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MICROPROPAGATION

Synergistic effect of cytokinins and auxins enables mass clonal multiplication of drumstick tree (*Moringa oleifera* Lam.): a wonder

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Abstract

The synergistic effect of plant growth regulators on axillary bud proliferation for mass clonal multiplication of *Moringa oleifera* Lam. (vern. drumstick) has been assessed for the first time. Treatment of decoated seeds with 1% (*w/v*) Bavistin for 60 min, 0.33% (*w/v*) streptocycline for 30 min, and 0.1% (*w/v*) $HgCl_2$ for 3.5 min resulted in complete removal of the surface contaminants. Maximum seed germination (89.13%) was obtained on quarter-strength Murashige & Skoog (MS) medium. Culture of nodal segments on MS + 6-benzyladenine (BA) at 3 mg L^{-1} resulted in multiple shoot proliferation with ~ 18 shoots per explant. All combinations of indole-3-acetic acid (IAA) + kinetin (Kn) resulted in elongated shoots, while only lower concentrations of BA (0.5 mg L^{-1}), along with IAA (0.5 to 2 mg L^{-1}), or Kn (0.5 to 5 mg L^{-1}), showed significant synergy in the shoot morphogenesis. In addition, the maximum (100%) rooting efficiency was attained on half-strength MS medium supplemented with different concentrations of IAA and indole-3-butyric acid (IBA). The rooted plants were successfully established in the greenhouse for acclimatization. Clonality of the raised plants was assessed using 15 random primers of Operon® technologies (OPT and OPF series), and eight primers resulted in significant amplification with distinct, identical, and reproducible bands that confirmed clonality of the micropropagated plants. The present study provides a comprehensive analysis of the synergistic effect of plant growth regulators (PGRs) on *in vitro* shoot regeneration and proliferation for clonal mass multiplication disease-free plantlets, which can be utilized to maximize the yield of healthy and genetically identical plants of drumstick tree, which is considered to be a miracle multipurpose tree.

Keywords Micropropagation · Growth hormones · Hardening · Acclimatization · Clonal fidelity · RAPD

Introduction

Moringa oleifera Lam. (Family: Moringaceae), commonly known as drumstick tree, has been widely used in food, pharmacy, and other economic utilities including bioremediation, water purification, and sustainable agricultural management (Gupta *et al* 2018). Drumstick tree is considered as a unique plant species that accommodates an exceptionally diverse nutrient and metabolite profile, which is rare to find in other plants (Razis *et al* 2014). For this reason, the plant has received unprecedented attention as the 'miracle tree' that is capable of providing complete nutrition as a natural source of nutraceuticals and food supplements.

Due to the multiple applications, drumstick tree has been overexploited in past decades, causing a potential threat to the long-term existence of its wild types, which requires conservation. Usually, stem cuttings are used for propagation of drumstick tree, but this method reduces the growth and yield of the plant (Islam *et al* 2005). The tree is also highly susceptible to insect infestations and fungal infections that lead to foliar and floral damage, which deteriorates yield and makes the plant unfit for preparation of any medicinal formulation.

Plant tissue culture is an excellent tool for both conservation and regeneration of infection-free plants. This technique serves as an efficient alternative to the traditional propagation strategies. The method also enables year-round maintenance and availability of plants and plant products to meet the industrial demands, using clonal mass multiplication of plants in a limited space and time (Jain *et al* 2012; Gupta *et al* 2018; Hu *et al* 2019). Micropropagation in drumstick tree has been

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reported by several researchers using different explants, but the shoot multiplication is limited (Förster *et al* 2013; Avila-Treviño *et al* 2017). Direct organogenesis from leaf explants has been reported by Jun-jie *et al* (2017). Vitrification in the regenerated shoots is a common problem in micropropagation of drumstick tree, and Hassanein *et al* (2018) reported the use of silver nitrate and salicylic acid in the culture medium for long-term maintenance of healthy shoots without any vitrification. Despite these results, studies to develop an efficient protocol for mass regeneration of genetically identical plants of drumstick tree are still limited. Therefore, in the present study, the effect of different cytokinins and auxins on shoot bud proliferation has been evaluated. Synergism among different combinations of plant growth regulators (PGRs), and their effect on shoot multiplication and elongation, and root induction/formation has also been evaluated. Micropropagated plantlets were subjected to acclimatization and hardening for maximum survival during field transfer. In addition, the regenerated plants were assessed for their clonality using randomly amplified polymorphic DNA (RAPD) markers to deliver maximum reproducibility.

Materials and Methods

Collection of plant material: Drumstick and nodal segments were collected from *Moringa oleifera* trees growing in the natural habitat at Jaipur, Rajasthan. Seeds harvested from the pods were decoated prior to any treatment. Decoated seeds and nodes were thoroughly washed under running tap water for 10 min, followed by rinsing with 5% (*v/v*) Tween®-20 (liquid detergent, HiMedia, Mumbai, India), for 5 min, and washing under running tap water to ensure complete removal of detergent and other contaminants.

