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In vitro micropropagation and flowering in *Ipomoea sepiaria* Roxb. An important ethanomedicinal plant

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ABSTRACT

Objective: To standardize a protocol for the micropropagation and *in vitro* flowering of *Ipomoea sepiaria(I. sepiaria)*, an important ethanomedicinal plant. **Methods:** The nodal cuttings were cultured on Murashige and Skoog (MS) medium supplemented with various concentrations of 6-benzyladenine (BA) or Kinetin (Kn; 1.0-4.0 mg/L) alone or in combination with α-naphthaleneacetic acid (NAA; 0.2-1.0 mg/L) for shoot proliferation. For rooting ½ MS medium supplemented with indole-3-butyric acid (IBA) or NAA (0.5-3.0 mg/L) was used. When the 45-day-old *in vitro* derived nodal cuttings were subcultured on MS medium supplemented with 3.0 mg/L BA and 0.5 mg/L NAA and various concentrations of abscisic acid (ABA; 0.5-3.0 mg/L), *in vitro* flowering was observed. **Results:** The highest shoot induction response in terms of percent cultures responding and number of shoots per explant was observed on 3.0 mg/L BA and 0.5 mg/L NAA. On this medium 100% cultures responded with an average number of 3.2 shoots per explant. The optimum rooting was observed on 2.0 mg/L IBA. Here 100% shoots rooted with an average number of 5.1 roots per shoot. The optimum *in vitro* flowering response (38%) was observed on 2.0 mg/L ABA. **Conclusion:** The present protocol is an efficient method for the rapid multiplication, flowering and conservation of this medicinal plant.

1. Introduction

Ipomoea sepiaria(I. sepiaria) is an ethnomedicinal plant belonging to Convolvulaceae family. It is one among the "Dasapushpa" (ten flowers) of aurvedic medicine[1]. It is distributed in streams and hedges near rivers of Indo-Malayan regions of Asia. The whole plant is medicinally useful. The powdered plant is used as a shampoo powder and stimulates hair growth. The plant is having cooling and rejuvenating effect[2]. It is useful in vitiated conditions of pitta, burning sensation, psychic disorders, strangury, hyperdipsia and general debility[3]. In traditional practices it is mainly used in the treatment of women sterility and pediatric diseases[4]. *I. sepiaria* is used in the treatment of ulcers and considered as a good antidote to arsenic[2] and also reported to have antiviral properties[5]. The powdered leaves and extracts provided good protection for black gram seeds against the pulse beetle, Callosobruchus maculatus by reducing insect oviposition and F1 adult emergence[6]. Significant aphidicidal activity of the hot and cold water extracts of this plant were tested against the bean aphid, Aphis craccivora[7].

Because of its ethnic and medicinal properties, the demand of the plant is increasing day by day. A reliable and efficient micropropagation protocol in this plant can result in superior differentiation, shoot development and entire plant regeneration which is essential for the propagation of selected traits within a specific genotype[8]. In addition to this, micropropagation is an important tool for the recovery and conservation of germplasm[9]. Other advantages of this technique involve rapid propagation rate, space exploitation, the enhancement of sanitary conditions of plants and the facilitation of international germplasm exchange. Plant tissue culture techniques play an important role in obtaining genetically uniform massive clones.

One of the most fascinating events in the lifecycle of angiosperms is the shift from vegetative phase to reproductive phase. This complicated process is often influenced by several aspects especially a combination of exogenous and endogenous factors. Virtually all these

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factors interact in various complex and unpredictable ways[10-14]. Important factors which influence the *in vitro* flowering include nature of plant growth regulators, light, carbon source and pH of the medium[15]. Similarly internal factors like position of the explant on the intact plant, genotype and genes expressed during flowering[16]. *In vitro* approach has proven to be a very useful strategy for the investigation of flowering physiology. It also helps to isolate potential flowering sites and use such tissues to test the effects of various parameters on flowering. There are no previous reports on micropropagation in this plant. Hence the present work is an attempt to develop an effective protocol for micropropagation of this plant using nodal explants and induction of *in vitro* flowering from shoots obtained from nodal segments.

