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CALLUS INDUCTION, REGENERATION OF SHOOTS AND ASSESSMENT OF CLONAL FIDELITY THROUGH ANDROGENESIS OF *Baliospermum montanum* (Willd.) Muell. Arg.

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ABSTRACT

An efficient reproducible protocol for *in vitro* propagation of *Baliospermum montanum* (Willd.) Muell.Arg. plantlets was achieved using androgenesis (anther culture), on MS medium, supplemented with different concentrations of auxins and cytokinins. This *in vitro* experimentation was conducted in the Plant Tissue Culture laboratory, PG and Research Department of Botany, Holy Cross College (Autonomous), Tiruchirappalli, Tamil Nadu, India, during the year 2021. Anthers excised from young flower buds were inoculated on basal MS medium supplemented with NAA (0.5 - 5.0 mg l^{-1}) in combination with BA (0.25 - 3.0 mg l^{-1}) for callus induction. A maximum of 67.5% callusing was recorded from the medium supplement with 2.0 mg l^{-1} BA and 4.0 mg l^{-1} NAA. The well developed hard, green calli were introduced into the regeneration medium containing increasing concentrations of BA (0.5 - 3.0 mg l^{-1}), BA (1.0 mg l^{-1}) + Ads (1.0 - 3.0 mg l^{-1}) and IAA (0.5 mg l^{-1}) + BA (1.0 - 3.0 mg l^{-1}). After 35 days of culture in regeneration medium, successful regeneration was achieved on MS medium supplemented with different concentrations of BA (0.5 - 3.0 mg l^{-1}). BA at 2.0 mg l^{-1} showed the highest response (89.0%), in which 4 shoots were observed. Regenerated shoots along with callus were transferred to the MS medium containing 2.0 mg l^{-1} BA and GA_3 (0.5 - 2.0 mg l^{-1}) for elongation. The maximum length of shoot (4.8 cm) after elongation was observed at 2.0 mg l^{-1} BA +1.0 mg l^{-1} GA_3 and transferred to rooting medium. A maximum of 54.0 % of rooting with a maximum of 7.0 roots / shoots were observed on half strength MS medium containing 2.0 mg l^{-1} of NAA. Well rooted plantlets of 23 days old culture were stabilized for few days and were transferred to vermiculite for hardening. The maximum of 51.0% rooted plantlets were hardened and successfully acclimatized in the soil. After six months of acclimatization the field grown plants, showed similar morphology and resembled the parent plant. Ultimately, the experimental outcome, substantiates the production of huge number of genetically identical clones in short period of time, that are more suitable for growth in changing environmental conditions. Hence, this study proves an excellent reproducible protocol for mass multiplication and conservation of this rare medicinal plant *B. montanum* through androgenesis, establishing a wide chance to the plant breeders in raising homozygous lines in short time and cost-effective means, levitating the farmers economy and agronomical status of the country.

(Key words: Androgenesis, *Baliospermum montanum,* callus induction, indirect organogenesis)

INTRODUCTION

Medicinal plants have gained a momentum in global modern research, to treasure its valuable therapeutic properties and continual futuristic existence in nature. About two decades ago, estimates have suggested that over half a million of dry raw material from plant sources, is indiscriminately and most destructively collected from the wild each year (Mudappa and Oommen, 1998). Habitat loss, over collection of plants, fragmentation and degradation due to population and developmental pressures are some of the major consequences of human activities, which has become a serious reason leading to a vulnerable state of many species to the point of extinction. As a result of the continuous exploitation of these plants in forests and the absence of regular developmental programs in the forestry and agricultural sectors, the forest resources are not able to meet the full demand of medicinal plants (Austin and Jegadeesan, 2000).

The plant sources of various drugs in wild are believed to be under serious threat of extinction.

