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High frequency of shoot regeneration on nodal explants of *Bacopa monnieri* - A high value medicinal plant

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Abstract: The paper highlights the significance of plant tissue methods and usage in producing planting materials of medicinal plant species, varieties and cultivars. Three experiments from nodal explants of Bacopa monnieri were demonstrated. In combination with MS medium, different concentration ofBAP (1.0, 1.5, 2.0, 2.5, 3.0, 3.5 and 4.0 mgl^{-1}) and NAA (1.0 mg/l) were used for callus induction, IAA (mg/l) and 2, 4-D (0.5, 1.0 mg/l) wereadditionally used in shoot regeneration experiment. The largest numbers of adventitious shoot buds were induced in 100% cultures from the explant when 6-benzylaminopurine was used at a concentration of 0.5mg/l. For root induction experiment differ concentration of sucrose (14.16, 18, 20.22 and 24 g/l) were used. The largest numbers of adventitious root were induced in the medium supplemented with sucrose at concentration of 20g/l. The success in mass clone propagation through tissue culture from selected individuals might improve rate of growth and quality of selected traits and resulting in short-term mass production. Rooting was achieved in microshoots on full strength basal liquid medium supplemented with sucrose (1%) and indole-3-butyric acid (0.5 mg/l). The obtained plantlets have been successfully acclimatized *ex vitro*.

Keywords: *Bacopa, Medicinal,* Herpestine. Bacosides, Memory chemicals, Nodal explants

Abbreviations: MS, Murashige and Skoog's medium; BAP, 6-benzylaminopurine; NAA, Naphthalene acetic acid; IBA, indole-3-butyric acid; 2, 4-D, 2, 4, Di-chloro acetic acid

Introduction

Bacopa monnieri is a perennial, creeping herb native to the wetlands of southern India, Australia, Europe, Africa, Asia, and North and South America. Bacopa is an important medicinal herb, a species of the family Scrophulariaceae,Tejavathi *et al*., (2001). Natural population stand of Brahmi is under extreme stress of unauthorized exploitation due to huge market demand, owing to which, the wild stock of this plant has been markedly depleted. (Aruna, *et al.,* 2010).The International Union for Conservation of Natural and National Resources has a long time ago listed Bacopa monnieri as a threatened species (Pandey *et al.,* 1993). It is placed second in the priority list of Indian medicinal plants, commonly called Brahmi, a small, amphibious plant growing in marshy areas throughout the Indian subcontinent. Brahmi is also known as "Medhya Rasayana" in Ayurveda as it has been reported to increase mental clarity and brain-stimulating activities Bacopa has traditionally been employed as a neurological tonic and cognitive enhancer, and it is currently being studied for its possible neuroprotective properties (Ailenimahender *et al.*, 2012).One of the important fact about this plant is that whole plant is extensively used as a drug to counteract the effects of various mental disorders, to enhance memory functions of brain, improve intelligence and cognitive functions (Augereau *et al*., 1986, Rastogi *et al*., 1994). It is in the list of one of the fewmedicinal plants remained, which has anti-pyretic, analgesic, anti-inflammatory,anti-cancer (Elangovan *et al*., 1995) and anti-oxidant properties. Itcontains saponins like Bacosides A, B, C and, alkaloids like nicotine, brahmine and herpestine, (Jain and Kulshreshtha, 1993). Bacosides A, B,C, and D which are biologically active triterpenoids, more commonly known as "memory chemicals" The potent nervine activity is reported to be present in bacosides A and B (Rastogi *et al*., 1994; Tiwari *et al*., 2001). Bacopa extract has anxiolytic, cognition-enhancing (Bhattacharya and Ghosal (1998), relaxing Dar and Channa (1997), antioxidant Mukherjee and Dey (1966), and immune modulator activities Dahanukar and Thatte (1997). The natural regeneration of this herb is hampered due to short viability (2 month) of seeds, frequent death of seedlings at atwo-leaf stage, and strict habitat requirements for marshyareas. A number of studies have been carried out on shoot regeneration from different explants, like leaf, internodal segments and nodal segments of B. monnieri (Tiwari *et al*., 1998; 2000; 2001; 2006). However, considerable variation has been reported regarding the capacity of regeneration. However, significant variation has been reported regarding the capacity of regeneration.Development of a highly efficient plant regeneration system is a prerequisite for production, Banerjee and Srivastava, (2008). In recent times, various biotechnological approaches have been optimized for