2. Materials and methods

2.1. Plant material and in vitro culture

Single node cuttings of *I. sepiaria* were collected from the Botanical garden of the Institute and were washed with soap solution for 5 minutes, rinsed 3 times in distilled water, immersed in 70% ethanol for 1 minute, washed 3 times in sterile distilled water. Finally disinfected with freshly prepared 0.1% (w/v) mercuric chloride ($HgCl_2$) for 3 minutes. The explants were again washed 3 times in sterilized distilled water to remove the traces of sterilant. After giving a final trimming at the cut ends, the nodal cuttings were cultured on Murashige and Skoog (MS) medium[17] supplemented with various concentration of 6-benzyladenine (BA) or kinetin (Kn; 1.0-4.0 mg/L) alone or in combination with α-naphthaleneacetic acid (NAA; 0.2-1.0 mg/L).

The percent response, average number of shoots per explant and average length of shoot were recorded after 45 days of culture. Elongated and well developed shoots above 2 cm height were excised and transferred to rooting medium containing 1/2 MS supplemented with different concentrations of indole-3-butyric acid (IBA; 0.5-3.0 mg/L). Plantlets having more than two roots were taken out from the culture tubes, washed thoroughly with tap water to remove agar and transferred to paper cups containing soil and sand (1:1) and covered with a plastic bag to maintain high humidity. The young plants were kept under 30%-40% natural light, sprayed with water twice a day. The acclimatized plants were transferred to their natural habitat and the survival rate was assessed after 3 months.

2.2. In vitro flowering

For *in vitro* flowering, nodal segments from *in vitro* formed shoots were isolated after 5 days and subcultured on MS medium supplemented with various concentrations of (0.2-1.5 mg/L) abscisic acid (ABA). Various parameters like percent response, average number of flowers and days to flowering were recorded after 50 days after culture.

2.3. Culture conditions

The pH of the medium was adjusted to 5.8 prior to adding 0.8% w/v agar before being sterilized by autoclaving at 120 ℃ and 104 kPa for 15 min. After the explant transfer, the test tubes were maintained at (25 ± 2) ℃ under 16-h photoperiod provided by cool white fluorescent tubes. Each treatment consisted of at least 12 cultures and all experiments repeated three times. Analysis of variance and Duncan's multiple range test were used for comparison among treatment means[18].

3. Results

3.1 Multiple shoot initiation and elongation

The nodal segments cultured on MS medium supplemented with various concentrations of BA or Kn (1.0-4.0 mg/L) alone or in combination with NAA (0.2-1.0 mg/L) showed bud break and shoot elongation. However, both these responses vary depending on plant growth regulator concentrations and combinations. The optimum percent response (78%) was observed on MS medium supplemented with 3.0 mg/L BA when used individually. However, the number of shoots was invariably one in all concentrations of BA. The average shoot number varies from 1.1 to 1.6 in various BA and Kn concentrations (Table 1, Figure 1A). The addition of NAA with BA improved the response further. 100% cultures responded with an average number of 3.2 shoots per explant on MS medium supplemented with 3.0 mg/L BA and 0.5 mg/L NAA (Figure 1B). There was very poor response when MS basal medium was used.

The bud break occurred usually after one week in most of the cultures. The shoot emerged from the axil of the explant within 2 weeks after culture initiation. The shoot growth was vigorous and healthy shoots were formed within 3-4 weeks after culture. The addition of other cytokinins like Kin or TDZ alone or in combination with auxin resulted in callus formation at the cut end of the explant (data not shown). Therefore only BA and NAA alone was used as plant growth regulators for shoot multiplication in the present investigation.

3.2 Rooting

Full strength MS medium produced callusing of the basal cut end and the roots were emerged from the calli. Hence ½ MS medium was used throughout the experiment for rooting. Shoots measuring a size of about 2.0 cm were excised and cultured on ½ MS medium supplemented with IBA or NAA in the range of 0.5-3.0 mg/L for root induction.

