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Organogenesis of *Abelmoschus moschatus* Medik. Using Aseptic Seedling Explants

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Authors' contributions

This work was carried out in collaboration between both authors. Author PT designed the study, wrote the protocol and corrected the manuscript. Author AV conducted the experiments managed the analyses of the study and wrote the first draft. Both authors read and approved the final manuscript.

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Short Research Article

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ABSTRACT

Aim: *Abelmoschus moschatus* have been extensively used in traditional medicine as well as in perfume industries. The primary goal of the present research was to develop an efficient plant regeneration protocol of *Abelmoschus moschatus* from aseptic seedling explants such as cotyledon, internode, leaf and root.
Methodology: The seeds of *Abelmoschus moschatus* were surface sterilized with 0.1% mercuric chloride and 70% ethanol were cultured on ½ MS basal media for developing aseptic seedlings Aseptic seedling explants were cultured on different concentrations of auxins for callus induction. Later callus was transferred on to different concentrations of cytokinins for shoot regeneration and for *in vitro*, rooting different concentrations of auxins were used. Finally, such *in vitro* developed plantlets were acclimatized.
Results: Half strength MS medium with 1% sucrose was used for raising aseptic seedlings. Maximum of 92% response of callus induction was obtained from leaf explants on MS medium +

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2 mg/L 2, 4-dichlorophenoxyacetic acid. An average of 2.4 shoots per callus were observed on MS + 2 mg/L benzyl-6-aminopurine from leaf explant. The regenerated shoots were best rooted on 1/2 MS + 0.5 mg/L indole-3-butyric acid. The regenerated plantlets were established with 70% survival.
Conclusion: An efficient plant regeneration protocol of *Abelmoschus moschatus* was developed.

Keywords: Abelmoschus moschatus; callus; leaf explant; shoot regeneration.

1. INTRODUCTION

Abelmoschus moschatus is a prostate herb widely cultivated for its seeds and essential oil along the tropical regions of Asia, Africa and South America [1]. The selection of this plant was based on its traditional usage. A survey of the literature revealed that the seeds of this plant (powdered form in lukewarm milk) have been recommended for use in various traditional systems of medicine for the treatment of intestinal complaints, constipation, dyspepsia and gonorrhea [1]. The seeds are used as stimulant, relaxant and also for casting out the poison of snakes. The seeds also serve as cardiac tonic, aphrodisiac, diuretic, antispasmodic [1]. Moreover, the leaf decoction has been effective against intestinal complaints and checks vomiting. The tincture of leaf powder is applied for skin diseases too.

The seeds traditionally used to treat neurodegenerative diseases, rheumatism, cystitis and diuretic. Seeds of the studied plant have been reported to possess biological and therapeutic potential such as diuretic activity, antilithiatic, anti-hysteric, hepato-protective, memory strengthening, anti ageing, antidepressant, anxiolytic, anticonvulsant, anti-spasmodic and anti-diabetic effect [1,2,3,4]. Phytochemical studies showed the presence of flavonoids, phenols, saponins, carbohydrates, terpenoids, myricetin, alkaloids and steroids [5].

The plant parts are used in Bangladesh by traditional healers [6]; used in tribal and traditional medicine of India [7]; for stomach pain and disorder in Trinidad and Tobago [8]. The essential oil is used in aromatherapy for treatment of depression and anxiety [9]. It contains flavonoids which are responsible for antioxidant, antimicrobial and free radical scavenging activity [10,11]. It also shows protective mechanism in respiratory troubles and asthma antispasmodic itch [12]. Sheik et al. [13] reported *A. moschatus* is an alternative source for central nervous system (CNS) drug development. *Abelmoschus moschatus* is usually propagated from its seeds but seeds lose their germination viability soon after harvesting. Thidiazuran induced regeneration of plantlets from cotyledonary nodes of *Abelmoschus moschatus* (Malvaceae) has been reported by Sharma and Shahzad [14]. Lithy et al. [15] reported *in vitro* propagation from cotyledonary nodes of germinated seedling of *Abelmoschus moschatus.* Optimization of protocols for callus culture and regeneration will help to enhance the production of secondary metabolite and transformation studies. There is no report on *in vitro* regeneration of plant via indirect organogenesis. The present work has been taken up with an objective to standardize a protocol for plant regeneration from aseptic seedling explants of *Abelmoschus moschatus*.

