

## Micropropagation from axenic seedlings of *Abelmoschus moschatus* Medic (*Kasturi bhendi*)

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### ABSTRACT

The musk-scented ambrette oil obtained from the seeds of *Abelmoschus moschatus* is highly valued in the perfumery industry. A protocol was designed to micropropagate *Abelmoschus moschatus* through apical and axillary nodal explants from axenic seedlings. Murashige and Skoog's medium (MS) supplemented with different combinations of  $\alpha$ -Naphthalene acetic acid (NAA), Indole-3-acetic acid (IAA), N<sup>6</sup>-Benzyladenine (BAP) and Kinetin (K) were used for shoot regeneration. The best quality of shoots with maximum number of leaves was regenerated in MS + 0.55 $\mu$ M IAA medium. Root initiation concomitant with shoot regeneration was observed in this medium. Callus was induced at the base of explants inoculated in media supplemented with cytokinins. The shoots obtained in different media were rooted in MS + 0.55 $\mu$ M IAA medium. The plantlets were hardened in coarse vermiculite-sand mixture irrigated with half strength liquid MS medium (1.5% sucrose). These were subsequently acclimatized and transferred to field conditions with 95.83% survival. The entire protocol could be completed in about 35 days and thus it is both economical and rapid.

**Key words:** *Abelmoschus moschatus*, *Hibiscus abelmoschus*, micropropagation, ambrette, axenic seedling, apical buds, nodal explants, IAA.

### INTRODUCTION

Traditionally, musk for perfume has been obtained from the pods of the male musk deer but only after the sacrifice of the animal. This makes it expensive, rare and unethical. The artificially synthesized musk odorant substances are abundant and cheap but have toxic side effects. The seeds of *Abelmoschus moschatus* Medic *syn.* *Hibiscus abelmoschus* Linn (commonly known as *Mushkdana*, *Kasturi bhendi*) are valued commercially as plant source of musk odorant substance<sup>1</sup>. These seeds are a healthier alternative to artificial musk, economical and an ethical substitute for animal musk.

The plant is native to India and is popularly known as Ambrette<sup>1</sup>. The seeds find application in Ayurvedic and Unani systems of medicine<sup>3</sup>. Different parts of this plant are used to treat various disorders of the alimentary tract, heart and skin<sup>3</sup>.

The distillation of the seeds of *Abelmoschus moschatus* yield ambrette oil<sup>2</sup>, with farnesol and ambrettolide being its major constituents. The extracts of the plant possess compounds like  $\beta$ -sitosterol, myricetin and their glucosides<sup>5</sup>. The essential oil of ambrette seeds promoted dermal papilla cell growth and therefore, probably, is a promising active ingredient for hair re-growth preparations<sup>6</sup>. Myricetin purified from the aerial parts of *A. moschatus* lowered plasma glucose levels and increased insulin sensitivity in streptozotocin-induced diabetic rats<sup>7</sup> thus revealing a potential for antidiabetic drug development<sup>8</sup>. Root extracts of *A. moschatus* showed larvicidal activity against mosquito larvae<sup>9</sup>. Reports suggest that ambrette oil is comparable to groundnut oil in all the parameters and is suitable for edible use<sup>4</sup>. Its stem bark is reported to yield a jute-like fibre with 78% cellulose<sup>10</sup>.

A large number of plants can be produced by micropropagation. Few protocols of micropropagation of this plant have been reported. In this report, we describe the micropropagation of *Abelmoschus moschatus*, using apical and axillary nodal explants obtained from axenic seedlings grown *in vitro*. All the experiments were done using ten replicates for each medium and each experiment was repeated thrice.

#### MATERIAL AND METHODS:

Fresh, germination quality, seeds of *Abelmoschus moschatus* were collected from various sources across India namely, Naik Krishi Udhog and Co., Pune; AayurMed Biotech P. Ltd, Mumbai and Gautam Global, Dehra Dun. The surface sterilization method suggested by Maheshwari P *et al.*<sup>11</sup> was modified for these experiments. The seeds were soaked in water for 30mins and washed with dilute liquid detergent (Teepol; Reckitt Benckiser, India) for 5mins, 1% v/v solution of antiseptic (Savlon; Johnson & Johnson Ltd, India) for 60s and 0.1% w/v solution of Mercuric chloride ( $\text{HgCl}_2$ , Suvudinath Laboratory, India) for 90s. The seeds were then rinsed thoroughly with sterile double distilled water (x5). Surface sterilized seeds were germinated in half strength Murashige and Skoog's medium<sup>12</sup> with 1.5% w/v sucrose (pure grade; SRL, India).