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Accordingly, rare plants are classified into four major categories as extinct, endangered, vulnerable and rare (Ved *et al.,* 2000). One such rare medicinal plant (under-shrub) which is under the threat of extinction is *Baliospermum montanum* (Willd.) Muell.-Ajg., locally known as "Danti" belongs to the family Euphorbiaceae. The tribal community of Andhra Pradesh, use the plant latex for body ache and joint pains. In Ayurvedic system of medicine the root is considered as pungent, purgative, anthelmintic, diuretic, widely used in the treatment of diseases related to skin, abdomen, piles, wounds, enlarged spleen itching inflammation, anemia, leucoderma, jaundice. Whereas leaves are used in the treatment of dropsy, poulticing wounds and given as decoction or infusion for asthma. The plant *B. montnaum* with its varied therapeutic properties, it is well attracted in pharmaceutical industries and is collected in large scale throughout the year. Crude drug of the plant is available in market, in the name 'Danti mula'. The plant and its parts, especially roots have much demand in traditional medicinal market with its high economic value of > Rs. 900 kg^{-1}. Due to its high medicinal and trade value, excessive collection of this plant has led to serious threat. Cultivation of this medicinally valuable plant is much needed in this era, which could be achieved through modern technology like plant tissue culture by producing large quantity of plantlets in short time (Jain, 1979; Leung, 1980; Anonymous, 1988).

Plant tissue culture techniques involving direct or indirect organogenesis, somatic embryogenesis and haploid production through various explant sources like leaf, shoot apex, hypocotyl, cotyledons, roots, anthers, etc., is an achievable method, recognized as a veritable tool in plant improvement in recent past (Reinert and Bajaj, 1977: Scowcroff, 1977: Vasil, 1980: Narayanasamy, 1994; Taksande *et al.,* 2018; Mane *et al.,* 2020). Haploid induction is one of the amazing division in Biotechnology Based Breeding Methods (BBBMs). Complete homozygous aspect of Doubled Haploids (DHs) has numerous applications in plant improvement through production of hybrids by attaining a hybrid vigor in short time (Niazian *et al.,* 2019; Samantaray *et al.,* 2021). Similarly, anther culture (androgenesis) is one of the methods in producing haploid and double haploid plant varieties (Hassan and Islam, 2021). The intricacies of medicinal plant production through micropropagation have been proficiently elaborated by Murashige (1978), Ammirato (1983) and Bajaj (1986). With the help of literature study on several reports, established a high efficacy of the application of plant tissue culture (*in vitro*) techniques in the regeneration and temporal monitoring of secondary metabolites (active principles) in plants could be achieved.

MATERIALS AND METHODS

Sterilization of glasswares

The glasswares used for tissue culture included culture tubes, conical flasks, beakers, autoclavable screwcap bottles, petridishes, pipettes, standard flasks and measuring cylinders. Firstly, the glasswares were soaked in sulfuric acid for four hours and washed under a running of tap water. Then, it was soaked in detergent for 30 minutes and washed thoroughly to remove the traces of detergent. Finally, it was rinsed with distilled water, dried in an hot-air oven and autoclaved at 15 lb pressure at 120°C for 20 minutes. After autoclaving, the sterilized glasswares were used for the preparation of culture medium under sterile condition.

Preparation of culture medium

Murashige and Skoog (MS) (1962) medium was used for the present work. Chemicals used in the preparation of medium components i.e., macro and micro nutrients were purchased of annular grade (Hi-media Laboratories, Bombay), also vitamins, amino acids and hormones were obtained from Sigma chemical company, USA and bacteriological grade agar for preparation of solid medium was purchased from Glaxo chemical company, Bombay. The nutrients of basal MS medium were prepared as stock solutions and its combinations are mentioned in Table-1 and Table 2. About 10 mg of the growth hormones were dissolved in their dissolving agent and made upto 10 ml of stock solution each using double distilled water. Hormonal stocks (thermostable) were added to the medium at the time of preparation according to the following formula;

$$\text{Hormonal stock solution to be added (ml)} = \frac{\text{Medium prepared (ml)} \times \text{Required concentration}}{1000}$$

All stock solutions listed in Table1 and Table 2, were stored in the refrigerator and were used in the preparation of culture medium which was maintained for a period of one month from the date of its preparation.

