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ORIGINAL ARTICLE

In vitro Propagation from Cotyledonary Nodes of Germinated Seedlings of *Abelmoschus moschatus*

Shejuty Shareen Lithy, Shahnaz Fatema Lisa, F. M. Safiul Azam, Shahnaz Rahman, Farjana Akter Noor, Mariz Sintaha, Alok Kumar Paul, Mohammed Rahmatullah

Department of Biotechnology & Genetic Engineering, University of Development Alternative, Dhanmondi, Dhaka-1205, Bangladesh.

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ABSTRACT

Regeneration of plantlets from cotyledonary nodes of *Abelmoschus moschatus* (Malvaceae) has been accomplished through the present research. This species is widely consumed for its edible parts- leaves, seed pods and seeds; and has extensive uses in traditional medicine. The plant contains essential oils and volatile compounds like myricetin, which possesses hypoglycemic activity. Our objective was to observe the effect of plant growth regulators *in vitro* to generate a method of propagation. We collected the mature seeds from the field and germination was carried out into petridishes following surface sterilization with 0.1% $HgCl_2$ for 6 minutes. The cotyledons of germinated seedlings were excised and inoculated onto Murashige and Skoog (MS) media containing different plant growth hormones including 6-benzylaminopurine (BAP), Kinetin (Kn), indole-3-acetic acid (IAA), α-naphthalene acetic acid (NAA), indole-3-butyric acid (IBA). After four weeks of inoculation, shoot regeneration occurred and they attained up to 3.95 cm in MS + BAP [1.0 mg/l]. Combination of BAP and Kn accelerated shoot proliferation with an average length of 5.08 cm. Multiplication of shoots (2.0 shoots/explant) was observed in MS + BAP [0.5 mg/l] + IAA [1.0 mg/l] with expanded and deep green leaves. Callogenic response was obtained when BAP and NAA was supplemented in MS medium. Roots successfully emerged when *in vitro* raised micro-shoots were separated from the clumps and culture in MS + IBA [1.0 mg/l]. The plantlets were acclimatized in to soil after proper hardening and 65% of them successfully survived after eight weeks of transplantation. This developed method of clonal propagation could play an important role in large-scale plant production of this herb and for *in vitro* conservation.

Key words: Clonal propagation, edible, myricetin, BAP + IAA, *Abelmoschus moschatus.*

Introduction

Abelmoschus moschatus (**Kasturidana** in Bengali, Ambrette in English) is an herbaceous trailing plant of tropical and subtropical countries. The plant belongs to the Malvaceae family and has edible parts. Leaves and unripe seed pods are eaten as vegetable; seeds are cooked or roasted, used for flavoring, chewed as nervine, stomachic and to sweeten the breath. Essential oils from the seeds are used in aromatherapy for the treatment of depression and anxiety. The seeds also showed antiplasmodic and aphrodisiac activity (Bown, 1995; Cornucopia, 1990; Manandhar, 2002; Grieve, 1984).

The plant parts are used in Bangladesh by traditional healers (Mollik *et al.*, 2010); used in tribal and traditional medicine of India (Jadeja and Nakar, 2010); for stomach pain and disorder in Trinidad and Tobago (Lans, 2007). Four natural volatile compounds, namely 1-(6-ethyl-3-hydroxypyridin-2-yl)ethanone (1), 1-(3-hydroxy-5,6-dimethylpyridin-2-yl)ethanone (2), 1-(3-hydroxy-6-methylpyridin-2-yl)ethanone (3), and 1-(3-hydroxy-5-methylpyridin-2-yl)ethanone (4) were isolated from the seeds of Ambrette (Du *et al.*, 2008). Myricetin, a compound found in this plant exhibited blood-glucose lowering activity (Liu *et al.*, 2006) and improved insulin sensitivity in rats (Liu *et al.*, 2007; Liu *et al.*, 2010).

Tissue culture of medicinal plants is a well established method in the context of plant biotechnology. It provides essential and innovative *in vitro* protocols to propagate medicinal plants as well as cereals, fruits, oils crops and fiber plants. It has become the most convenient and rapid way to make clones of plants from any of its parts through avoiding the natural reproductive arrangements. Such methods could assist to amplify the clonal stock of elite genotypes by enabling rapid multiplication. The technique offers a viable tool for mass

Corresponding Author: Professor Dr. Mohammed Rahmatullah, Pro-Vice Chancellor, University of Development Alternative House No. 78, Road No. 11A (new) Dhanmondi R/A, Dhaka-1205, Bangladesh. Phone: 88-02-9136285; Fax: 88-02-8157339; E-mail: rahamatm@hotmail.com

multiplication, germplasm conservation and genetic improvement of rare and endangered medicinal plants for meeting the pharmaceutical needs (Sahoo and Chand, 1998).