Comparatively IBA gave better results than NAA.

Root induction medium containing ½ MS with IBA produce roots in most of the explants within 2 weeks (Figure 5, Table 2). 1.0 mg/L NAA produced optimum response of 67% cultures responded with an average number of 1.8 roots per shoot. However, the highest response was observed on MS medium supplemented with 2.0 mg/L IBA. Here 100% cultures responded with an average number of 5.1 roots per shoot (Figure 1E). The roots were healthy and whitish in colour.

Table 1

Effect of different concentration of BAP, Kn alone or in combination with NAA on shoot proliferation from nodal explants of *I. sepiaria*.

Plant growth regulators (mg/L)		% response	Average no of shoots	Average shoot length (cm)
BA	NAA			
1.0	0.0	65^{e}	1.3 ± 0.02^{c}	0.9±0.03^{b}
2.0	0.0	73^{d}	1.4±0.04^{c}	1.0±0.07^{b}
3.0	0.0	78^{d}	1.6 ±0.05^{c}	1.1±0.09^{b}
4.0	0.0	69^{d}	1.4 ± 0.04^{c}	1.4±0.06^{a}
3.0	0.2	92^{b}	2.1±0.07^{b}	1.6±0.07^{a}
3.0	0.5	100^{a}	3.2±0.03^{a}	1.8±0.08^{a}
3.0	1.0	90^{b}	1.9±0.08^{b}	1.7±0.07^{a}
Kn	NAA			
1.0	0.0	43^{f}	1.1±0.04^{d}	0.5±0.07^{b}
2.0	0.0	55^{e}	1.3 ± 0.02^{c}	0.6±0.09^{b}
3.0	0.0	66^{e}	1.4±0.04^{c}	0.9±0.09^{b}
4.0	0.0	75^{d}	1.6 ±0.06^{c}	1.1±0.06^{b}
3.0	0.2	68^{d}	1.3 ± 0.03^{c}	1.1±0.08^{b}
3.0	0.5	87^{c}	1.7±0.04^{b}	0.9±0.09^{b}
3.0	1.0	83^{c}	1.5±0.06^{c}	0.5±0.05

Values in the column with the same letters are not statistically different according to the Duncan's multiple range test ($P \leq 0.05$).
In each experiment 10 explants were cultured and all the experiments were repeated three times.

Table 2

Influence of IBA or NAA on rooting of in vitro formed shoots of *I. sepiaria* on ½ MS medium after 45 days.

IBA (mg/L)	% response	No. of roots/plantlet	Average root length (cm)
0	0	–	–
0.5	66^{d}	1.3± 0.6^{c}	1.2±0.3
1.0	74^{c}	3.6± 0.7^{b}	1.4±0.4
2.0	100^{a}	5.1 ± 0.4^{a}	2.1±0.2
3.0	94^{b}	3.2± 0.7^{b}	1.8±0.3
NAA (mg/L)			
0.5	44^{f}	1.2± 0.3^{c}	1.2±0.4
1.0	52^{e}	1.6± 0.2^{c}	1.4±0.4
2.0	59^{e}	1.9 ± 0.5^{c}	2.1±0.2
3.0	63^{d}	1.7± 0.3^{c}	1.8±0.3

Values in the column with the same letters are not statistically different according to the Duncan's multiple range test ($P \leq 0.05$).
In each experiment 10 explants were cultured and all the experiments were repeated three times.

Table 3

Effect of ABA on in vitro flowering in *I. sepiaria*.

ABA (mg/L)	% response	Average no of flowers	Days to flowering
0.0	0.0	0.0	0.0
0.5	6^{d}	1.1±0.03^{c}	48.6±0.56^{b}
1.0	32^{b}	2.3±0.07^{b}	40.2±0.45^{b}
2.0	38^{a}	3.2±0.05^{a}	34.6±0.32^{a}
3.0	22^{e}	2.1±0.02^{b}	45.3±0.40^{b}

Medium: MS supplemented with 3.0 mg/L BA and 0.5 mg/L NAA. Observations were taken 45 days after culture.
Values in the column with the same letters are not statistically different according to the Duncan's multiple range test ($P \leq 0.05$).
In each experiment 10 explants were cultured and all the experiments were repeated three times.

