered plant explants from the outside of the research center tainted with
254 microorganisms. Various parameters (Type of explant, age, treatment time) led to death of explants [19,
255 20] hence ethyl alcohol and sodium hypochloride were not suitable and efficient sterilizants in case of
256 *Bacopa monnieri* L.

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

261 Media + Sucrose 30 g/L + Agar 7 g/L. Our results were similar to that of previous studies [21, 22], the
262 impact of sucrose was broke down as regenerative potential and development of recovered shoots amid
263 culture period. The most surprising rate of recovery was seen in the medium enhanced with 2% sucrose
264 with most surprising number and length of recovered shoots.

265 The explants were cultured on MS media with different combination of 2, 4-D (2, 4-dichlorophenoxyacetic
266 acid), KIN (Kinetin) and BAP (6-Benzyl amino purine) to induce callus and enlargement after 12-14 days
267 of inoculation. However, callus formation (callus was globular white and was of pale yellow in color)
268 started after 20- 25 days at the ends of the explants. Based on results, callus induction requires the
269 presence of auxins or cytokinins or both in the media based on the source of explants. Previous studies
270 suggested that by addition of 1 μ L BAP and 2, 4-D, initiated a thin layer of granular callus after 4 weeks of
271 culture. However, all the explants turned brown to black at the base after 6 weeks of culture [9]. Atefeh et
272 al., also reported, 2, 4-D (2.5 mg/L) and KIN (0.5 mg/L) in MS medium was best media for callus
273 induction in cumin (*Cuminum cyminum*) [23]. Highest percentage of callus induction was acquired with
274 media, supplemented with 2.4-D and KIN in *Juniperus excels L.* [24] and *Kelussia odoratissima* Mozaff
275 [25].

276 The started explants were cultured on MS media and enhanced with various mix of IAA (Indole-3-acidic
277 acid), KIN (Kinetin) and BAP (6-Benzyl amino purine) to start root and root length (Table 3). Be that as it
278 may, root commencement was begun following 5-6 days of inoculation. Following 10 days, 1-2 cm length
279 of single and various roots were formed. The explants cultured with the plant development hormones, 0
280 mg/L of IAA, 0.0 mg/L of KIN and 0.5 mg/L of BAP indicated great and fast development of root ($6.85 \pm$
281 0.97) and root length (1.17 ± 0.11). Based on results, MS media with any additional growth regulator
282 showed 100% root formation. MS media with 2 mg/L of indole-3-butyric acid gave 87% of root growth
283 [10]. Singh et al., 1999 and Rani et al., 2000 also reported that root growth in MS media with BAP (0.5
284 mg/L) within 6 days of culture and 90% of growth rate in 2.46 μ m IBA [26,27] .

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

289 the MS media containing combination of NAA (1 mg/L), KIN (1 mg/L) and BAP (1 mg/L). Previous studies
290 on medicinal plants (*Paederia foetida*, *Centella asiatica* and *Rauwolfia serpentine*) have shown effects of
291 hormones (alone and in combination) on axillary bud induction and bud length [9, 11, 26]. MS media
292 supplemented with BAP (1.0 mg /L) in *Paederia foetida* and *Centella asiatica* [26] and benzyladenine
293 and NAA in *Rauwolfia serpentine* [11] showed optimum axillary bud proliferation, growth and length.
294 The cultured explants were demonstrated numerous shoots inside about fourteen days of inoculation in
295 MS media with BAP (6-Benzyl amino purine), KIN (Kinetin) and GA3 (Gibberellin A3). After progressive
296 sub culturing, MS media with combination of BAP (2.0mg/L), KIN (1.0 mg/L) and GA3 (0.5 mg/L) was
297 given best numerous shoots induction (8.25 ± 0.57) and number (4.09 ± 0.09). Our results suggested that
298 MS media is very effective for shoot multiplication. These results were associated with previous studies
299 [1, 28-30].

300

301 **Conclusion**

302 In conclusion, higher number of roots and increased root length was observed in MS Media + Sucrose
303 (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
304 BAP (2.0 mg/L) rapid callus growth was observed which turned pale yellow and of globular appearance.
305 Highest shoot length was observed in MS + NAA (1.0 mg/L) + (KIN 1.0 mg/L) + BAP (1.0 mg/L) medium.
306 Maximum number of multiple shoots was found in MS + BAP (2.0 mg/L) + KIN (1.0 mg/L) + GA3 (0.5
307 mg/L).