increasing the level of bioactive molecules inmedicinal plants or microbes Grotewold (2008); Schafer and Wink (2009). Success of regeneration depends not only on the type of the explant chosen, but also the way explants are placed on the culture medium Srivastava *et al*. (1994). Keeping in view the medicinal potential and commercial value, B. monnieri has been identified as one of the major priority species of medicinal plants for further research and development. The aim of the present study was to develop an efficient and cost effective in vitro plant regeneration protocol through nodal segment culture of the medicinally important herb Bacopa and optimization of various parameters.

Materials and Methods

Preparation of explants and medium: Explants were collected from in vivo grown healthy vegetative plants from Botanical garden of Banaras Hindu University. Nodal segments, were used as explants for direct organogenesis. A 4-week-old proliferating shoot cultures were used as source of material. Nodal explants (5 mm in length) used in all experiments. The fourth node were excised, kept in running tap water (1 h), then washed with teepol (1 to 2 drops) and finally rinsed with water till the detergent was thoroughly removed. Entire plantlets were used as explants to establish cultures on Murashige and Skoog's (1962) medium (MS). Apical portions of healthy twigs bearing leaves up to the fifth node were excised, kept in running tap water (40 min), then washed with Tween -20 (1 to 2 drops) and finally rinsed with water to confirm complete removal of detergent. Further treatments were carried out in a laminar air flow cabinet. Explants were surface sterilized with 0.1% $HgCl_2$ (w/v) for 2-3 min and later thoroughly washed with sterile double distilled water (three to four times) to remove traces of $HgCl_2$. Entire leaves were excised from stem in sterile Petri dishes. MS medium supplemented with sucrose (3%) along with different concentrations of BAP and IAAwas used to establish optimum culture conditions for callus formation and root differentiation.

Optimization of Medium composition for shoot androot differentiation: The following treatments were imposed to the nodal explants for shoot and root differentiation:

a) For callus induction,(i) MS + 5.0 mg/l BAP + 1.0 mg/l NAA(MSC_1); (ii) MS + 1.0 mg/l BAP + 1.0 mg/l NAA(MSC_2); (iii) MS + 2.0 mg/l BAP + 1.0 mg/l NAA(MSC_3); (iv) MS + 3.0 mg/l BAP + 1.0 mg/l NAA(MSC_4) ; (vi) MS + 4.0 mg/l BAP + 1.0 mg/l NAA(MSC_5).

b) For shoot induction, (i)MS basal as control(MS); (ii) MS + 0.5 mg/l BAP + 0.5 mg/l NAA(MS_1); (iii) MS + 0.5 mg/l BAP + 1.0 mg/l NAA (MS_2); (iv) MS + 1.0 mg/l BAP + 1.0 mg/l IAA(MS_3); (v) MS + 1.5 mg/l BAP + 1.0 mg/l IAA (MS_4);(vi) MS + 0.5 mg/l BAP + 0.5.0 mg/l 2,4-D(MS_5).(vii) MS + 0.5 mg/l BAP + 1.0 mg/l 2, 4-D ((MS_6).

c) For root induction (i) MS + sucrose (14 gm/l) + Agar (7gm/l) (MSR_1); (ii) MS + Sucrose (16 gm/l) + Agar (7gm/l) (MSR_2). (iii)MS + Sucrose (18 gm/l) + Agar (8gm/l) (MSR_3). (iv) MS + sucrose (20 gm/l) + Agar (7gm/l) (MSR_4)(v) MS + sucrose (22 gm/l) + Agar (7gm/l) (MSR_5);(vi) MS + sucrose (24 gm/l) + Agar (7gm/l) (MSR_6).