2. MATERIALS AND METHODS

2.1 Preparation of Culture Media and Growth Conditions

MS (Murashige and Skoog) [16] medium was prepared with 3% (w/v) sucrose and supplemented with different plant growth regulators such as auxins 2,4-D (2,4-dichlorophenoxyacetic acid), NAA (naphthalene-3-acetic acid), IAA (indole-3-acetic acid), IBA (indole-3-butyric acid) and picloram (4 – amino,3,5,6 – trichloro picolinic acid) and cytokinins such as BAP (benzyl-6-aminopurine) and KN (kinetin). The pH of the medium was adjusted to 5.7 with 1N NaOH or 1N HCl before gelling with 0.8% (w/v) plant tissue culture agar (Himedia, India). In all the experiments, the chemicals used were of analytical grade (Merck and Sd-fine Chemicals, India). About 15 ml of culture medium was poured into each culture tubes (150 × 25 mm) (Borosil, Mumbai, India), mouth tightly covered with aluminium foil and autoclaved at 104 kPa and 121°C for 15 min. All the cultures were incubated at 25 ± 2°C under 16 hours photoperiod of 45 - 50 µ mol/m2/s irradiance provided by cool white fluorescent tubes (Philips, India) and with 60 - 65% relative humidity. All subsequent subcultures were done at four week intervals.

2.2 Surface Sterilization of Seed and Establishment of Aseptic Seedling Culture

Abelmoschus moschatus (Kasturi benda) seeds were collected from Central Institute for Medicinal and Aromatic Plants, Hyderabad. These seeds were stored at room temperature until use. Healthy seeds were taken in a 250 ml clean sterile Erlenmeyer flask and washed in agitated solution of liquid soap (Tween 20) solution for 30 minutes and washed under running tap water followed by sterile double distilled water. Further operations were carried out under laminar air flow chamber; the seeds were subjected to treatment of 70% ethanol for 60 seconds followed by a sterile distilled water wash. Seeds were surface sterilized with chemical disinfectants like mercuric chloride (0.5, 1, 2, 3%), hydrogen peroxide(10, 20, 30%) etc. at different concentration for 1, 2, 3, 5, 7 and 10 minutes of interval then followed by three rinses with distilled water. These surface sterilized seeds were individually inoculated on ½ strength MS basal medium supplemented with 1% (w/v) sucrose (Sdfine Chemicals, India) and solidified with 0.7% agar. The surface sterilized seeds were implanted vertically on the culture medium for aseptic seed germination. Germinated seeds were used for culture initiation which also served as explants source for subsequent experiments.

2.3 Callus Induction

30 days old aseptic seedling explants of cotyledon, internodes, leaf and root were used for callus induction. Explants were cultured on MS medium supplemented with 2,4-D (0.1 - 5.0 mg/L), NAA (0.1 - 5.0 mg/L), IAA (0.1 - 5.0 mg/L) and Picloram (0.1 - 5.0 mg/L). Percentage of callus induction and nature of response were recorded after 30 days of culture.

2.4 Shoot Regeneration from Callus

For *in vitro* shoot regeneration, callus was made into pieces and transferred onto shoot induction medium supplemented with BAP (0.1-3 mg/L) and KN (0.1-3 mg/L). Percentage of shoot regeneration from callus, number of shoots per callus and shoot length were recorded after 40 days of culture.

2.5 *In vitro* Rooting

After shoots elongated (3 - 5 cm) it was excised and transferred to full and half-strength MS containing 1% (w/v) sucrose and 0.7% (w/v) agar. The medium was further supplemented with IAA (0.1 - 2.0 mg/L), IBA (0.1 - 2.0 mg/L) and NAA (0.1 - 2.0 mg/L) individually. Percentage of root induction, number of roots per shoot and root length was recorded after 30 days of culture.

2.6 Acclimatization

Plantlets with well-developed roots were removed from the culture medium and after washing the roots gently under running tap water, plantlets were transferred to plastic pots (10 cm diameter) containing autoclaved soil and farmyard manure (1:1). Each plantlet was irrigated with half-strength MS basal salt solution devoid of sucrose and inositol every fourth day for two weeks. The potted plants were covered with porous polythene sheets for maintaining high humidity and were maintained inside the culture room conditions. The relative humidity was reduced gradually, and after 30 days the plantlets were transplanted to pots (25 cm diameter) containing forest humus and garden soil (1:1). The pots were transferred to greenhouse for further growth and development. Well acclimatized *in vitro*-raised plants were transferred to its original habitat for its survival ability. Further the morphological characteristics, growth characteristics, and floral features were examined.

2.7 Statistical Analysis

Experiments were set up in a randomized block design and each experiment usually had 15 replications and was repeated at least three times. Observations were recorded on the percentage of response (number of explants responding for callus formation, organogenesis and root development by total number of explants multiply by 100) and number of shoots per callus and roots per shoot respectively. The analysis of variance (ANOVA) appropriate for the design was carried out to detect the significance of differences among the treatment means. Means followed by the same letter not significantly different by the Tukey test at 0.05% probability level.