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309

310 **Conflicts of interest**

311 All the authors were declared that there is no conflict of interest.

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315

316 **Table 1.** Upshot of various concentrations of sucrose (g/L) along with MS media on root
 317 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 (10g/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

318
 319 Data was expressed as mean ± standard deviation (N = 30 explants/concentration). MS:
 320 Murashige and Skoog.

321
 322
 323 **Table 2.** Effect of various combinations of plant growth regulatory hormones on *invitro* callus
 324 induction (days) of *Bacopa monnieri* L.

SI 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

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

328

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

331

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

332

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

335

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

338

SI 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 \pm 0.25	1.36 \pm 0.03
2.	0.00	1.00	0.50	10.12 \pm 1.08	8.50 \pm 0.16
3.	1.00	1.00	1.00	18.09 \pm 1.45	10.50 \pm 0.32
4.	1.00	1.00	2.00	12.21 \pm 1.12	6.90 \pm 0.14
5.	2.00	1.00	1.00	11.05 \pm 1.11	5.60 \pm 0.11
6.	1.00	2.00	0.00	15.14 \pm 1.75	4.80 \pm 0.21
7.	3.00	1.00	1.00	16.23 \pm 1.82	7.20 \pm 0.19

339

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

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

344

SI 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

345

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

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353

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

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361 (a)

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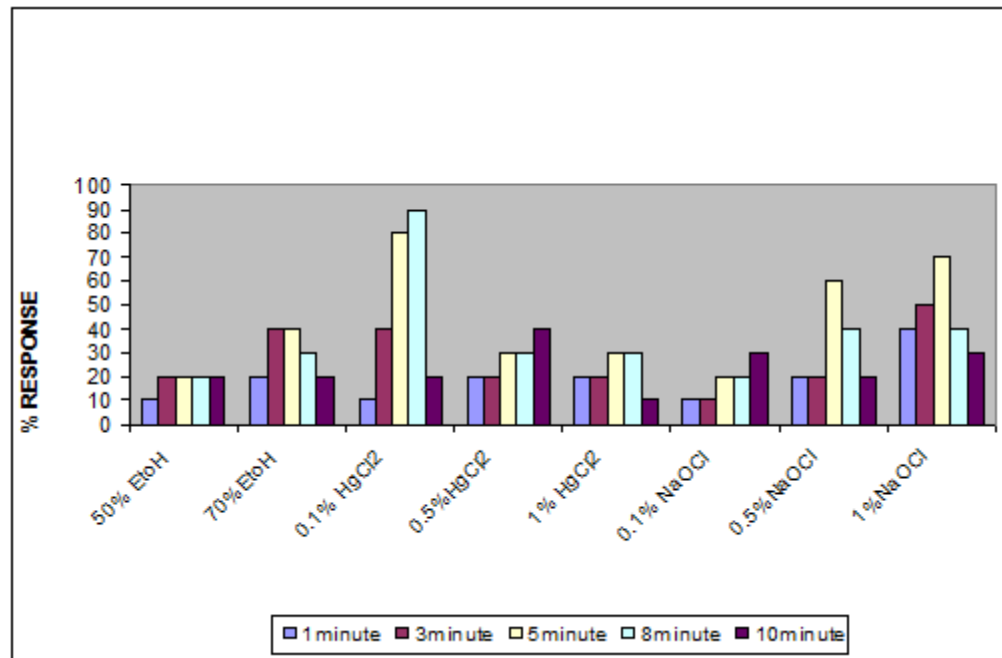
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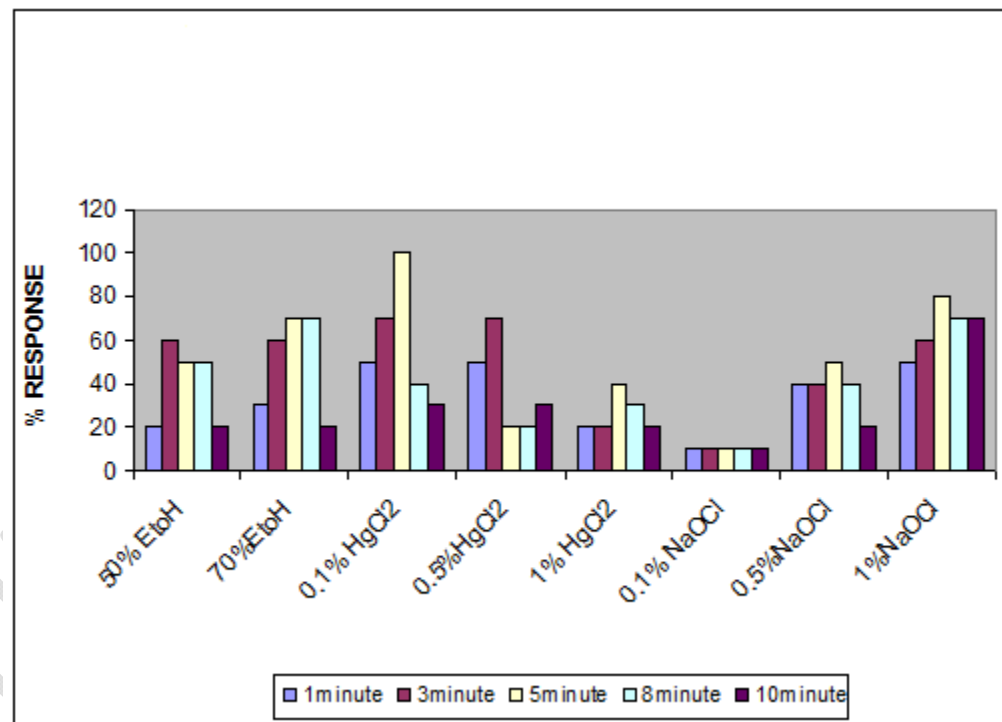
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(b)