0.1N NaOH or 0.1 N solutions were used to adjust the pH of all the media. The medium was solidified with the addition ofagar (0.8%) (Andrés *et al*., 2010) and distributed likewiseinto culture vessels. All culture media were sterilized by autoclaving at 121 °C for 20 min at 15 atm. The aseptic operations were conceded in a horizontal laminar air flow cabinet; the working table was cleaned with 70% ethyl alcohol. All of the sterilized instruments and medium were irradiated with UV light for 20 min. The cultures were incubated under a 16 hrs. Photoperiod in cool white florescent light (55/μmol M/S) and maintained at a constant temperature of 25 ± 2°C. The 83-85% humidity was maintained in the culture room. The cultures were maintained by sub culturing at four weeks intervals to fresh medium with the same composition. Explant with proliferated shoot buds, formed under optimal condition were transferred to elongation medium. The number of elongated shoot was counted after three week of culture. In all experiments, 10replicates were maintained and the experiment was repeated thrice to minimize experimental error.

Table-1: Media combination for in vitro callus induction

Medium code	MS+ Supplement
MS	MS basal as control
MSC_1	MS + 1.0 mg/l BAP + 1.0 mg/l NAA
MSC_2	MS + 5.0 mg/l BAP + 1.0 mg/l NAA
MSC_3	MS + 1.5 mg/l BAP + 1.0 mg/l NAA
MSC_4	MS + 2.0 mg/l BAP + 1.0 mg/l NAA
MSC_5	MS + 2.5 mg/l BAP + 1.0 mg/l NAA

Table-2: Shoot induction from callus of nodal segments under different combination of media

Medium code	% shoot induction	Average no. of shoot	Average shoot length(cm)
MS	-	-	-
MSS_1	63	5	5.5
MSS_2	86	11	10.5
MSS_3	81	7	6.4
MSS_4	78	6	6.5
MSS_5	68	6	5.5
MSS_6	60	5	6

Table-3: Medium optimization for root induction and differentiation

medium	% root	Average No. of root	Average root length (cm)
MS	55	2	1
MSR1	65	4	1.5
MSR2	79	7	2
MSR3	85	8	4
MSR4	72	6	3
MSR5	72	5	3

Callus Initiation from nodal explant: Nodal explants were cultured with each concentration of callus media containing BAP and NAA, and their combinations. Media jars were incubated in Biotronette Mark III Environmental Chamber incubator (Bio-Line) under 16 hr. photoperiod maintained with fluorescent and incandescent lights at room temperature. Explants were kept in the callus media for 2 weeks. Observations were recorded weekly until week 6 and no media was changed during that time.

Shoot induction: Calli were transferred to MS medium supplemented with different concentrations of BAP. Samples were