Preparation of Murashige and Skoog medium

Basal MS medium was used as the culture medium. One litre of medium was prepared along with the addition Na_2EDTA, appropriate amount of growth regulators and all other constituents listed in Table 1. About 3 % (W/V) sucrose was added to the final concentration of medium as a carbon source. pH of the medium was adjusted to 5.8 using 0.1 N NaOH or 0.1 N HCl. Then the solution was made upto 1 litre using distilled water, was divided into necessary aliquots according to the structured hormonal proportions and was solidified with 0.8% bacteriological grade agar. The melted media were distributed to culture tubes (150 x 24 mm^2) at the rate of 10 ml $tube^{-1}$, plugged with non-absorbent cotton and bundled as 10 culture tubes $bundle^{-1}$ (each bundle is considered to be a replicate of a particular concentration of combination).

Source of plant material and explants

Experimental plant material

The experimental plant *B. montanum* cuttings were obtained from Andhiyothiya Sangh (ANSA), Tiruchirappalli, Tamilnadu, India. The plant was grown and maintained in the college herbal garden for further use. About one month-old well growing plants raised from the stem cuttings were

used as a source, for the collection of *in vivo* explants.

Source of explant

For the present investigation, anthers of *B. montanum* were excised from young flower buds before anthesis and used as explants. Collection of flower buds (*in vivo*) was made in the early morning hours and surface sterilization of flower buds was carried out.

Sterilization of explants

Surface sterilization was carried out under laboratory condition by washing with tap water for 5-10 minutes to remove the soil particles and other extraneous fine particles. Then, it was sterilized with 0.1 % Bavistin for 15 minutes and rinsed thoroughly using distilled water. It was then, treated with 5 % $HgCl_2$ for 3 minutes in laminar air-flow chamber and washed three to four times using sterile water. After washing, explants were transferred to the sterilized petridish containing sterile water.

Inoculation and incubation

At the time of inoculation, explants were trimmed to a desirable size and introduced into the culture medium, was carried out aseptically in the laminar air-flow chamber. Culture tubes were labelled after inoculation with relevant information and maintained at 25 ± 2°C with a 16 hrs photoperiod (photon flur of 15 $\mu molm^{-2}s^{-1}$) provided by cool white fluorescent tubes.

Sterilization of medium

The culture tubes containing the culture medium were autoclaved for 15 minutes at 120°C under 15 lb pressure. The autoclaved medium was taken to culture room, allowed to cool for a minimum period of 24 hours before any inoculation work.

Callus formation

Anthers were carefully excised from the sterilized flower buds under aseptic conditions. About 4-5 anthers were cultured in MS medium with 3 % (w/v) sucrose, 800 mg l^{-1} PVP containing different concentrations of NAA (0.5 - 5.0 mg l^{-1}) in combination with 0.25 - 3.0 mg l^{-1} BA. A pinch of charcoal was also added and solidified with 0.8 % (w/v) agar prior to autoclaving. The effect of growth regulators on callus induction was analysed and further subculturing was done at 15 days interval on the same medium.

Regeneration and elongation of shoots

Hard, green calli obtained from the above culture were inoculated on MS medium containing various concentrations of BA (0.5 mg l^{-1} - 3.0 mg l^{-1}) in combination with IAA (0.5 mg l^{-1}) and BA (1.0 mg l^{-1} + Ads (1.0 - 3.0 mg l^{-1}) Regenerated shoots were transferred to MS medium supplemented with 10.0 mgl^{-1} BA + lAA (0.5 mg l^{-1}) + GA_3 (1.0 mg l^{-1}) for shoot elongation.

Rooting of elongated shoots

The elongated shoot lets were treated with full, half and quarter strength of MS medium fortified with NAA (0.2, 0.5, 1.0 and 3.0 mg l^{-1}). Full strength MS basal medium with auxins like IAA and IBA (1.0 mg l^{-1} - 3.0 mg l^{-1}) were also tried for rooting.