376

377

378 **Fig.2.** Effect of various types of surface sterilizing agents (EtOH: Ethanol; HgCl₂: Mercuric
379 chloride; NaOCl: sodium hypochlorite) in different concentrations [EtOH (50 and 70%); HgCl₂
380 (0.1 and 0.5%); NaOCl (0.1, 0.5 and 1%)] at different time intervals (1, 3, 5, 8 and 10 minutes)
381 without hot water (a) and with hot water (b) of *Bacopa monnieri* (L.) explants to obtain aseptic
382 culture, expressed as response (%).

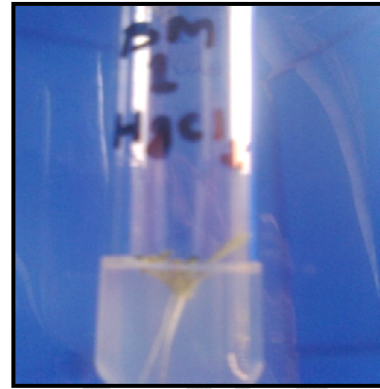
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(a)



MS + Agar (7gm/L) + Sucrose (30gm/L)

(b)



MS + Agar (7gm/L) + Sucrose (10g/L)

(c)



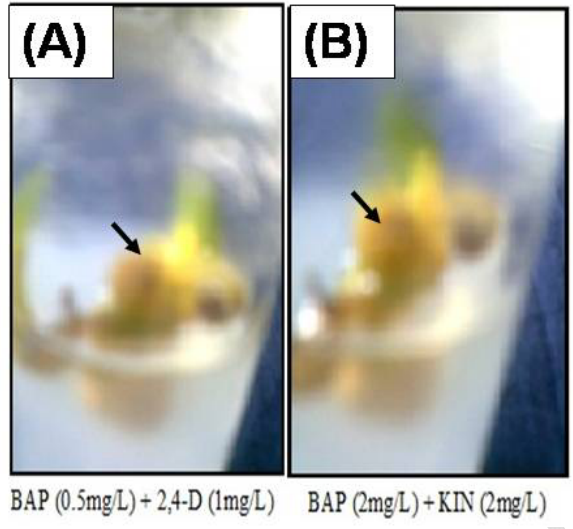
MS + Agar (7gm/L) + Sucrose (20gm/L)

(d)