Optimization of sterilization method: The plant is known to be susceptible to various infections of flies, aphids, and other insects; therefore, the explants collected from the wild were subjected to treatment with microbicidal agents prior to surface sterilization in the aseptic conditions. Surface sterilization was done with mercuric chloride (HiMedia) solution (0.1%, *w/v*) for 2 to 4 min. Explants treated with $HgCl_2$ alone for 2 to 4 min were used as the control. Each treatment step followed thorough washing of the explants with sterile distilled water. Different combinations of microbicidal agents used in the sterilization step are summarized in Table 1.

Culture media and growth conditions: All of the micropropagation experiments in the present study were completed on Murashige and Skoog (MS) culture medium (Murashige and Skoog 1962), which contained 3% (*w/v*) sucrose (HiMedia) and 0.8% (*w/v*) plant culture tested agar (HiMedia), and the pH was maintained at 5.8 ± 0.05. All the PGRs used in the study were obtained from Sigma-Aldrich®, Bangaluru, India. Explants for shoot multiplication were inoculated in Erlenmeyer flasks (100 mL, Borosil, Mumbai, India), which contained about 40 mL media, and those for rooting were transferred to boiling tubes (32 × 200 mm, 100 mL, Borosil), which contained 50 mL media in each. The culture media was sterilized by autoclaving at 15 psi, pressure and 121 °C for 20 min.

All of the cultures were maintained in growth chambers at 25 ± 1°C with 85% relative humidity, and a 16/8 h photoperiod with an illumination of 25 μmol m^{-2} s^{-1} photosynthetic photon flux density.

Establishment of primary cultures: Decoated seeds were inoculated in 20 replicates of each on one-fourth and half-strength MS medium, both containing 3% sucrose (*w/v*) followed by incubation in the dark until germination. The optimum medium for *in vitro* seed germination was identified by recording the number of seeds germinated on both media. The germinated seeds were decapitated and transferred to MS medium supplemented with varying concentrations (0.5 to 5 mg L^{-1}) of different PGRs and incubated in the growth chamber.

Effect of different plant growth regulators: All the growth hormones used were procured from Duchefa Biochemie, Haarlem, Netherlands. Nodal segments derived from *in vitro* germinated seeds were inoculated on MS medium supplemented with 6-benzyladenine (BA), Kinetin (Kn), 6-(γ,γ-Dimethylallylamino)purine (2iP), 1-Naphthaleneacetic acid (NAA), indole-3-acetic acid (IAA), and indole-3-butyric acid (IBA) alone, with a concentration range of 0.5 to 5 mg L^{-1}. Combinations of auxins and cytokinins (BA + Kn, BA + IAA, Kn + IAA) were also used for axillary shoot regeneration. Combinations were prepared by varying the concentration of one hormone (0.5 to 5 mg L^{-1}), while keeping the other hormone concentrations constant.

Five flasks with four explants in each were maintained in a culture room, and the number of responding explants, number of shoots per explant, and shoot length (cm) was recorded for every hormone concentration. The observations were made after 4 wk of incubation.

Rooting of *in vitro*-regenerated shoots: Healthy elongated shoots (> 2.5 cm) were transferred to half-strength MS basal medium supplemented with IBA and IAA at different concentrations (0.1 and 0.2 mg L^{-1}), either alone or in combinations. Ten replicates for each type of media were maintained in the growth chamber with previously mentioned culture conditions. The number of rooted plants (% response) and number of roots per shoot along with the average root length, shoot length, and number of nodes induced on each rooting medium after 14 and 28 d of incubation was recorded.

Table 1. Contamination response of *Moringa oleifera* under different surface sterilization procedures

Treatment	Bavistin		Streptocycline		Ethanol		Bleach		$HgCl_2$		% contamination	Control $HgCl_2$ (0.1%, *w/v*) alone	
	Conc. (%, w/v)	Time (min)	Conc. (%, w/v)	Time (min)	Conc. (%, *v*/v)	Time (sec)	Conc. (%, v/v)	Time (min)	Conc. (%, w/v)	Time (min)		Time (min)	% contamination
Decoated seeds													
T1	1	30	0.25	30	–		–		0.1	2	90	2	100
T2	1	30	0.25	30	–		–		0.1	3	100		
T3	1	60	0.33	30	–		–		0.1	2.5	12.5	2.5	100
T4	1	60	0.33	30	–		–		0.1	3	18.75	3	100
T5	1	60	0.33	30	–		–		0.1	3.5	0		
(A) Mature nodal segments													
T6	0.1	45	–		–		–		0.1	5	100	3	100
T7	0.1	45	–		–		–		0.1	3	100		
T8	–		–		70	30	–		0.1	2.5	100	3.5	100
T9	2.5	40	–		70	30	–		0.1	2.5	100	4	100
T10	1	60	0.25	60	–		–		0.1	4	0		
(B) Immature nodal segments													
T11	0.1	45	–		–		–		0.1	3	100	2	100
T12	0.1	45	–		–		–		0.1	2.5	100		
T13	2.5	40	–		–		–		0.1	2.5	100	2.5	100
T14	–		–		50	60	–		0.1	2.5	100		
T15	1	30	0.2	30	–		25	5	0.1	2.5	20	3	100
T16	1	60	0.25	60	–		–		0.1	3	0		

Hardening and acclimatization of rooted plantlets: Rooted plantlets were carefully removed from the culture vessels to ensure there was not any damage to the plantlets. The roots were gently washed with tap water for complete removal of medium remnants, and the plants were potted in small pots containing a mixture of garden soil-organic mixture (Tree House, Jaipur, India) (2:1, *w/w*). To maintain maximum humidity, the pots were covered with transparent plastic bags and kept for primary hardening in green house. The growth of the plantlets was recorded every week. After 1 mo of primary hardening, the plants were subjected to secondary hardening. Holes in the plastic bags were made at regular intervals, followed by complete removal of bags after 30 d. Plants were monitored for their growth in the secondary hardening, and the number of plants that survived both primary and secondary hardening were recorded.