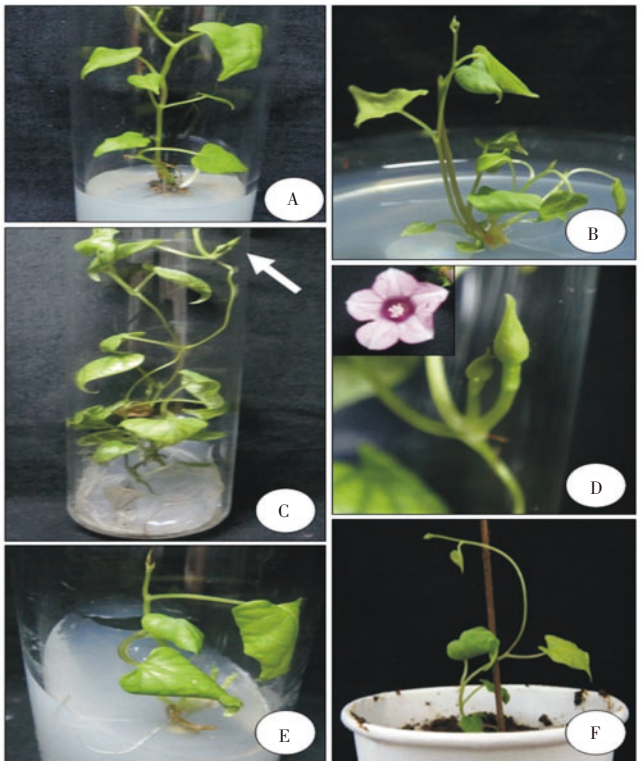
3.3 Hardening

Plantlets with 2 or 3 leaves and well developed roots were taken from the culture tubes and washed in running tap water to remove the traces of agar. The plantlets were subsequently immersed in 1% fungicide (Bavistin) solution and then transplanted to paper cups containing sterilized garden soil and sand (1:1; Figure 1F). The moistened ½ MS medium for a week and gradually transferred into field.

About 80% of the regenerated plants survived following transfer from vermiculite to natural soil and no detectable variation with respect to morphology or growth characteristics was observed. The period of transition during the process of hardening after transfer from the *in vitro* to the *ex vitro* condition is considered to be the most crucial step in plant tissue culture. The plant has a heterotrophic mode of nutrition and therefore lack adaptation or exposure to the outside environment, during laboratory to land transfer micropropagated plants are first placed in the hardening chamber with high humidity.

3.4 In vitro flowering

For *in vitro* flowering specific media combination is necessary. Similarly 45 day old nodal cuttings taken from *in vitro* cultures were only responded. Such single nodal cuttings measuring a size of about 2.0–3.0 cm were cultured on MS medium supplemented with 3.0 mg/L BA, 0.5 mg/L NAA and various concentrations of abscissic acid (ABA, 0.5–3.0 mg/L) for *in vitro* flower induction. The optimum flowering was observed on MS medium supplemented with 3.0 mg/L BA, 0.5 mg/L NAA and 2.0 mg/L ABA. On this medium 38% cultures produced flowers with an average number of 3.2 flowers per shoot (Figure 1C, D). Here the first flower bud appeared about 35 days after culture. Flower buds were appeared in the developing shoot only after 40 days. Flowers were emerged from the tip region of the developing shoot. A single bunch consisted of two flower buds (Figure 1D).