A. moschatus is usually propagated from its seeds but it is difficult to obtain viable seeds round the year. The seeds lose their germination viability soon after harvesting. Considering the benefits of tissue culture, we focused on the *in vitro* study of plant growth regulators to establish a protocol for clonal propagation of this species from cotyledonary segments because it avoids the chance of somaclonal variation. We anticipated that this research work will to lead conservation and exchange of germplasm, and commercial plant production as like as our pervious works on *Wedelia chinensis* (Agarwala *et al*., 2010), *Lippia javanica* (Ara *et al*., 2010), and *Stevia rebaudiana* (Sadeak *et al*., 2009).

Materials and Methods

Explants source and Preparation:

Seeds of ripped fruit were harvested from the mature *Abelmoschus moschatus* plant, grown in the Garden of Medicinal Plant at the Department of Biotechnology and Genetic Engineering, University of Development Alternative. Then they were placed for germination under laminar air flow into sterile petridishes with the addition of sterile distilled water (5 ml/petridishes, and 20 seeds/petridishes) to soak the seeds (9 cm) following a wash with running tap water for 30 minutes. The petridishes used for germination were prepared by placing two filter papers in each petriplates with a lead cover before autoclaving. Sprouted cotyledonary nodes (5-7 mm) of seedlings were excised to use as explant after 15 days of germination in petridishes. Explants were surface sterilized with 0.1% $HgCl_2$ for 6 minutes and rinsed with sterile distilled water for four times before implanting on basal Murashige and Skoog (MS) (Murashige and Skoog, 1962) medium containing different concentrations (details in Result section) of 6-benzylaminopurine (BAP), kinetin (Kn), combination of BAP and Kn, combination of BAP and indole-3-acetic acid (IAA), □-naphthalene acetic acid (NAA), indole-3-butyric acid (IBA); sucrose (3%) and agar (0.7%). The pH of the medium was adjusted to 5.8 and the medium poured into test-tubes (25 × 150 mm) and sealed with cotton plugs before autoclaving for 20 min at 121ºC and 1.05 kg/cm^2.

Culture of explants:

Sterile explants were inoculated to culture in the prepared medium and maintained at 26 ± 2ºC with a photoperiod of 12/12 h under an illumination of 40-50 μmol $m^{-2}s^{-1}$ provided by cool white fluorescence lamps.

Establishment into soil:

Acclimatization was carried out for *in vitro* raised plantlets by putting them in poybags containing garden soil and cowdung at the ratio of 2:1 to ensure proper hardening through gradual exposure to sunlight and relative humidity.

Data assembly and scrutiny

Ten explants were cultured per treatment and randomly seven from each treatment were taken for data analysis. Weekly growth observations were made from 1st to 4th week of inoculation after setup of each treatment and experimental data were recorded. The parameters were:

a. Percentage response (%).
b. Average length of shoot in cm and number of leaves/shoot.
c. Average number of multiple shoot/explant.
d. Average number of roots/shoot.

Results:

Effect of Cytokinins:

Explants responded with the tallest shoots when the MS medium was fortified with BAP (1.0 mg/l) within 4th week and attained up to 3.95cm. These shoots showed short internodes and had expanded leaves with an average of 2.75/shoot. Lower concentration of BAP (0.5 mg/l) showed the highest response (85%) in shoot regeneration, and shoot proliferation was reduced as a function of the increase of concentrations of this cytokinin [Table 1] [Figure 1]. Callogenesis was observed in every treatment of BAP at the bases of explants. The excised nodes from *in vitro* raised shoots were used as explants source for subsequent experiments.

While supplementing Kn alone in MS medium, the tested concentrations (0.5 and 1.0 mg/l) did not show positive results on regeneration. As the micro-shoots were underdeveloped, we did not carry on further experiment with this hormone. Growth of shoots was very short; leaves were too small and did not survive more than three weeks in both of the medium compositions.

Table 1: Effect of BAP (mg/l) on shoot regeneration of *A. moschatus*

MS + BAP (mg/l)	Percentage of explants responded (%)	Average length of shoot (cm) (mean±SE)	Average number of leaves/shoot (mean±SE)	Growth	Size of callus
0.5	85	1.22±0.16	1.75±0.47	++	**
1.0	57	3.95±1.95	2.75±1.31	+++	*
1.5	71	1.10±0.37	1.33±0.87	+	**

+ Poor response, ++ Good response, +++ Better response; * poor growth, ** moderate growth

Fig. 1: Shoot regeneration of *A. moschatus* in MS + BAP [1.0 mg/l].