Data collection and analysis: The data was statistically analyzed using one-way analysis of variance (ANOVA) to test the significance among the observations recorded. Fisher's least significant difference (LSD) test was used to identify significantly different means. All the tests were conducted at the 5% level of significance ($P < 0.05$).

Clonal fidelity analysis using RAPD markers: The genetic similarity among the regenerated plantlets was assessed using RAPD markers. DNA was extracted from 16 randomly selected micropropagated plantlets by the cetyl trimethylammonium bromide (CTAB) method (Doyle 1990). A total of 15 random primers belonging to T and F Operon® series (Operon Technologies, Alameda, CA), were tested to ascertain the clonal fidelity of the plantlets. The polymerase chain reaction (PCR) was carried out in a total reaction volume of 25 μL, which contained the DNA sample, 2.5 μL of 10X Taq Buffer B, 200 μM dNTPS each, 2.5 mM $MgCl_2$, 0.75 U of Taq polymerase (Bangalore Genei, Bangaluru, India), 0.8 μM primer, and deionized water (Merck Millipore, Bangaluru, India). The PCR cycle was performed in C1000 Touch™ Thermal Cycler (Bio-Rad, Dalkeith, UK), and the amplification conditions were as follows: initial denaturation at 95°C for 5 min followed by 35 cycles of 95°C for 60 s, 37°C for 45 s, and 72°C for 45 s, with a final extension at 72 °C for 5 min. The amplicons were separated using 1.2% (*w/v*) agarose (Himedia), with horizontal gel electrophoresis (Bangalore Genei). The gel was stained with ethidium bromide (Himedia) and photographed using the Gel Documentation System (Gel Doc XR+ Imager, Bio-Rad).

Results and Discussions

Effect of sterilization methods: Different treatments were used to sterilize the decoated seeds, from which T5 was found to be the most effective disinfection method compared to the other treatments (Table 1). The combination of Bavistin and Streptocycline significantly reduced the contamination from the decoated seeds. Pre-treatment with fungicide and antibiotic reduced microbial load from the explant and enhanced the efficiency of $HgCl_2$. The seeds remain in direct contact with environment, which increases the susceptibility to microbial attack, and removal of the seed coats prior to surface sterilization significantly reduced the endophytic infection. Decoating the seeds for complete removal of contaminants was also reported in *Moringa* by Avila-Treviño *et al* (2017) and Hassanein *et al* (2018).

Nodal segments excised from field-grown trees were divided in two groups, (A) mature, and (B) immature nodal segments. Out of all treatment regimes, T10 reduced the contamination significantly in 'group A,' and T15 and T16 aided in significant reduction of contamination (< 20%) in 'group B' (Table 1). Although the contamination was significantly reduced in nodal segments, the prolonged treatment from disinfectants caused browning followed by death of explant tissues, which in turn resulted in reduced bud break. Förster *et al* (2013) also reported lower survival rates and response of nodes after surface sterilization. Therefore, due to lower survival rates (< 40%) of disinfected nodes, *in vitro*-germinated seedlings were used as the explant source for the present study.

Primary culture: Primary cultures were established using *in vitro* germination of the decoated seeds. The decoated seeds cultured on one-fourth strength MS basal medium that contained 3% (*w/v*) sucrose showed the maximum germination efficiency of 89.13%. The seed germination percentage was greatly reduced (58.26%), on half-strength MS basal medium and 3% (*w/v*) sucrose. In previous studies, a germination frequency as high as 84% was reported on MS basal medium and filter paper beds (Stephenson and Fahey 2004; Steinitz *et al* 2009; Avila-Treviño *et al* 2017).

Germinated seedlings were inoculated on MS medium supplemented with different PGRs. Apart from cotyledonary node elongation, multiple shoot proliferation from the decapitated seeds inoculated on MS medium supplemented with different concentrations of cytokinins and auxins was also observed (Table 2, Fig. 1 *a*, *b*). Shoot multiplication from decapitated seeds of *Moringa* was also reported previously, with a maximum regeneration of six shoots (Steinitz *et al* 2009), which affirmed the tendency of *Moringa* seeds to regenerate multiple shoot after decapitation. Shoot morphogenesis from decapitated seedlings can occur either from meristems in the axil of the cotyledonary node or through *de novo* apical shoot meristems. Similar conclusions were also drawn by Avila-Treviño *et al* (2017), after the apical shoot was removed from the seedlings of drumstick.