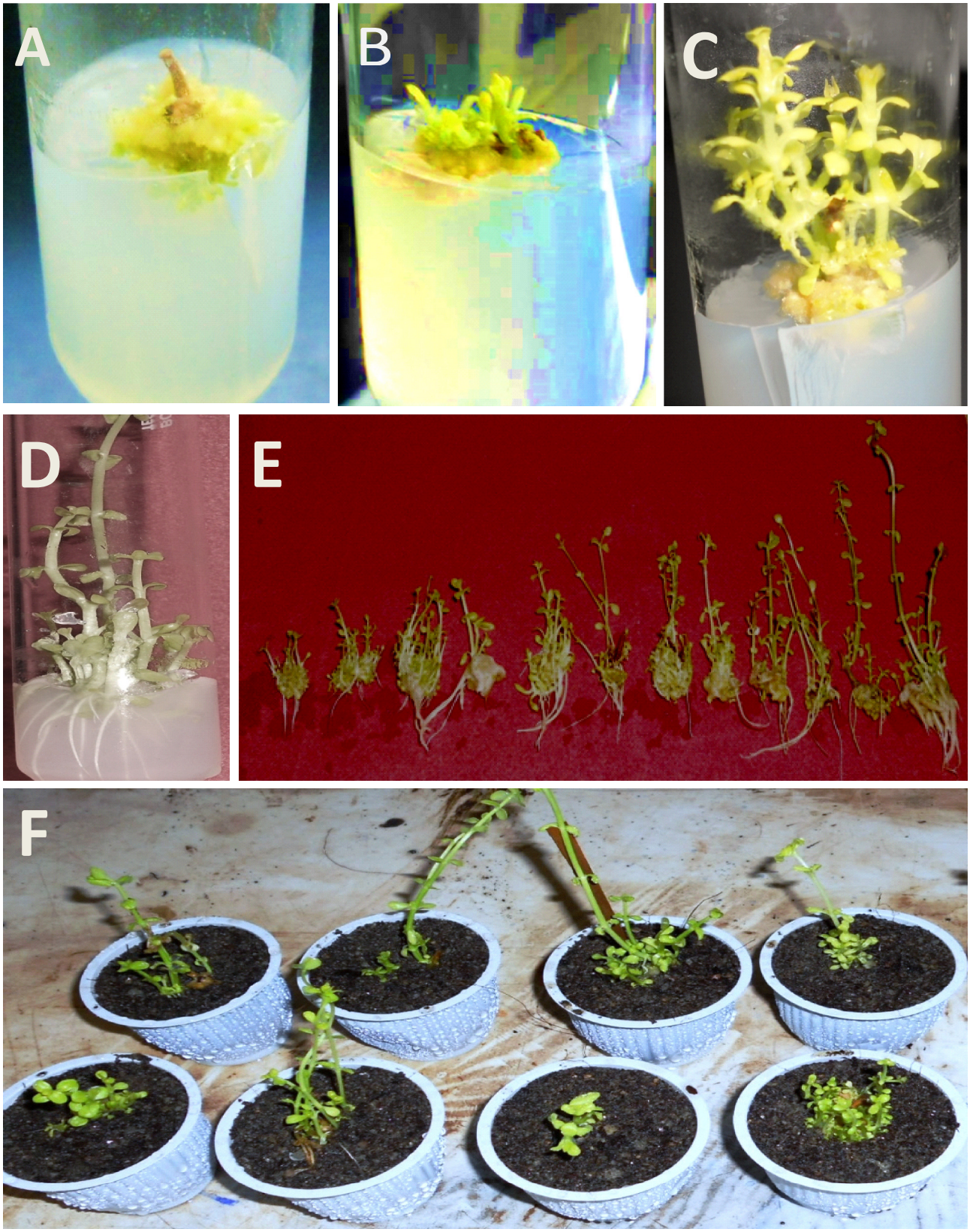

Fig. 1: Regeneration of *Bacopa monnieri* from nodal explants. **A**. Callus development in MS+ 4 mg/l BAP. **B** and **C** Multiple shooting from callus in 5.0 mg/l BAP + 1.0 mg/l IAA. **D**. Rooting in MS + Sucrose (20 gm/l). **E**. rooted Baccopa before hardening. **F.** Hardening and acclimatization of *Bacopa* microshoots in sand/ Humus soil (1:1)

incubated in the light for 16 h (light 2000 lux, temperature 23 ±1 C, humidity 60–70%) followed with 8 h in darkness at 16 ±1 C. The number and length of the shoots produced from calli were established after 30 days of culture.

Shoot multiplication: The callus-derived shoots were transferred to MS medium supplementedwith different concentrations of cytokines in combination with auxin (NAA). Using the conditions described for shoot induction, 45 shoots were cultured. Thenumber and length of shoots were determined after 30 days of culture; the experiments were repeated three times.

Rooting of elongated shoots: When plantlets grown to 2 cm or so at length, they were cut at the ground of plantlets andtransferred into the rooting medium. Two kinds of rooting medium were used in which one isMS medium and another is MS medium supplemented with 0.5 mg L^{-1} IBA. Elongated shootswerecultured in rooting medium for 2-3 weeks. Rooting in the medium containing IBA is fasterthan that in the medium without phytohormones. In general, elongated shoots cultured in medium containing IBA for 15 d, or in MS medium for over 20 d. Then rooted plantlets were acclimatized with open cap before they were transferred into soil. Subsequently, plantlets were grown into maturity with a survival percentage of 90-95 %.