The apical and axillary nodal explants of the axenic seedlings were inoculated in MS medium or MS medium supplemented with different concentrations of IAA (Indole-3-acetic acid; Suvudinath Laboratory, India), NAA ( $\alpha$ -Naphthalene acetic acid extra pure; Himedia, India), BAP (N<sup>6</sup>-Benzyladenine extra pure AR; SRL, India) and K (Kinetin pure; Himedia, India) with Sucrose 3% w/v (pure, SRL, India).

Based on the available literature on *A. moschatus* shoot regeneration, various media were devised to induce shoots namely, MS; MS + 0.55 $\mu\text{M}$  NAA + 18 $\mu\text{M}$  BAP; MS + 0.55 $\mu\text{M}$  NAA + 18 $\mu\text{M}$  K; MS + 0.55 $\mu\text{M}$  IAA + 18 $\mu\text{M}$  K; MS + 0.55 $\mu\text{M}$  IAA + 3 $\mu\text{M}$  K; MS + 0.55 $\mu\text{M}$  IAA + 3 $\mu\text{M}$  BAP; MS + 0.55 $\mu\text{M}$  IAA; MS + 3 $\mu\text{M}$  K; MS + 1 $\mu\text{M}$  BAP; MS + 3 $\mu\text{M}$  BAP.

The pH of all the media was adjusted to 5.75 using 1N NaOH or 1N HCl. The media were then solidified using 0.9% Agar (Himedia, India), prior to autoclaving at 1.05 Kg/cm<sup>2</sup> and 121° for 20mins. Explants were inoculated in 10ml of medium in Borosil glass test tubes (150 x 25mm) for seed germination and further for shoot initiation and root induction. The explants were cultured in Plant Tissue Culture laboratory conditions of cool white fluorescent light (40.5  $\mu\text{mol}/\text{sq.m}/\text{s}^2$ ) with a photoperiod of 16hrs light and 8hrs dark at 22°  $\pm$  2.

The shoots thus obtained in ten days were transferred for root initiation and development in MS + 0.55 $\mu\text{M}$  IAA medium. The shoots that developed a dense network of roots were retained in same medium till they attained the height of 8–9cm. The shoots that had just initiated roots or had failed to root in ten days were subcultured in fresh MS + 0.55 $\mu\text{M}$  IAA medium. The plantlets that had reached the required height were hardened in a sterilized mixture of coarse vermiculite and sand (filled to a height of about 60mm, in clear glass jars of 1L capacity) and wetted with half strength of MS liquid medium. These were maintained for five days in controlled Plant Tissue Culture laboratory conditions. Subsequently they were shifted to room temperature and natural light for next five days for acclimatization. The plantlets were then exposed to non-sterile conditions, though retained in the vermiculite sand mixture for another five days before they were potted in soil.

All quantitative results were tested for significance using ANOVA (1% level of significance) and the comparison of the means was done using the Duncan Multiple Range Test<sup>15</sup>. The colour of the leaves obtained in a medium was qualitatively expressed by determining the statistical parameter 'Mode' for all thirty replicates of the medium.

#### RESULTS AND DISCUSSION

The axenic seedlings were grown for 15 to 30 days to obtain 3 to 5 nodes per seedling. The shoots were initiated from nodal explants in 1 to 2.57 days in different shooting media (Table 1, Row A; Figure 1-B). The fastest rate of shoot initiation was observed in MS medium.