Effect of cytokinins on shoot bud multiplication: Nodal segments derived from *in vitro* regenerated shoots were

Table 2. Effect of different PGRs on *Moringa oleifera* Lam. multiple shoot morphogenesis from decapitated seeds

MS medium supplemented with PGRs (in mg L^{-1})	% response	No. of shoots produced
BA (0.5)	25	10
Kn (3)	75	15
2iP (5)	33.33	10
IAA (5)	33.33	3
NAA (1)	33.33	6
NAA (3)	66.67	3
IBA (0.5)	33.33	6

BA 6-benzyladenine; *Kn* Kinetin; *2iP* 6-(γ,γ-Dimethylallylamino)purine; *NAA* 1-Naphthaleneacetic acid; *IAA* indole-3-acetic acid; *IBA* indole-3-butyric acid

inoculated on MS medium fortified with various concentrations (0.5 to 5 mg L^{-1}) of BA, Kn, and 2iP. Multiple shoot morphogenesis was observed on all the concentrations of BA and Kn except at 5 mg L^{-1} Kn, whereas, there was no significant shoot multiplication on any of the concentrations of 2iP (Table 3).

Shoot multiplication from nodal segments followed swelling and callusing at the cut end at lower concentrations of BA (0.5, 1, and 2 mg L^{-1}) (Fig. 1*c*), while at high BA concentrations (3 and 5 mg L^{-1}), prominent shoot proliferation through callus was observed (Fig. 1*d*). The maximum response (88.33 ± 7.26%) was observed on MS + 0.5 mg L^{-1} BA and declined with increased concentrations of the hormone. At higher BA levels (3 mg L^{-1}), indirect organogenesis was observed with a maximum of 17.62 ± 2.35 shoot buds per explant, but the shoot buds were not able to elongate. Regeneration of dwarf shoots (< 1 cm) from callus can be attributed to the differential signal transduction of the endogenous growth hormone(s) and due to the presence of massive intervening callus (Rasool *et al* 2013). Direct regeneration of multiple shoots using nodal explants in drumstick trees has been reported by several researchers (Islam *et al* 2005), but indirect organogenesis using BA has been addressed for the first time in the present study.

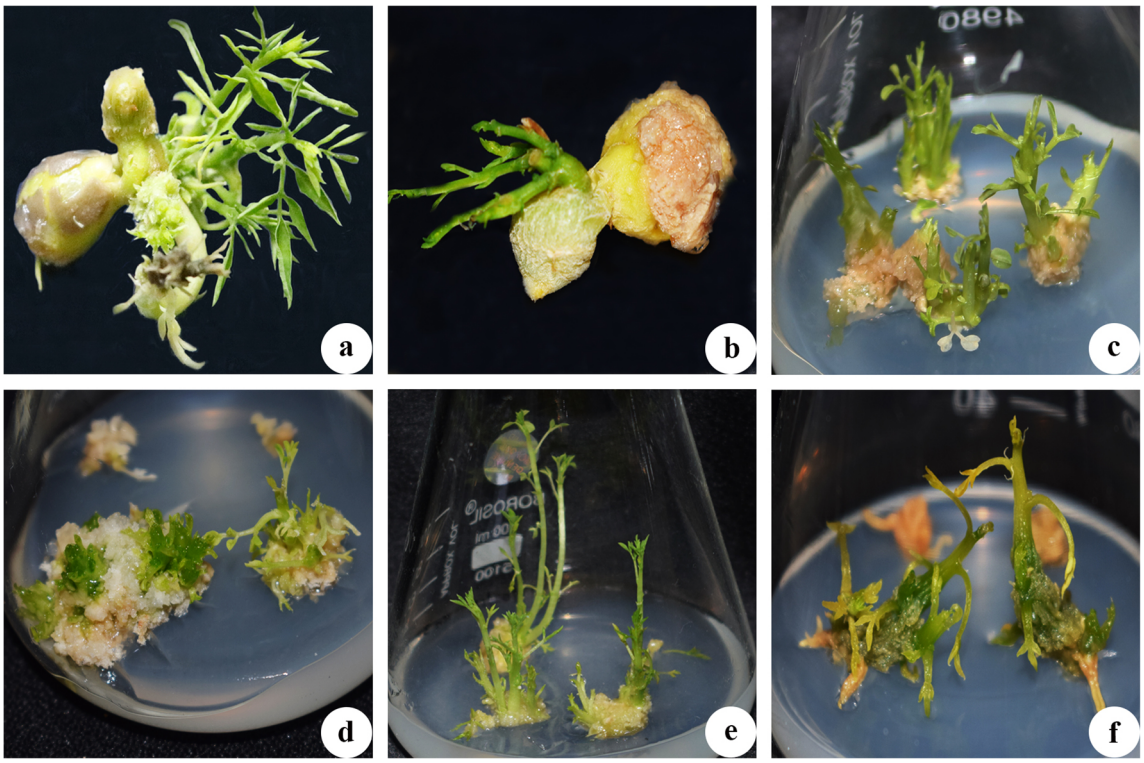

Figure 1. Multiple shoot proliferation/regeneration from decapitated *Moringa oleifera* Lam. seeds on Murashige and Skoog (MS) medium supplemented with (*a*) 6-benzyladenine (1 mg L^{-1}), (*b*) 1-Naphthaleneacetic acid (1 mg L^{-1}), (*c*) Shoot bud proliferation from cultured nodal segments on MS + BA (1 mg L^{-1}), (*d*) Shoot bud proliferation through callus on MS + BA (3 mg L^{-1}), (*e*) Shoot elongation on MS + Kinetin (3 mg L^{-1}), and (*f*) Multiple shoots from nodal segments on MS + Indole-3-acetic acid (1 mg L^{-1}).