Firuge 1 . A. 45-day-old shoot developed from nodal cuttings on MS medium supplemented with 3.0 mg/L Kn and 0.5 mg/L NAA.
B. Multiple shoot induction from nodal cuttings on MS medium supplemented with 3.0 mg/L BA and 0.5 mg/L NAA. Four shoots were emerged from a single explant.
C. Induction of flower bud (arrow) on MS medium supplemented with 3.0 mg/L BA, 0.5 mg/L NAA and 2.0 mg/L ABA 45 days after culture.
D. An enlarged view of the flower buds and a single flower (inset) at the time of anthesis on on MS medium supplemented with 3.0 mg/L BA, 0.5 mg/L NAA and 2.0 mg/L ABA 45 days after culture.
E. A rooted shoot on ½ MS medium supplemented with 2.0 mg/L IBA 30 days after culture
F. A transplanted plant three months after transfer to soil. The plant has developed new leaves.

4. Discussion

In the present study maximum multiple shoot induction (3.2 shoots/explant) was observed on MS medium supplemented with 3.0 mg/L BA and 0.5 mg/L NAA. For shoot induction and elongation BA plays a very crucial role. BA induced shoot multiplication has been reported in several systems like *Caralluma bhupenderiana*[19]. However, the addition of an auxin along with cytokinin considerably increased percent cultures responding as well as shoot number and average shoot length[20,21,22].

For root induction in *I. sepiaria*, shoots half strength medium is preferred over full strength medium since the latter produced callusing at the basal cut end of the shoots. Lower mineral content is thought to be more efficient for *in vitro* rooting[23]. Controlled starvation of the plants in culture is often considered as a useful method for root induction in several systems. Our result is in agreement with previous observations by several workers[24-26].

In the present study ABA in combination with BA and NAA were responsible for *in vitro* flowering. Cytokinins in medium are considered as the main component which induce flowering. Flower induction and development in response to exogenous cytokinins have been observed in a few herbaceous plants[27-29]. According to Chailakhyan and Butenko[30] cyokinin is a conformity with the observations made on the present studies where presence of cytokinin either BAP or kinetin proved essential in the medium for *in vitro* flowering. However, in the present study we obtained highest *in vitro* flowering on a medium containing a combination of ABA, BA and NAA. ABA either with an auxin or cytokinin or in combination with auxin and cytokinin induced *in vitro* flowering in several systems like Panax ginseng[31] *Torenia* sp.[32], *Perilla* sp.[33]. The promotive role of ABA on flower induction *in vitro* could be attributed to their role in water stress management. ABA is considered as a stress hormone often accumulate in floral parts of plants and functions through a set of ABA regulated genes, which in turn lead to accumulation of osmo-protectants like proline[34]. The presence of ABA responsive elements (ABRE) in the genes regulated by dehydration indicated a putative role of ABA in flower induction[35]. In addition, there were indirect evidences that stress induced compounds like abscisic acid (ABA)[32], may influence flowering.

In the present investigation, only 45 day old *in vitro* grown nodal cuttings produced *in vitro* flowering. The nodal cuttings subcultured before 45 days did not induce flowering. This is in agreement with earlier studies in some systems where juvenile explants do not flower due to inability to produce flowering factor(s) or the inability of meristems to respond to flowering factors[13,36]. In *Rosa* sp. Wang *et al.*[37] noted that the total time from original culture and subculture before flower induction were two very important factors for *in vitro* flower induction. Many reports are there to support the influence of age of explants in *in vitro* flowering. Nodal explants of Mulberry incubated in MS medium supplemented with BAP produce *in vitro* inflorescences only after 45 days of culture and sex expression of this dioecious plant modified by the application of ethrel and silver nitrate[38]. In Ceropegia the flowering occurred only after the incubation for more than 4 weeks on the same media[39].

To our knowledge, this is the first report on micropropagation of I. sepiaria. The protocol described here is reproduciable and could be used for the large scale multiplication and propagation of this important medicinal plant. The micropropagated shoots were rooted and transplanted to soil

successfully. Another important observation noticed during our study was the induction of flowering on ABA containing medium. This will give further light to the developmental studies of floral differentiation. The *in vitro* flowering protocol described here bears immense importance and would further facilitate selective hybridization using pollen from rare stocks and explore the possibility of recombining genetic material via *in vitro* fertilization.

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