RESULTS AND DISCUSSION

In vitro androgenesis (anther culture) provides a prolific and desirable method in the production of homozygous hues over the course of few months, rather than preferring conventional techniques in the production of several generations through whole plant (Choo *et al.,* 1985; Morrison and Evans, 1988; Snape, 1989; Srivastava and Chaturvedi, 2008). Reports have proved that growth regulators have a significant role in *in vitro* anther culture. For the androgenic induction, auxins have been widely used and its concentration differs from species to species, also the addition of cytokinin in the induction medium improves the androgenic capability (Kasha *et al.,* 1990; Gosal *et al.,* 1997).

Callus formation

Anthers excised from young flower buds of *B. montanum* were used for callus induction. Basal MS medium supplemented with NAA (0.5 - 5.0 mg l^{-1}) in combination with BA (0.25 - 3.0 mg l^{-1}) was used for callus induction. Swelling of anther was noticed on 5th day of inoculation (Figure 1a), whereas, callus initiation was observed from 7th day onwards (Figure 1b). Further proliferation of callus was observed with the maximum callusing of 67.5%, recorded in the basal MS medium supplemented with 2.0 mg l^{-1} BA and 4.0 mg l^{-1} NAA (Figure 1c). Comparatively, a lesser callus induction frequency of 50.17% in hybrid rice varieties (Ali *et al.,* 2021), and 25% in *Citrus aurantium.,* (L.) (Jin *et al.,* 2022) were reported in approximately 3-5 weeks duration. This shows that maximum frequency and less duration of callus induction was obtained in the present investigation. Greenish, loosely arranged calli were observed at the initial stage and during subsequent subcultures compact, green calli were recorded. The combination of BA at 2.0 mg l^{-1} with increasing concentrations of NAA (0.5 - 4.0 mg l^{-1}) showed an increase in response (Table 3), whereas other hormonal combinations did not exhibit any renewable results. There was an alternate report to the present study, which showed a proficient embryogenesis in *Cucumis sativus* (L.) using different nutrient combinations supplemented with either 2,4-D or NAA. About 24 regenerated plantlets of 21 calypso and 17 green long haploid cultivars of *C. sativus,* produced from an optimal embryogenic calli induction from anther explant in B5 medium (Gamborg's medium) supplemented with 2.0 µ M 2,4-D+1.0 µ M BAP; embryo differentiation B5 medium with 0.09 M sucrose + 0.25 µ M NAA + 0.25 µ M Kn; embryo maturation in B5 medium with 5.0 µ M ABA and germination of plantlets in B5 medium with 0.09 M sucrose (Ashok *et al.,* 2003). This substantiates that, at each stage of callus proliferation in anther culture several combinations of growth regulators are required based on the plant species and medium used.

Elongation of regenerated shoots

Regenerated shoots along with callus at the basal

end were transferred to the MS medium containing 2.0 mg l^{-1} BA and GA_3 (0.5 - 2.0 mg l^{-1}) for elongation. The maximum of 4.8 cm shoot length was observed at 2.0 mg l^{-1}BA +1.0 mg l^{-1} GA_3 after shoot elongation (Table 4; Figure 1e).

Regeneration of shoots

The 35 days old sub cultured calli was selected for regeneration. A well developed hard, green calli was introduced into the regeneration medium containing increasing concentrations of BA (0.5 - 3.0 mg l^{-1}), BA (1.0 mg l^{-1}) + Ads (1.0 - 3.0 mg l^{-1}) and IAA (0.5 mg l^{-1}) + BA (1.0 - 3.0 mg l^{-1}). Successful regeneration was achieved on MS medium supplemented with different concentrations of BA (0.5 - 3.0 mg l^{-1}) after 35 days of culture in regeneration medium with a reduction in percentage of response (24.4%), when compared to the indirect organogenesis and BA at 2.0 mg l^{-1} showed the highest response of 89.0% in the regeneration of shoots (Plate 1d). Similarly, in about 4-6 weeks of interval *in vitro* derived *C. aurantium* plantlets were obtained at the rate of 50-60% (Jin *et al.,* 2022). Though the regeneration time was similar but the regeneration rate was found to be higher in the current investigation, which reveals a positive way to present results. The number of shoots $callus^{-1}$ was 4.0 and found to be the maximum (Table 5).