3. RESULTS AND DISCUSSION

3.1 Aseptic Seed Germination

In the process of establishment of callus culture, aseptic seed germination was initiated. Aseptic seed germination protocol was optimized by subjecting to 60 seconds treatment with ethanol

and followed by 0.1% mercuric chloride for 3 minutes (data not shown). Lithy et al. [15] previously reported that surface sterilization was done using 0.1% mercuric chloride for 6 minutes for 15 days old seedling explants of *Abelmoschus moschatus*. Seed germination was initiated after 5 days of inoculation on ½ strength MS medium. 85% of seeds germinated after 30 days of incubation. Those 30 days old aseptic seedlings were used as explants source for callus initiation on MS medium supplemented with auxins.

3.2 Callus Induction

Callus was induced from cotyledon, root, leaf and internode of aseptic seedling explants. Among various explants of *Abelmoschus moschatus* leaf explants were more responsive followed by cotyledons, internode and root. Leaf explants gave maximum 92% of response compared to other explants.

Induction of callus was maximum with leaf explants on 2 mg/L 2, 4-D and developed into profuse green friable callus with 92% response (Table 1). Lower concentrations than 1 mg/L 2, 4-D induced less amount of callus. Increase in concentration above 2 mg/L reduced the percentage of response and nature of callus also varied. Compared to NAA, IAA and Picloram, 2, 4 – D gave maximum response for all explants. Similar reports were also reported in *Caralluma lasiantha* [17]. In *Abelmoschus esculentus* MS medium supplemented with 2,4 –D and BAP provided effective callus induction from hypocotyl explant [18].

Leaf explant cultured on 0.1 mg/L NAA resulted in rooting with green scanty callus. As the concentration increased rhizogenesis decreased and produced greenish white callus, where rhizogenesis and callusing increased with increasing concentration of IAA and callusing further decreased after 2 mg/l IAA. Leaf explant cultured on picloram resulted in profuse light brown friable callus.

Callus obtained from cotyledon and internode on 2 mg/L 2, 4-D medium resulted in greenish yellow friable callus with a maximum response of 87 and 78% respectively. Embryonic callus was produced on 2 mg/L 2, 4-D for root explant of *Abelmoschus moschatus,* whereas Daniel et al. [19] reported embryonic callus induction with combination 2,4-D and KN from cotyledon leaf explant of *Abelmoschus esculentus*. Different stages of somatic embryos were observed on 2 mg/L 2, 4-D for root explant.

Table 1. The effect of ½ strength MS medium supplemented with different concentrations of auxins on callus induction aseptic seeding explants of *Abelmoschus moschatus*

Plant growth regulator concentration (mg/L)				Type of explants			
				Cotyledon	Internode	Leaf	Root
2, 4 – D	NAA	IAA	Picloram	% of response	% of response	% of response	% of response
0.1				61^{e}	55^{d}	73^{d}	42^{f}
0.5				69^{d}	57^{d}	81^{c}	56^{d}
1.0				73^{e}	69^{b}	84^{bc}	67^{b}
2.0				87^{a}	78^{a}	92^{a}	75^{a}
3.0				69^{d}	64^{c}	81^{c}	69^{b}
	0.1			54^{f}	38^{g}	60^{f}	23^{i}
	0.5			60^{e}	46^{f}	73^{d}	35^{g}
	1.0			68^{d}	59^{cd}	79^{c}	46^{e}
	2.0			73^{c}	64^{c}	87^{b}	33^{g}
	3.0			71^{cd}	53^{e}	73^{d}	20^{f}
		0.1		42^{h}	20^{i}	52^{g}	30^{h}
		0.5		51^{f}	34^{h}	68^{e}	42^{f}
		1.0		64^{e}	45^{f}	87^{b}	51^{d}
		2.0		59^{ef}	39^{g}	81^{c}	67^{b}
		3.0		47^{g}	31^{h}	74^{d}	49^{e}
			0.1	69^{d}	45^{f}	62^{f}	39^{fg}
			0.5	72^{c}	57^{d}	79^{c}	61^{c}
			1.0	79^{b}	68^{b}	85^{bc}	69^{b}
			2.0	81^{b}	72^{b}	72^{d}	71^{ab}
			3.0	75^{c}	64^{c}	69^{e}	63^{c}

Means followed by the same letter not significantly different by the Tukey test at 0.05% probability level

3.3 Organogenesis

Morphogenesis was observed from callus derived from cotyledon, internode and leaf explants. Organogenesis was not seen from callus derived from root explant. Leaf explant derived callus shown an average 2.4 shoots on MS + 2 mg/L BAP (Table 2). An average of 1-2 shoots were observed for cotyledon and internode explant. Sub culturing after four weeks further increased in the shoot number to 11 per callus. Anisuzzaman et al. [20] reported the combination of 2 mg/L BAP + 0.1 mg/L IBA for shoot regeneration of *A. esculentus*. Similar result was reported by Irshad et al. [18] in *A. esculentus* L. cv. Wufu. Shoot regeneration was not observed for callus produced from root explant.