Table 1: Shoot Initiation

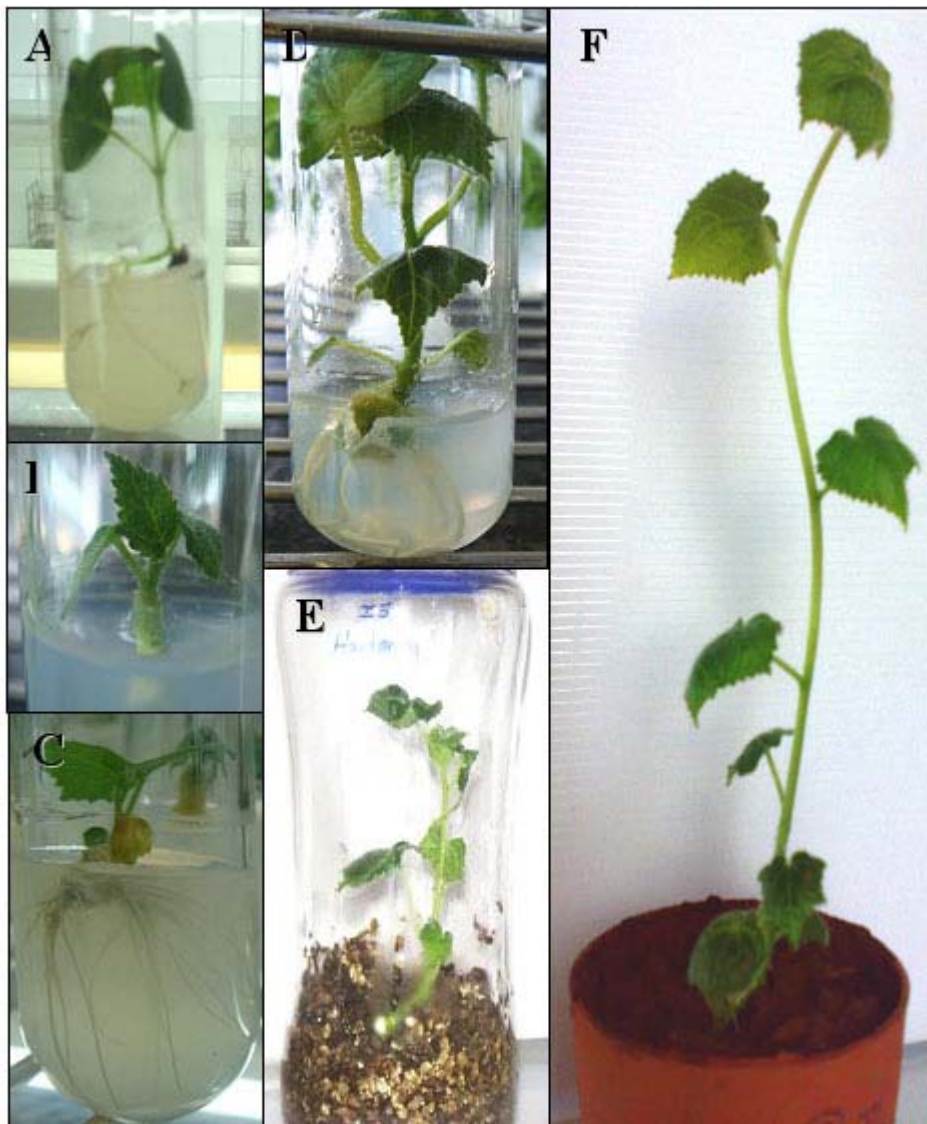
Media → Parameters ↓	MS a	NB18 b	NK18 c	IK18 d	IB3 e	IK3 f	I g	K3 h	B1 i	B3 j	
RowA	Shoot	1 ± 0	2.57±	1.70±	1.96±	2.33±	1.57±	2.13±	1.60±	1.93±	2.13±
	Initiation (Days)		1.45	0.65	0.88	1.99	0.94	1.73	0.97	1.20	1.70
RowB	Average No. of Leaves per shoot.	2.67±	1.91±	2.14±	1.75±	1.69±	2.60±	5.26±	2.90±	2.13±	1.61±
	Average Size of Leaves (mm)	1.60	0.66	0.78	0.76	0.71	0.89	2.06	0.96	0.62	0.68
RowC	Callus Initiation (% Response)	5.94±	3.63±	4.72±	4.61±	4.61±	7.14±	5.30±	5.74±	7.73±	6.53±
	Callus Initiation (% Response)	1.35	1.09	1.63	1.03	2.00	2.02	1.74	1.62	2.47	2.35
	Callus Initiation (% Response)	-	83.33%	80%	60%	60%	6.67%	-	20%	43.33%	60%
RowD	Concomitant Root Initiation (% Response)	-	5.28±	6.50±	6.33±	6.44±	8.50±	-	9.17±	7.54±	7.17±
	Concomitant Root Initiation (Days)		1.20	0.78	2.11	1.82	0.70		1.33	1.33	1.20
	Concomitant Root Initiation (% Response)	43.33%	-	-	-	-	26.67%	70%	20%	-	-
RowE	Multiple Shoots (% Response)	4.23±	-	-	-	-	7.50±	5.33±	8.83±	-	-
	Avg. No. of Shoots per Node	0.92	-	13.33%	-	6.66%	0.70	1.39	0.75	6.66%	-
	Avg. No. of Shoots per Node	-	-	2	-	2.5	-	-	-	2	-



Within ten days, the shoots showed difference in quality and quantity of foliage. The shoots in MS + 0.55µM IAA medium showed maximum number of leaves but larger leaves were observed in MS + 1µM BAP medium (Table 1, Row B). The colour of the leaves in different media varied from pale yellow to dark green. The shoots in MS, MS + 0.55µM IAA, MS + 0.55µM IAA + 3µM K and MS + 3µM K media showed fresh dark green leaves.

Sporadic instances of multiple shoot formation were observed in MS + 0.55µM NAA + 18µM K; MS + 1µM BAP; MS + 0.55µM IAA + 3µM BAP media with an average of 2.5 shoots per node (Table 1, Row E). Several of these shoots were malformed.