Rooting, hardening and acclimatization of plantlets

After shoot elongation, about 7 roots $plant^{-1}$ were observed with the root length of 6.6 cm and 54.0% of rooting response. The well rooted plantlets of 23 days old culture were stabilized for few days and were transferred to vermiculite for hardening. The maximum of 51.0% rooted plantlets were hardened (Figure 1g). The survival of *in vitro* derived plantlets was noted on 20th day and the plants were shifted to the soil. The successfully acclimatized plantlets showed further growth in the soil (Figure 1h) were 65.4 % (Table 6). There was diverse approach followed in regeneration of *in vitro* derived *C. aurantium* plantlets under *in vitro* and *in vivo* conditions when compared to the present study. Jin *et al.* (2022) reported that *in vitro* derived *C. aurantium* plantlets were firstly grown in hormone free MS medium, then transferred to MS medium supplemented with 500 mg l^{-1} malt extract + 50 mg l^{-1} sucrose + 0.5 mg l^{-1} GA_3 at the regeneration rate of 70-80 %. This shows that before hardening the *in vitro* derived plantlets of different species required different media supplement and the regeneration rate also differed accordingly.

Medicinal plants are of great interest to the researchers in the field of biotechnology, as most of the drug industries depends on various parts of plants for the production of pharmaceutical compounds. Globally, the primary requisite for such an approach is dependent on the production of large quantity of plants within a short time, is achievable through non -conventional methods like plant tissue culture techniques, that helps in mass production and conservational aspects. Optimization of media, chemical composition and proper dosages of each nutrient are very important factors for success on *in vitro* doubled haploids production that influences androgenic responses in crop plants. The present scientific investigation on anther culture of *B. montanum* provides a reliable protocol in achieving haploid and homozygous diploid production. A low concentration of cytokinin and high concentration of auxin were suitable for callus induction and BA alone was effective for shoot regeneration, also, shoot elongation was high with BA 2.0 mg l^{-1} + GA_3 1.0 mg l^{-1} combination and the elongated shoots showed best rooting on half strength MS medium at 2.0 mg l^{-1} NAA. The findings and methods standardized in this current work is very effective in conservation of *B. montanum* and also for the mutant varieties that parallelly helps for further advancement in biotechnological research in the improvement of crop regeneration through anther culture. Reports on *in vitro* propogation of euphorbiaceae members and other medicinal plants through anther culture are very scanty and this study would provide a great acquaintance to raise other species of the same family, agricultural crop varieties and plants under threat, which could be conserved using this achievable regeneration protocol. Subsequently, this anther culture technique will provide a boon in other breeding programs, enabling a rapid plant development by raising the agronomy and economic status of a country.

Table 1. Constituents of MS medium and preparation of stock solutions

Constituents of MS Medium			Stock Solution		
S.No.	Constituents (nutrients)	mg l^{-1}	Stock (mg l^{-1})	Solvent (D. H_20) in ml	Stock $litre^{-1}$ of media. (in ml)
	Macro Nutrients : Stock - I (30 X)				
1.	NH_4NO_3	1660	49800		
2.	KNO_3	1900	57000		
3.	$Mg\ SO_4.7H_2O$	370	11100	600	20
4.	KH_2PO_4	170	5100		
	Stock - II (30 X)				
5.	$CaCl_2.2H_2O$	440	17600	200	5
	Micro Nutrients: Stock - III (100 X)				
6.	H_3BO_3	6.20	620		
7.	$MnSO_4.H_2O$	22.30	2230		
8.	$ZnSO_4.\ 7H_2O$	8.60	860	500	5
9.	KI	0.83	83		
10.	$N_a2\ MoO_4.\ 2H_2O$	0.25	25		
11.	$C_uSO_4.\ 5H_2O$	0.025	2.5		
12.	$CoCI_{2.}\ 6H_2O$	0.025	2.5		
	Iron Sourcee : Stock - IV (20 X)				
13.	$FeSO_{4.}\ 7H_2O$	27.8	556	200	10
14.	Na_2 EDTA. $2H_2O$	37.3	746		
	Vitamins and Amino Acids				
15.	Glycine	0.100	5 (50 X)	50	1.0
16.	Nicotinic acid	0.050	50(100X)	50	0.5
17.	Pyridoxine	0.050	50(100X)	50	0.5
18.	Thiamine - HCL	0.010	200(100X)	50	0.5
19.	Myoinositol	-	100		0.5