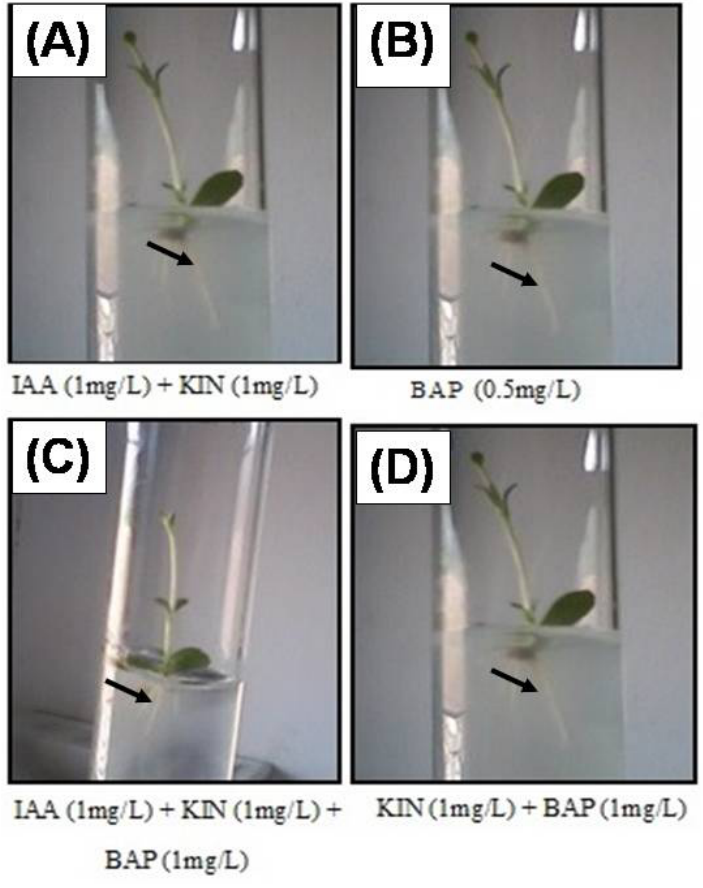
MS + Agar (7gm/L)

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



406

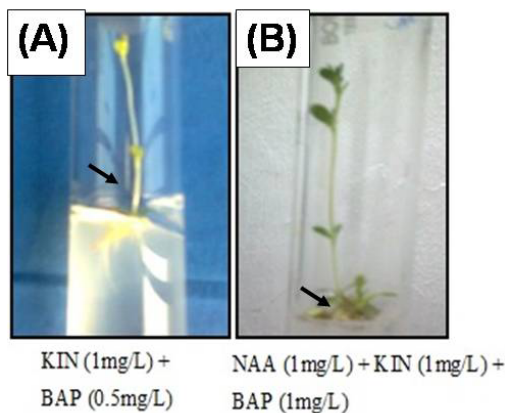
407 **Fig.4.** Effect of different combinations of plant growth regulatory hormones with different
 408 concentrations (a): BAP: 6-Benzyl amino purine (0.5mg/L) plus 2,4-D: 2,4-
 409 dichlorophenoxyacetic acid (1mg/L); (b): BAP: 6-Benzyl amino purine (2.0mg/L) plus KIN:
 410 Kinetin (2mg/L) on callus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



412

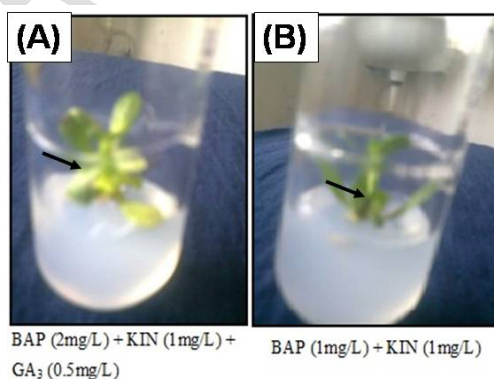
413 **Fig.5.** Effect of different combinations of plant growth regulatory hormones with different
 414 concentrations (a): IAA: Indole-3-acetic acid (1.0mg/L) plus KIN: Kinetin (1.0mg/L); (b): BAP:
 415 6-Benzyl amino purine (0.5mg/L); (c): IAA: Indole-3-acetic acid (1.0mg/L) plus KIN: Kinetin
 416 (1.0mg/L) plus BAP: 6-Benzyl amino purine (0.5mg/L); (d): KIN: Kinetin (1.0mg/L) plus BAP:
 417 6-Benzyl amino purine (0.5mg/L) on root initiation and root length of *Bacopa monnieri* L. Black
 418 colour arrow indicates root induction and root length of *Bacopa monnieri* L.

419



420

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



427

428 **Fig.7.** Effect of different combinations of plant growth regulatory hormones with different
 429 concentrations (a): BAP: 6-Benzyl amino purine (2.0mg/L) plus KIN: Kinetin (1.0mg/L) plus
 430 GA₃: Gibberellin A₃ (0.5mg/L); (b): BAP: 6-Benzyl amino purine (1.0mg/L) plus KIN: Kinetin
 431 (1.0mg/L) on multiple shoot induction and number, of *Bacopa monnieri* L. Black colour arrow
 432 indicates multiple shoot induction of *Bacopa monnieri* L.

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