Table 3. Effect on axillary bud proliferation from nodal segments of *Moringa oleifera* Lam. cultured on Murashige and Skoog (MS) medium supplemented with different cytokinins and auxins

MS medium supplemented with PGRs (in mg L^{-1})						% Response ± SE#	Avg. no. of shoots per explant ± SE#	Avg. shoot length** ± SE#	Avg. no. of nodes per shoot ± SE#
BA	Kn	2-iP	IAA	NAA	IBA				
–	–	–	–	–	–	83.33 ± 10.54	NM	5.81 ± 0.51	7.63 ± 0.41
0.5	–	–	–	–	–	88.33 ± 7.26	$7 \pm 0.45^{f\text{-}y*}$	NM	NM
1	–	–	–	–	–	82.40 ± 5.60	$8.85 \pm 0.82^{u\text{-}z,\alpha\text{-}\varepsilon}$	NM	NM
2	–	–	–	–	–	80.14 ± 11.08	$12.09 \pm 1.92^{\tau\eta}$	NM	NM
3	–	–	–	–	–	75.87 ± 8.70	$17.62 \pm 2.35^{\lambda}$	NM	NM
5	–	–	–	–	–	74.67 ± 8.86	$13.92 \pm 1.04^{\eta\text{-}\kappa}$	NM	NM
–	0.5	–	–	–	–	84.45 ± 7.54	$4.89 \pm 0.77^{a\text{-}t}$	$3.7 \pm 0.7^{a\text{-}f}$	$5.8 \pm 1.7^{a\text{-}p}$
–	1	–	–	–	–	100 ± 0	2.14 ± 0.26^{a}	$5.4 \pm 0.6^{e\text{-}m,p}$	$8 \pm 0.7^{r\text{-}t}$
–	2	–	–	–	–	69.44 ± 19.45	$3.4 \pm 0.4^{a\text{-}n,pt}$	$4.4 \pm 0.6^{a\text{-}h}$	6.5 ± 0.5
–	3	–	–	–	–	90 ± 10	$7.67 \pm 1.86^{m\text{-}z,\alpha\text{-}\tau}$	3.1 ± 0.5^{ab}	$5.8 \pm 0.5^{a\text{-}p}$
–	5	–	–	–	–	64 ± 15.68	NM	$4.0 \pm 0.3^{a\text{-}e}$	4.8 ± 0.8^{abd}
–	–	0.5	–	–	–	75 ± 14.43	NM	2.5 ± 0.96	6.33 ± 1.86
–	–	1	–	–	–	91.67 ± 8.34	NM	NM	NM
–	–	2	–	–	–	56.67 ± 12.02	NM	NM	NM
–	–	3	–	–	–	82.5 ± 11.8	$4.6 \pm 0.93^{a\text{-}t,w}$	3.33 ± 0.33	6 ± 0
–	–	5	–	–	–	66.67 ± 33.34	NM	NM	NM
–	–	–	0.5	–	–	93.75 ± 6.25	$3.2 \pm 0.37^{a\text{-}j,mnpt}$	$5.9 \pm 0.5^{g\text{-}p}$	4.7 ± 0.5^{a}
–	–	–	1	–	–	75 ± 14.43	$10.57 \pm 1.53^{\alpha\text{-}\eta}$	NM	NM
–	–	–	2	–	–	100 ± 0	$11.5 \pm 1.32^{\varepsilon\text{-}\theta}$	$5.2 \pm 0.5^{d\text{-}k,mp}$	$5.5 \pm 0.6^{a\text{-}l,p}$
–	–	–	3	–	–	52.78 ± 23.73	$11.33 \pm 1.76^{\delta\text{-}\eta}$	NM	NM
–	–	–	5	–	–	87.5 ± 12.5	$7.25 \pm 0.25^{i\text{-}z*\alpha\text{-}\varepsilon}$	9.3 ± 1.3^{r}	9.7 ± 1.3^{t}
–	–	–	–	0.5	–	NM	NM	NM	NM
–	–	–	–	1	–	66.67 ± 19.25	NM	NM	NM
–	–	–	–	2	–	NM	NM	NM	NM
–	–	–	–	3	–	83.34 ± 16.67	NM	NM	NM
–	–	–	–	5	–	NM	NM	NM	NM
–	–	–	–	–	0.5	77.78 ± 22.22	NM	NM	NM
–	–	–	–	–	1	NM	NM	NM	NM
–	–	–	–	–	2	100 ± 0	NM	NM	NM
–	–	–	–	–	3	NM	NM	NM	NM
–	–	–	–	–	5	87.5 ± 12.5	NM	NM	NM
0.5	–	–	0.5	–	–	100 ± 0	$6 \pm 1.29^{a\text{-}x}$	2.2 ± 0.1	4 ± 0.2
	–	–	1	–	–	87.5 ± 12.5	$6.85 \pm 0.82^{e\text{-}y*}$	2.3 ± 0.2	3.8 ± 0.4
	–	–	2	–	–	90 ± 6.12	$6.29 \pm 0.95^{e\text{-}w*}$	2.2 ± 0.1	4 ± 0.3
	–	–	3	–	–	87.5 ± 12.5	$6 \pm 1.08^{a\text{-}z,\alpha\beta}$	2.4 ± 0.2	4.9 ± 0.4
	–	–	5	–	–	90 ± 6.12	$7.38 \pm 1.05^{k\text{-}z\varepsilon}$	2.4 ± 0.2	3.7 ± 0.1
1	–	–	0.5	–	–	85 ± 10	$8.13 \pm 0.93^{r\text{-}z,\alpha\delta\varepsilon}$	NM	NM
	–	–	1	–	–	75 ± 7.91	$7.43 \pm 0.95^{l\text{-}z\varepsilon}$	NM	NM
	–	–	2	–	–	85 ± 10	$6.5 \pm 0.87^{d\text{-}w*}$	NM	NM
	–	–	3	–	–	95 ± 5	$7.12 \pm 0.63^{h\text{-}y*}$	NM	NM
	–	–	5	–	–	88.33 ± 7.26	$7.1 \pm 0.62^{g\text{-}y*}$	NM	NM
2	–	–	0.5	–	–	87.5 ± 7.22	$8.09 \pm 0.99^{q\text{-}z,\alpha\delta\varepsilon}$	NM	NM
	–	–	1	–	–	87.5 ± 7.22	$9.83 \pm 1.17^{y\text{-}z,\alpha\text{-}\tau}$	NM	NM
	–	–	2	–	–	66.67 ± 22.05	$7.33 \pm 0.33^{a,k\text{-}z*\varepsilon}$	NM	NM
	–	–	3	–	–	93.75 ± 6.25	$9.13 \pm 0.67^{v\text{-}z,\alpha\text{-}\tau}$	NM	NM
	–	–	5	–	–	83.75 ± 9.87	$7.3 \pm 1.01^{j\text{-}z*\varepsilon}$	NM	NM