Table 2. Preparation of growth hormones

S. No.	Hormones	Dissolving Agents
1.	2,4 - Dichlorophenoxy Acetic acid [2,4 - D]	50% Ethyl alcohol
2.	Gibberlic acid (GA_3)	50% Ethyl alcohol
3.	6, Benzyladenine [6 - BA]	1N NaOH
4.	Kinetin (Kn)	1N NaOH
5.	Adenine sulphate (Ads)	1N NaOH
6.	Zeatin (Zn)	1N NaOH
7.	Indole - 3 Butyric Acid (IBA)	1N NaOH
8.	Naphthaleneacetic Acid [NAA]	1N NaOH
9.	Indole Acetic Acid [IAA]	1N NaOH

Table 3. Effect of increasing concentrations (0.5 - 5.0 m gl^{-1}) of NAA in combination with BA (2.0 mg l^{-1}) on callus formation from anthers of *B. montanum*

Growth Hormones (mg l^{-1})		Percentage of Callusing (%)
NAA	BA	
0.5	2.0	37.5
1.0	2.0	43.3
2.0	2.0	50.0
3.0	2.0	60.0
4.0	2.0	67.5
5.0	2.0	47.5

Table 4. Effects of GA_3 (0.5 - 2 mg l^{-1}) in combination with BA (2.0 mg l^{-1}) on elongation of shoots derived from anther callus of *B. montanum*

Growth Regulator (mg l^{-1})		Length of Shoot (cms)
BA	GA_3	
2.0	0.5	$3.5 \pm 1.9_{ab}$
2.0	1.0	$4.8 \pm 1.1_{a}$
2.0	2.0	$2.9 \pm 0.4_{b}$

The results are expressed in terms of Mean ± SD. Mean values within a column with same alphabets are not significantly different (P = 0.05) according to Duncan's Multiple Range Test and the significance was determined at 1 % level. Each treatment consisted of 10 shoots and the experiment was repeated thrice.

Table 5. Effect of increasing concentrations (0.5 - 3.0 mg l^{-1}) of BA on regeneration of shoots from anther derived callus of *B. montanum*

BA (mg l^{-1})	Percentage of callus showed shoot initiation (%)	No. of shoots/ Callus
0.5	33.0 ± 1.5	$1.8 \pm 0.3_{b}$
1.0	48.0 ± 1.3	$2.2 \pm 0.8_{ab}$
2.0	89.0 ± 1.6	$4.0 \pm 1.1_{a}$
3.0	24.4 ± 1.1	$2.2 \pm 1.1_{ab}$