3.4 *In vitro* Rooting

Micro shoots that have attained a length of 5-6cm were excised and placed on rooting medium. In the present investigation the shoots that were regenerated on MS medium supplement with BAP were aseptically transferred onto ½ strength MS medium fortified with different concentrations of IAA, IBA and NAA.

A few strong, healthy, branched 7-8 roots were obtained from ½ strength MS medium supplemented with IBA 0.5mg/L (Fig. 1). While ½ strength MS + 0.5 mg/L NAA gave only 3 roots. Whereas ½ strength MS different concentration of IAA produced 1- 2 roots. Among different auxins used IBA was more effective for root induction than NAA and IAA (Fig. 2). Similar result was reported in *A. esculentus* L. cv. Wufu by Irshad et al. [18].

3.5 Acclimatization

The ultimate success of *in vitro* propagation lies in the successful establishment of plants in soil. The rooted *in vitro* plantlets were transferred to polythene cups containing autoclaved soil and farmyard manure (1:1) for acclimatization at 25 ± 2°C for ten days during which elongation and growth of the plant was observed. Later, these plantlets were transferred to pots containing forest humus and garden soil (1:1). The potted plants were finally transferred to original habitat after four weeks. The *in vitro*-grown plants established at survival rate of 80% and 70% in polythene cups and field conditions, respectively. The growth characteristics of *in vitro*-raised plants did not show any significant morphological variations from those of the natural habitat. All *in vitro* regenerated plants grew normally and produced multiple branching with roots at nodes and after their transfer to the original habitat.

Table 2. The effect of MS medium supplemented with different concentrations of cytokinins on shoot regeneration of *Abelmoschus moschatus* after 30 days

Plant growth regulator concentration (mg/L)		Types of explants					
		Cotyledon		Internode		Leaf	
BAP	KN	% of response	No. of shoot per callus	% of response	No. of shoot per callus	% of response	No. of shoot per callus
0.1		28	0.51 ± 0.14^{c}	23	0.28 ± 0.11^{d}	53	0.84 ± 0.14^{c}
0.5		37	0.94 ± 0.13^{a}	29	0.44 ± 0.17^{c}	62	1.14 ± 0.13^{bc}
1.0		42	1.06 ± 0.16^{a}	37	0.67 ± 0.14^{b}	75	1.45 ± 0.17^{b}
2.0		57	1.14 ± 0.17^{a}	45	0.94 ± 0.16^{a}	83	2.40 ± 0.10^{a}
3.0		45	0.73 ± 0.11^{b}	36	0.67 ± 0.13^{b}	77	1.37 ± 0.12^{b}
	0.1	20	0.37 ± 0.13^{c}	-	-	58	0.49 ± 0.18^{d}
	0.5	29	0.44 ± 0.14^{c}	28	0.29 ± 0.18^{d}	64	0.56 ± 0.13^{d}
	1.0	35	0.53 ± 0.15^{c}	42	0.45 ± 0.14^{c}	69	0.91 ± 0.15^{c}
	2.0	44	0.58 ± 0.19^{bc}	39	0.68 ± 0.13^{bc}	55	1.21 ± 0.13^{b}
	3.0	40	0.47 ± 0.11^{c}	31	0.34 ± 0.16^{cd}	47	0.74 ± 0.11^{cd}

Values represent mean ± standard error of 15 replicates per treatment in three repeated experiments. Means followed by the same letter not significantly different by the Tukey test at 0.05% probability level