Table 3. (continued)

MS medium supplemented with PGRs (in mg L^{-1})						% Response ± SE[#]	Avg. no. of shoots per explant ± SE[#]	Avg. shoot length** ± SE[#]	Avg. no. of nodes per shoot ± SE[#]
BA	Kn	2-iP	IAA	NAA	IBA				
3	–	–	0.5	–	–	66 ± 12.98	$10 \pm 1.12^{z,\alpha\text{-}\tau}$	NM	NM
	–	–	1	–	–	78 ± 11.02	$9.47 \pm 0.76^{x\text{-}z,\alpha\text{-}\tau}$	NM	NM
	–	–	2	–	–	85 ± 15	$8.18 \pm 1.13^{s\text{-}z,\alpha\delta\varepsilon}$	NM	NM
	–	–	3	–	–	75 ± 13.69	$7.91 \pm 1.31^{o\text{-}z,\alpha\delta\varepsilon}$	NM	NM
	–	–	5	–	–	75 ± 14.43	$8 \pm 2^{p\text{-}z,\alpha\text{-}\tau}$	NM	NM
5	–	–	0.5	–	–	51.25 ± 16.63	$7.71 \pm 1.44^{n\text{-}z,\alpha\gamma\varepsilon}$	NM	NM
	–	–	1	–	–	68.75 ± 17.12	$6.25 \pm 1.01^{b\text{-}x*}$	NM	NM
	–	–	2	–	–	55.42 ± 9.06	$9.14 \pm 0.63^{w\text{-}z,\alpha\text{-}\tau}$	NM	NM
	–	–	3	–	–	39.58 ± 7.47	$8.67 \pm 0.33^{t\text{-}z,\alpha\text{-}\tau}$	NM	NM
	–	–	5	–	–	58.33 ± 9.28	$6.13 \pm 1.03^{a\text{-}x}$	NM	NM
–	0.5	–	0.5	–	–	95 ± 5	NM	$3.8 \pm 0.7^{a\text{-}e}$	4.8 ± 0.7^{abd}
–	1	–		–	–	84 ± 9.8	NM	$4.3 \pm 0.5^{b\text{-}e}$	$6.2 \pm 0.4^{b\text{-}p}$
–	2	–		–	–	92 ± 4.9	NM	3.2 ± 0.2^{ab}	$5.4 \pm 0.4^{a\text{-}d,ij}$
–	3	–		–	–	92.7 ± 4.5	NM	3.1 ± 0.2^{ab}	5.1 ± 0.3^{ad}
–	5	–		–	–	100 ± 0	NM	3.4 ± 0.3^{ab}	5.1 ± 0.3^{ad}
–	0.5	–	1	–	–	95 ± 5	NM	$3.9 \pm 0.3^{a\text{-}e}$	$6.6 \pm 0.2^{f\text{-}r}$
–	1	–		–	–	92 ± 8	NM	$5 \pm 0.3^{c\text{-}h}$	$7.3 \pm 0.3^{o\text{-}r}$
–	2	–		–	–	100 ± 0	NM	3.1 ± 0.4^{ab}	$5.6 \pm 0.5^{a\text{-}j}$
–	3	–		–	–	82.9 ± 10.2	NM	2.5 ± 0.3^{ab}	$6.4 \pm 0.2^{d\text{-}r}$
–	5	–		–	–	100 ± 0	NM	2.1 ± 0.1^{a}	$5 \pm 0.3^{a\text{-}d,ij}$
–	0.5	–	2	–	–	81 ± 4.8	NM	$6.8 \pm 0.8^{j\text{-}q}$	$6.9 \pm 0.4^{h\text{-}r}$
–	1	–		–	–	87 ± 5.4	NM	$6.6 \pm 0.5^{i\text{-}p}$	$6.6 \pm 0.5^{g\text{-}r}$
–	2	–		–	–	100 ± 0	NM	$5.8 \pm 0.5^{f\text{-}p}$	$7.1 \pm 0.4^{l\text{-}r}$
–	3	–		–	–	100 ± 0	NM	$7 \pm 0.6^{l\text{-}q}$	$7.2 \pm 0.5^{l\text{-}r}$
–	5	–		–	–	95 ± 5	NM	$7 \pm 0.5^{k\text{-}q}$	$7.1 \pm 0.3^{k\text{-}r}$
–	0.5	–	3	–	–	91 ± 5.6	NM	8 ± 0.6^{qr}	$7.7 \pm 0.4^{q\text{-}s}$
–	1	–		–	–	69 ± 8.7	NM	$7.1 \pm 0.5^{n\text{-}q}$	$7.2 \pm 0.2^{n\text{-}r}$