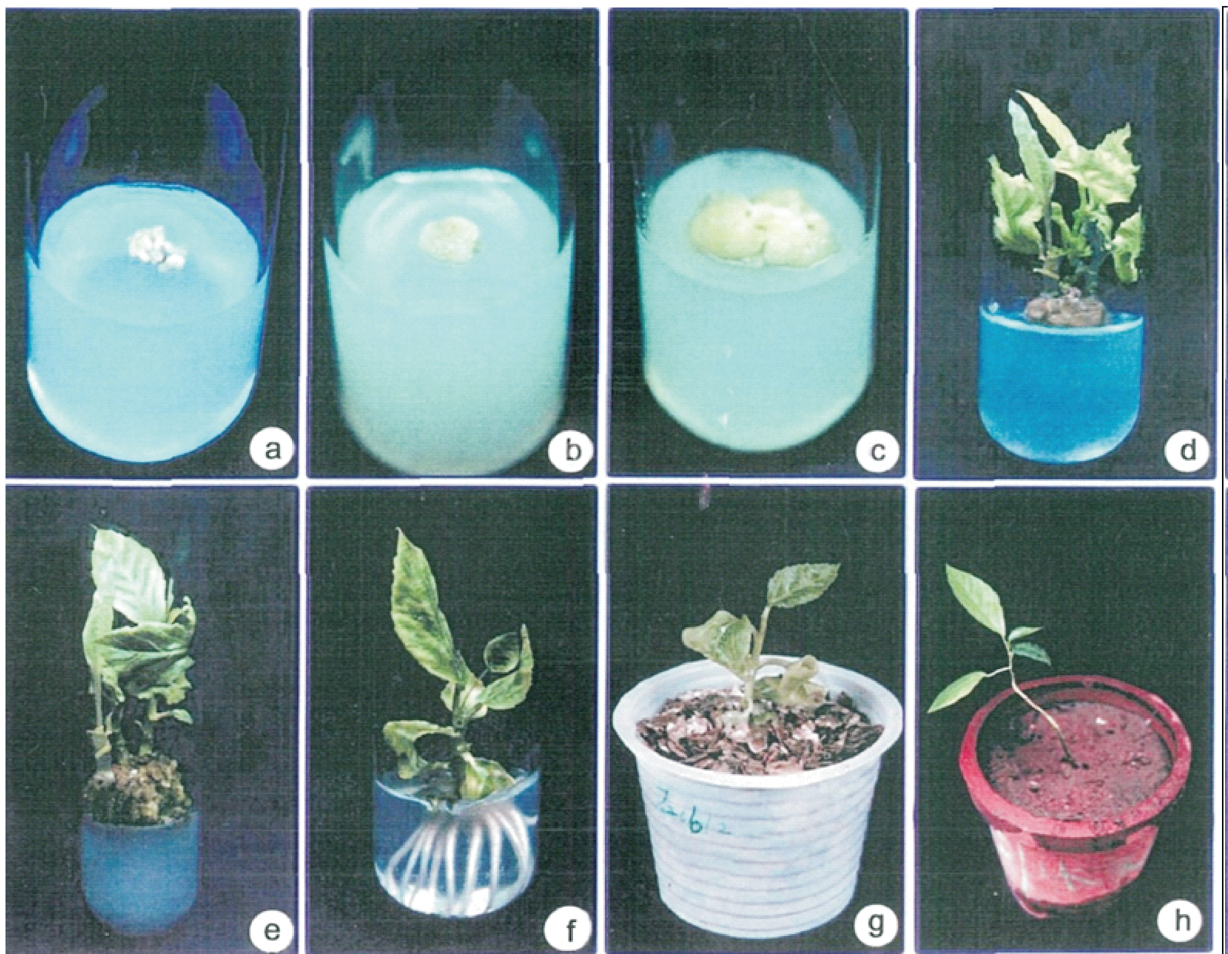
The results are expressed in terms of Mean ± SD. Mean values within a column with same alphabets are not significantly different (P = 0.05) according to Duncan's Multiple Range Test and the significance was determined at 1 % level. Each treatment consisted of 10 samples and the experiment was repeated thrice.

Table 6. Number of roots, length of roots and percentage of rooting, hardening and acclimatization of plantlets derived from anther culture of *B. montanum*

Method of Regeneration	Explants	Percentage of Rooting(%)	No. of Roots	Length of Roots (cm)	Percentage of Hardening(%)	Percentage of Acclimatization(%)
Anther Culture	Anther	54.0 ± 1.5	7.0 ± 1.0	6.6 ± 0.4	51. 0 ± 1.6	65.4 ± 1.1

The results are expressed in terms of Mean ± SD. Each treatment consisted of 10 shoots and the experiment was repeated thrice.