When the combination of BAP and Kn was attempted in MS medium, it exhibited *in vitro* shoot multiplication of this species. Average multiplication was 1.11/explants with 80% response when they were cultured in MS + BAP (1.5 mg/l) + Kn (0.5 mg/l) [Figure 2]. The longest shoot (5.08 cm) was obtained in MS + BAP (1.0 mg/l) + Kn (0.5mg/l) with an average of 8.20 leaves/shoot [Table 2] after three weeks of inoculation. The shoot growth was faster than the other treatments with callus formation at the tuft of the shoot bases. We had leaf abscission in all the combinations of BAP and Kn when the propagules were maintained up to fourth week.

Table 2: Combine effect of BAP (mg/l) and Kn (mg/l) in MS medium for shoot multiplication of *A. moschatus.*

MS+Hormone (mg/l)		Percentage of explants responded (%)	Average number of multiple shoot (cm) (mean±SE)	Average length of shoot (cm) (mean±SE)	Average number of leaves (mean±SE)	Callusing	Growth
BAP	Kn						
0.5	0.5	70	0	0.90±0.07	1.57±0.52	---	++
	1.0	90	0	0.88±0.08	2.33±0.33	---	++
	1.5	50	0	0.77±0.07	2.00±0.40	---	++
	2.0	50	0	0.86±0.03	2.20±0.48	---	++
1.0	0.5	50	0.80±0.48	5.08±1.37	8.20±1.43	*	+++
	1.0	70	0	1.55±0.36	7.42±2.06	*	++
	1.5	100	0.25±0.24	2.91±0.32	7.08±0.87	---	++
	2.0	70	0	1.63±0.15	5.00±1.21	---	++
1.5	0.5	80	1.11±0.61	1.35±0.22	6.88±2.58	**	++
	1.0	80	0	0.98±0.09	3.50±0.07	**	++
	1.5	100	0	0.94±0.08	4.00±0.89	*	++
	2.0	80	0.37±0.37	0.91±011	5.00±2.69	**	++
2.0	0.5	70	0	1.07±0.15	4.00±0.84	*	++
	1.0	90	0	0.93±0.15	1.88±0.42	*	++
	1.5	60	0	0.95±0.07	2.50±0.56	---	++
	2.0	100	0	0.82±0.06	1.60±0.42	---	++

-No response, + Poor response, ++ Good response, +++ Better response; * poor growth, ** moderate growth

Effect of Cytokinin-Auxin Combination:

During this part of the experiment, we examined the effect of auxin-cytokinin (BAP-IAA) ratio on proliferation and multiplication of shoot in *A. moschatus*. Highest response on shoot proliferation was 90% in MS + BAP (0.5 mg/l) + IAA (2.0 mg/l) with profuse callogenesis [Figure 3]. In case of multiplication, combination of lower cytokinin with higher auxin showed an average of 2.0 shoots per explant. The

combinations of BAP and IAA supplemented with MS medium resulted in the best shoot growth coupled with expanded and deep green leaves in comparison with other hormones (single or combinations) tested in the entire experiment [Table 3].

Fig. 2: Effect of BAP [1.5 mg/l] and Kn [0.5 mg/l] supplemented with MS medium on shoot multiplication.

Table 3: Combine effect of BAP (mg/l) and IAA (mg/l) on shoot proliferation of *A. moschatus.*

MS + **Hormon** (mg/l)		Percentage of explants responded (%)	Average number of multiple shoot(cm) (mean±SE)	Average length of shoot (cm) (mean±SE)	Average number of leaves (mean±SE)	**Callusing**	Growth
BAP	IAA						
0.5	0.5	57	1.75±1.03	2.75±0.54	6.50±1.65	***	++
	1.0	42	2.0±0.0	3.43±1.68	6.66±0.87	**	+++
	2.0	90	0	2.81±1.12	3.88±1.08	***	++
1.0	0.5	80	0	1.36±0.31	2.50±0.94	---	++
	1.0	80	0	1.12±0.06	2.62±0.59	---	++
	1.5	70	0	0.88±0.07	2.00±0.57	---	+
	2.0	70	0	1.0±0.15	2.00±0.61	**	+
2.0	0.5	70	0	1.50±0.37	3.14±1.50	*	++
	1.0	60	0	1.11±0.08	2.16±0.90	*	++
	1.5	60	0	1.41±0.26	4.33±1.03	*	++
	2.0	60	0	1.08±0.05	2.00±0.61	*	++

-No response, + Poor response, ++ Good response, +++ Better response; * poor growth, ** moderate growth, *** best response

Fig. 3: Shoot elongation in MS + BAP [0.5 mg/l] and IAA [2.0 mg/l].