–	2	–		–	–	77 ± 10.2	NM	$6.2 \pm 0.5^{h\text{-}p}$	$6.6 \pm 0.3^{e\text{-}p}$
–	3	–		–	–	91.7 ± 8.3	NM	$7.2 \pm 0.7^{o\text{-}q}$	8.5 ± 0.3st
–	5	–		–	–	80 ± 11.5	NM	$7.1 \pm 0.8^{m\text{-}q}$	$7.3 \pm 0.7^{p\text{-}s}$
0.5	0.5	–	–	–	–	100 ± 0	$4.4 \pm 0.37^{a\text{-}e,ijmnpt}$	2.8 ± 0.2^{ab}	$6.4 \pm 0.3^{c\text{-}p}$
	1	–	–	–	–	78.57 ± 10.53	$4.16 \pm 0.3^{a\text{-}e,impt}$	$2.7 \pm 0.3^{a\text{-}d}$	$7 \pm 1^{i\text{-}r}$
	2	–	–	–	–	83.93 ± 11.8	$4.31 \pm 0.41^{a\text{-}e,ijmnpt}$	$3 \pm 0.6^{a\text{-}e}$	$7 \pm 0^{j\text{-}r}$
	3	–	–	–	–	68.75 ± 11.97	$4.20 \pm 0.41^{a\text{-}n,pt}$	2.8 ± 0.3^{ab}	$5.7 \pm 0.3^{a\text{-}p}$
	5	–	–	–	–	80.95 ± 5.15	$3.75 \pm 0.37^{a\text{-}d,impt}$	$3.4 \pm 0.2^{a\text{-}e}$	$6 \pm 0.8^{a\text{-}p}$
1	0.5	–	–	–	–	93.33 ± 6.67	$15.5 \pm 1.84^{r\text{-}z,\alpha\delta\varepsilon}$	NM	NM
	1	–	–	–	–	100 ± 0	$17.36 \pm 2.32^{l\text{-}z\varepsilon}$	NM	NM
	2	–	–	–	–	100 ± 0	$16.33 \pm 2.2^{d\text{-}w*}$	NM	NM
	3	–	–	–	–	100 ± 0	$11.21 \pm 1.11^{h\text{-}y*}$	NM	NM
	5	–	–	–	–	86.67 ± 8.16	$11.24 \pm 1.21^{g\text{-}y*}$	NM	NM

[#] Different *letters* in *superscript* indicate significantly different values at $P < 0.05$;

**Shoot length was recorded only for > 1 cm long shoots;

NM – Not measured

BA 6-benzyladenine; *Kn* Kinetin; *2iP* 6-(γ,γ-Dimethylallylamino)purine; *NAA* 1-Naphthaleneacetic acid; *IAA* indole-3-acetic acid; *IBA* indole-3-butyric acid

Nodal segments cultured on MS + Kn (0.5 to 5 mg L^{-1}) showed both shoot multiplication and elongation (Fig. 1*e*) with a lower shoot count (Table 3). The nonsignificant regeneration pattern indicated differential signaling of BA and Kn during morphogenesis. Use of Kn alone for shoot multiplication was not preferred by earlier researchers in medicinal herbs and trees including drumstick (Takayama and Misawa 1982; Jain *et al* 2009; Jun-jie *et al* 2017) due to its lower regeneration potential. The effect of another cytokinin (2iP) was also studied on the nodal explants and 2iP did not show any significant shoot proliferation or elongation, compared to BA and Kn (Table 3).