Figure 1.Production of plantlets through Indirect Organogenesis from anthers of *B. montanum*



a.Swelling of anthers

b.Callus initiation

c. Proliferated callus

d. Shoot regeneration

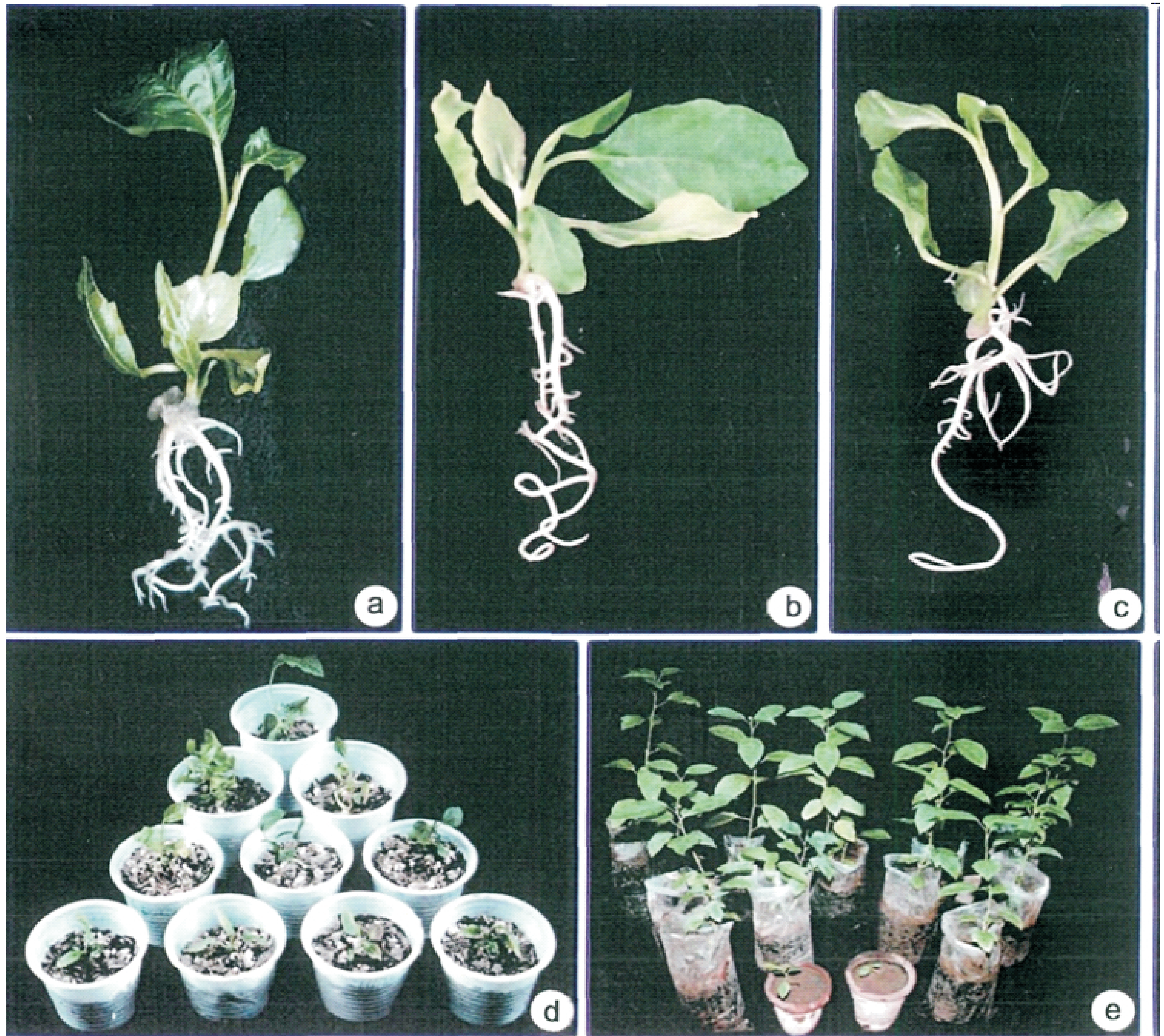
e.Elongation of shoots

f. Rooting of shoot

g. Hardened plantlet

h. One month old acclimatized plantlet

Figure 2. Rooting pattern of shoots on MS Medium containing NAA, IAA and IBA on rooting of regenerated shoots, hardened and acclimatized *in vitro* derived plantlets of *B. montanum*



a. Rooting of shoot in NAA
b. Rooting of shoot in IAA
c. Rooting of shoot in IBA
d. *In vitro* derived hardened plantlets
e. *In vitro* acclimatized plantlets

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