Media that included cytokinins showed callusing at the base of the shoots. This callus was excised from the base before transferring the shoots



**Fig. 1: A, Seed germination in MS½ medium; B, Shoot induction without callus formation at the base of the explants in MS + 0.55µM IAA medium; C, Concomitant root initiation with shoot induction in MS + 0.55µM IAA medium; D, Root induction in MS + 0.55µM IAA medium; E, Hardening in Vermiculite-sand mixture wetted with MS½ Liq. medium; F, Plantlet in soil**

into rooting medium. However, shoots in some media did not show any callus production during shoot induction such as MS medium and MS + 0.55 $\mu$ M IAA medium (Table 1, Row C; Figure 1-B).

Good shoots with concomitant root initiation were observed in MS and MS medium supplemented with 0.55 $\mu$ M IAA and/or 3 $\mu$ M K, in ten days (Table 1, Row D; Figure 1-C). The number of explants that showed concomitant root initiation was maximum (70%) in MS + 0.55 $\mu$ M IAA medium.

The shoots from different media were transferred for root initiation and development in MS + 0.55 $\mu$ M IAA medium. The average days required to initiate roots varied from  $2.20 \pm 0.44$  to  $26.84 \pm 4.38$  days (Table 2, Row F; Figure 1-D). The best response was observed in shoots developed in MS + 0.55 $\mu$ M IAA medium. The plantlets that attained a height of 8cm to 9cm with good roots were transferred into vermiculite-sand mixture for hardening. Plantlets regenerated in MS + 0.55 $\mu$ M IAA medium attained the requisite height in the shortest period of time ( $5.76 \pm 1.24$  days; Table 2, Row G). Hardening for five days (Table 2, Row H; Figure 1-E) was followed with acclimatization for another ten days and then the plantlets were transferred to field conditions. Plantlets regenerated in MS + 0.55 $\mu$ M IAA medium showed maximum percentage of survival in field conditions (95.83%; Table 2, Row I; Figure 1- F).

Nodal explants obtained from axenic seedlings in the above protocol showed rapid shoot initiation in MS medium in one day. The earlier micropropagation studies reported that explants derived from green house grown plants initiated shoots in seven days in MS + 4mg/L BAP + 0.1mg/L NAA medium<sup>11</sup> and cotyledonary nodal explants of *A. moschatus* initiated shoots in MS + 0.01mg/L Thidiazuran (TDZ) in eight days<sup>13</sup>.

Medium with 1 $\mu$ M TDZ l<sup>-1</sup> was reported to initiate multiple shooting in *Hibiscus cannabinus* (Kenaf)<sup>14</sup>. Multiple shoots in *A. moschatus* were reported by Renu S. *et al.*<sup>13</sup> and Maheshwari P *et al.*<sup>11</sup>, using MS + 0.01mg/L Thidiazuran (TDZ) and MS + 4mg/L BAP + 0.1mg/L NAA respectively. Sporadic multiple shoot formation was observed in the current studies in MS + 0.55 $\mu$ M NAA + 18 $\mu$ M K;

MS + 1 $\mu$ M BAP and MS + 0.55 $\mu$ M IAA + 3 $\mu$ M BAP media. However, these shoots could not be subcultured.

The present studies also indicated that nodal explants initiated callus at the base of the shoots in the presence of increasing concentrations of cytokinins. Reports in literature indicate that explants of *A. moschatus* in medium with NAA alone, regenerated shoots accompanied with callus or root initiation<sup>11</sup>. Renu S. *et al.* reported that kinetin in the medium promoted callusing in cotyledonary nodal explants of *A. moschatus*<sup>13</sup>.

Regenerated shoots of *A. moschatus* were rooted using NAA<sup>11</sup> and IBA<sup>13</sup>. IAA has been used by other investigators to initiate roots in tissue culture<sup>16</sup>. In the present study, along with shoots regenerated in MS + 0.55 $\mu$ M IAA medium a good and strong network of roots was also induced. Furthermore, it was observed that media supplemented with NAA promoted callus induction at the base of shoots. (Table 1, Row C). Thus, IAA was considered appropriate for root induction.

The plantlets were transferred to field conditions after hardening and acclimatization in a period of 15 days.

MS medium and MS + 0.55 $\mu$ M IAA medium were very good for regeneration of plantlets. However MS + 0.55 $\mu$ M IAA medium could be chosen to be the best medium in this study considering parameters such as the number of leaves in a period of ten days, absence of callus, rate of root induction and root quality. These plantlets could also be transferred for hardening in the least possible time with the best survival in field conditions.

The ambrette seeds are economically very important for their use in perfumery. This technique would enable the production of a large number of plants, rapidly and economically, from a small sample of seeds.

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