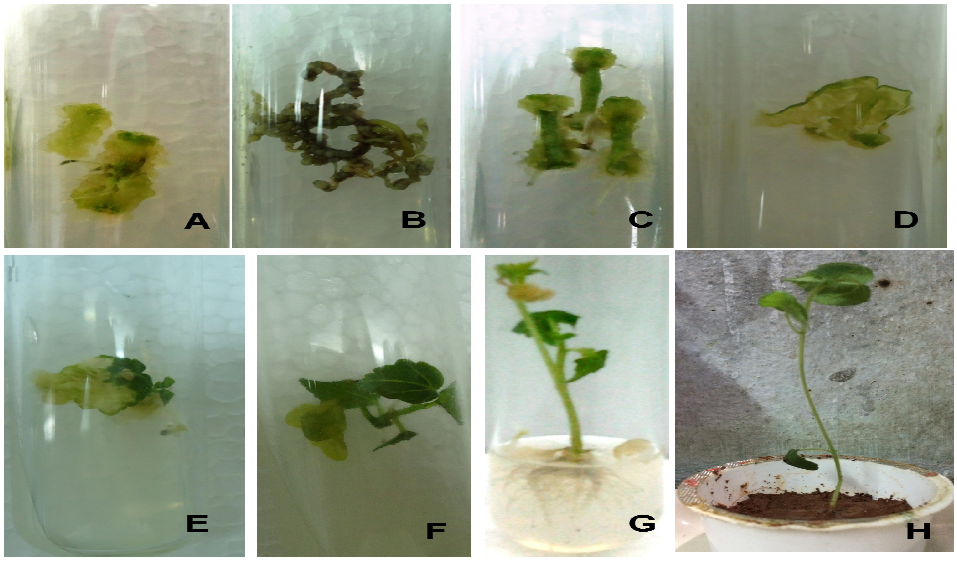

Fig. 1. *In vitro* shoot regeneration of *Abelmoschus moschatus*

A. *Callus induction of cotyledon explants cultured on MS medium supplemented with 2,4-D 2 mg/L after 15 days.*
B. *Callus induction of root explants cultured on MS medium supplemented with 2,4-D 2 mg/L after 15 days.*
C. *Callus induction of internode explants cultured on MS medium supplemented with 2,4-D 2 mg/L after 15 days.*
D. *Callus induction of leaf explants cultured on MS medium supplemented with 2,4-D 2 mg/L after 15 days.*
E. *Shoot regeneration from leaf explants cultured on MS medium supplemented with BAP 2 mg/L after 10 days*
F. *Shoot regeneration from leaf explants cultured on MS medium supplemented with BAP 2 mg/L after 20 days*
G. *In vitro rooting of in vitro raised shoots on ½ MS medium fortified with IBA 0.5 mg/L after 25 days.*
H. *In vitro rooted plants transferred to pots containing forest humus and garden soil 1 weeks.*

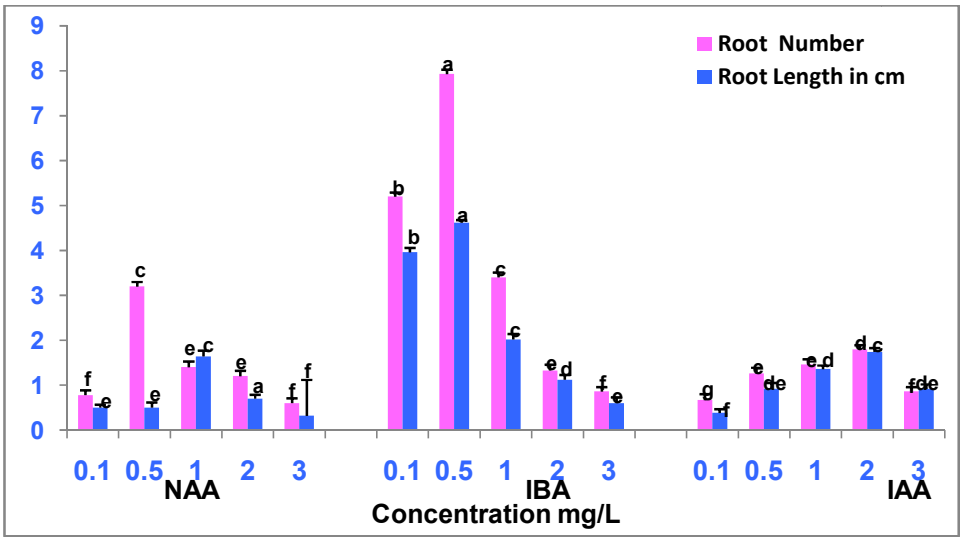

Fig. 2. *In vitro* rooting of *in vitro* raised shoots of *Abelmoschus moschatus* on ½ strength MS medium supplemented with different concentration of auxins after 30 days

Mean ± standard error of 15 replicates per treatment in three repeated experiments. Means followed by the same letter not significantly different by the Tukey test at 0.05% probability level

4. CONCLUSION

An efficient plant regeneration protocol was developed which could help in further improvement of crop and production of transgenic plants using gene manipulation techniques.

CONSENT

It is not applicable.

ETHICAL APPROVAL

It is not applicable.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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