Acclimatization: After one month incubation on root induction medium, a total of 60 plantlets from 0.5 mg/ l IBA containing medium were removed from jar and agar was washed from roots of plantlets. Thereafter plantlets were transferred to sand bed in green house and plantlets were maintend in a temperature range of 23-25 ^{0}C for proper growth and development. MS1/2 (containing only micro and macro elements) was sprayed regularly after atime interval of five day. Finally after two week of acclimatization, plantlets were transferred to plastic pots containing a mixture of sand and humus soil (1:1 v/v) (Aruna G et al. 2010). The rate of survival observed after 25 days transferring to pot.

Result and Discussion

The nodal explants were found to be suitable for *in vitro* proliferation of *B. monnieri*. Among the different MS basal medium tested and supplemented with sucrose (3%) and different concentrations of auxin, MS3 medium wasperceived moreefficient for shoot regeneration from entire nodal explants in comparison to all other medium tested in shoot regeneration experiment. All the explants in this experimental study were placed vertically in solidified mediumto ensure contact with the medium, as this is known to promote efficient regeneration in many herbaceous plants, Effectiveness of MS medium for optimum shoot multiplication in different Bacopa species was reported by Tiwari *et al*., (1998, 2000, 2001). Adventitious shoot bud regeneration was observed in all the combinations of medium evaluated, but there was variation in the induction of shoot buds from the explants.In *B. monnieri*, among four different concentration of BAP (0.5, 1.0, 1.5, 4.0 mg/l), concentration of 4 mg/l was recorded as optimum concentration for bettercallus differentiation and developmentfrom nodal explants (Table-1). Cytokinins are known to be very effective in promoting shoot proliferation and their role in shoot organogenesis is well established (Evans *et al*.,1983, 2000).

Among the different MS media used and supplement with different concentration of BAP and NAAand 2,4,D, MS2 media was observed to be more effective for shoot induction ,shoot length and average no of shoots. In MS basal medium percent shoot induction was observed to 60%. Percentage response of three observed parameters; % shoot induction, average shoot length, average number of shoots was minimum in basal MS medium noticeably showing the need of plant growth regulators. Among different concentration of BAP used, 0.5mg/l concentration has shown better response while other concentration of BAP used (1.0 and 1.5) in combination with NAA showed comparatively lower responses. High frequency of adventitious shoot formation in presence of BAP from leaf explants in B. monnieri was reported by Joshi et al. (2010). MS5 and MS6 media (BAP+2, 4, D) did show their responses on tissue differentiation and shoot bud formation and shoot differentiation but their effect was not very prominent (Table-2). Hence, it may be assumed that, BAP alone was effective in inducing differentiation and proliferation of callus and it probably inhibits the effect of NAA on callus formation. Further study was conducted to optimize suitable concentration of root induction medium. For this, different concentration of sucrose was used for optimization of root induction experiment. MS medium was separately supplemented with 14.16.18.20.22.24 gm/l sucrose and tasted for root induction. When sucrose concentration was increased up to 20gm/l there was a gradual increase in no of root and root length and marked decrease in number of root and root length was observed above this concentration, although concentration up to 24 mg were used and tested in the experiment (Table-3). A dual role of sucrose as carbon source and osmotic agent was observed in Solanum melongena by Mukherjee *et al*., (1991), but in Amygdalus communis shoot proliferation was observed only with 5 and 6% sucrose by Gurel and Gulsen (1998).

The results clearly demonstrated the potential of nodal explant of Baccopa monnieri and mayhave major implications on both basic and applied aspects of plant tissue cultureand standard protocols have been developed for high rate production of plants from nodal explants of Baccopa monnieri. The observations revealed that all of the nodal explants responded quickly, but there was variation in the number of shoot buds, shoot length and number of shoot and roots, being produced. Direct shoot bud formation started within the start of second week in all shooting experiment.Moreover, continued work by the use of biotechnology will contribute in improving vegetative propagation of this medicinal plant and playing an important role in breeding programs.

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