Combinations of BAP and NAA showed callogenic responses, and the growth of callus was high in all the combinations of lower cytokinin and higher auxin [Figure 4, Table 4].

Rooting:

Single micro-shoot was separated from each clump and culture in ½ MS medium containing IBA for root induction. Highest percentage of response was obtained when MS was enriched with IBA (0.5 mg/l) where average length of root was 3.0 cm [Table 5]. Roots emerged within 3 weeks of culture with an average of 8.80 roots/shoot in ½ MS + IBA (1.0 mg/l) [Figure 5].

Table 4: Combine effect of BAP (mg/l) and NAA (mg/l) on callus induction in *A. moschatus.*

MS + Hormone	Percentage of	Average length	Average number of leaves	**Callusing**	Growth

(mg/l)		explant responded	of shoot (cm)	(mean±se)		
BAP	NAA	(%)	(mean±se)			
0.5	0.5	90	0.34±0.13	0.88±0.30	***	+++
	1.0	80	0	0	***	++
1.0	0.5	100	0.34±0.20	0.70±0.69	***	+++
	1.0	70	0	0	***	++

++ Good response, +++ Better response; *** best growth

Fig. 4: Callus induction in MS + BAP [1.0 mg/l] and NAA [1.0 mg/l].

Table 5: Effect of IBA (mg/l) on root induction of *A. moschatus.*

½ MS+ IBA (mg/l)	Percentage of explants responded (%)	Average length of shoot (cm) (mean±SE)	Average number of leaves/shoot (mean±SE)	Average no of root (mean±SE)	Average length of root (mean±SE)	Growth
0.5	100	3.94±1.17	5.00±0.07	6.00±1.58	3.00±0.69	+++
1.0	80	3.44±0.32	4.20±0.37	8.80±1.28	1.23±0.21	++
1.5	40	2.68±0.26	4.40±1.07	1.0±0.63	1.10±0.18	+

+ Poor response, ++ Good response, +++ Better response

Fig. 5: Effect of IBA [0.5 mg/l] enriched with MS medium on *in vitro* root induction of *A. moschatus*.

After successful rooting, the plantlets were transplanted into soil with proper hardening and acclimatization. We observed 65% survival rate after eight weeks of field observation and no morphological variation was seen in these plants.

Discussion:

BAP has an effective role in shoot survival and for the development of axillary shoots from aseptically germinated seedlings *in vitro*. Its effectiveness has been reported in varying tissues from juvenile to mature in several species like *Acacia mangium* (Ahmed, 1989), *Bougainvillea glabra* (Sharma *et al.,* 1981), *Primus serotina* (Tricoli *et al.,* 1985), *Eucalyptus* spp. (Gupta *et al.,* 1981), *Paederia foetida* (Alam *et al.*, 2010), *Artocarpus heterophyllus* (Azam *et al.,* 2009), and *Centella asiatica* (Singh *et al.,* 1999). The effectiveness of BAP on shoot regeneration was also observed in the present study. Use of cotyledon for direct shoot organogenesis was demonstrated by the present study, which was also described previously by Chen *et al.* (2010), Donaldson and Simmonds (2000), Shyamkumar *et al.* (2003), Jha *et al.* (2004), and Jeyakumar and Jayabalan (2002).

Sharma and Shahzad (2008), Ara *et al.* (2010) reported that the combination of cytokinins was useful for multiplication of *A. moschatus* and *L. javanica,* respectively. Our present results also revealed that BAP and Kn has effect on multiple shoot proliferation. In *Acacia seyal*, Al-Wasel (2000) reported that the combination of

auxin-cytokinin could exhibit shoot multiplication which was in agreement with results of our present experiment.

The results obtained by Sharma and Shahzad (2008) are in agreement for employing IBA for root induction in this species. Use of IBA in rooting media has been reported by Azam *et al.* (2010) and Alam *et al.* (2010) in *Musa sp.* and *P. foetida*, respectively. The hypothesis on root induction by IBA has been validated in the present study.

Conclusion:

The defined protocol here demonstrates the feasibility of *in vitro* clonal propagation of *A. moschatus* which could play a potentially excellent role for conservation of this plant's genetic resource, as well as serve to make plants available for pharmaceutics and additives, and large-scale plantation.

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