Although a range of responses was observed with each of the cytokinins used alone, none of the hormone regimes resulted in optimum shoot growth and development, and different combinations of cytokinins (BA (0.5 to 5 mg L^{-1}) + Kn (0.5 to 5 mg L^{-1})) were also tested.

Effect of auxins on shoot bud regeneration: Explants cultured on MS medium supplemented with IAA (0.5 to 5 mg L^{-1}) showed two types of responses: (*i*) formation of new shoot buds from the node and (*ii*) elongation of the shoots regenerated from node (Fig. 1*f*). The shoots regenerated on IAA were vitrified and responded well after subculture on the same medium. The maximum number of shoots (11.5 ± 1.32) was obtained on 2 mg L^{-1} IAA (Table 3), with a little or reduced callus at the base. Higher IAA concentration (5 mg L^{-1}) facilitated elongation of shoots with an average shoot length of 9.27 ± 1.32 cm and 9.67 ± 1.28 for the average number of nodes per shoot (Table 3).

The average shoot length at lower concentrations of IAA (0.5 to 2 mg L^{-1}) was comparable with that recorded at the same concentration of Kn (Table 3), but at higher IAA concentrations (3 to 5 mg L^{-1}), shoot length was significantly higher than the respective counterparts using MS + Kn (3 to 5 mg L^{-1}). In contrast, the average number of nodes per explant was higher at lower concentrations of Kn (0.5 to 1 mg L^{-1}) than IAA, which can be attributed to the cumulative effect of both endogenous and supplemented levels of auxin in the explant (Rani and Raina 2000).

Profuse callusing followed by gradual senescence of the explants cultured on MS medium supplemented with NAA (0.5 to 5 mg L^{-1}) was observed after 2 wk of culture. MS medium containing 1 mg L^{-1} NAA induced tuberous roots along with callusing from the cut end of the nodal explant. These nodes did not show any bud break and did not survive for long. Formation of tuberous roots could be attributed as a metabolic consequence in presence of NAA, as no other auxin used in this study led to tuberization of roots. *In vitro* root tuberization has also been reported in *Trichosanthes kirilowii* cultured on half-strength MS medium that contained 2.7 μM NAA and 5% (*w/v*) sucrose (Zhao *et al* 2018).

Effect of different combinations of PGRs: No PGR in MS medium alone was found to be suitable for development of healthy shoots. Therefore, combinations of cytokinin with other cytokinins and auxin were used to attain optimum development of regenerated shoots. The effect of three combinations (BA + IAA, Kn + IAA, and BA + Kn) on axillary bud proliferation, growth, and development was studied further.

A total of 45 combinations with varying concentrations of cytokinins (BA and Kn) and IAA and 10 combinations of BA and Kn were tested for axillary bud proliferation (Table 3). Callus formation at the base of the nodal segments followed by shoot proliferation was noted on all combinations of BA and IAA (Table 3). The percentage of responding explants decreased with increasing concentrations of BA and IAA. Shoot count also decreased with increased IAA concentrations, while the BA concentration was kept constant. High shoot count (10) was observed on MS medium supplemented with BA (3 mg L^{-1}) and a lower concentration of IAA (0.5 mg L^{-1}) (Table 3), whereas the maximum shoot length (2.4 ± 0.2 cm) was observed with 0.5 mg L^{-1} of BA and IAA (3 mg L^{-1} and 5 mg L^{-1}) (Table 3, Fig. 2*a*). Callus formation also accelerated at increased concentrations of BA and IAA. As previously discussed, the maximum shoot count and shoot length was attained at higher BA and IAA concentrations, when used alone, respectively. When used in combination, both shoot proliferation and elongation was attained, which can be attributed to the synergistic effect of BA and IAA. Synergism between BA and IAA was greatly affected by the varied concentrations of BA and declined with the increasing BA concentrations (1 to 5 mg L^{-1}), irrespective of the IAA counterpart (Fig. 2*b–c*), and MS + BA (0.5 mg L^{-1}) + IAA (0.5 to 5 mg L^{-1}) was the most effective (Table 3).

Similarly, combinations of Kn and IAA resulted only in shoot elongation, with the maximum shoot length of 8 ± 0.6 cm on MS + Kn (0.5 mg L^{-1}) + IAA (3 mg L^{-1}), and the maximum average number of nodes per shoot (8.5 ± 0.3) on MS + Kn (3 mg L^{-1}) + IAA (3 mg L^{-1}) (Table 3). The average shoot length and nodes per shoot increased significantly with the higher concentration of IAA than that of Kn. In addition, higher concentrations of IAA (2 to 5 mg L^{-1}) resulted in the formation of callus at the base of the nodal segments, whereas swelling with negligible callusing at the base was found in those cultured on MS that contained lower concentrations of IAA (0.5 to 1 mg L^{-1}) and Kn (0.5 to 5 mg L^{-1}). Explants cultured on MS + IAA (2 mg L^{-1}) + Kn (3 mg L^{-1}) resulted in the formation of multiple axillary buds at the nodes of the elongated shoots. It was also found that the explants showed significant shoot elongation along with little shoot bud proliferation on MS medium augmented with IAA or Kn alone, and when used in combination, their cumulative effect resulted only in shoot elongation (Fig. 2*f*).

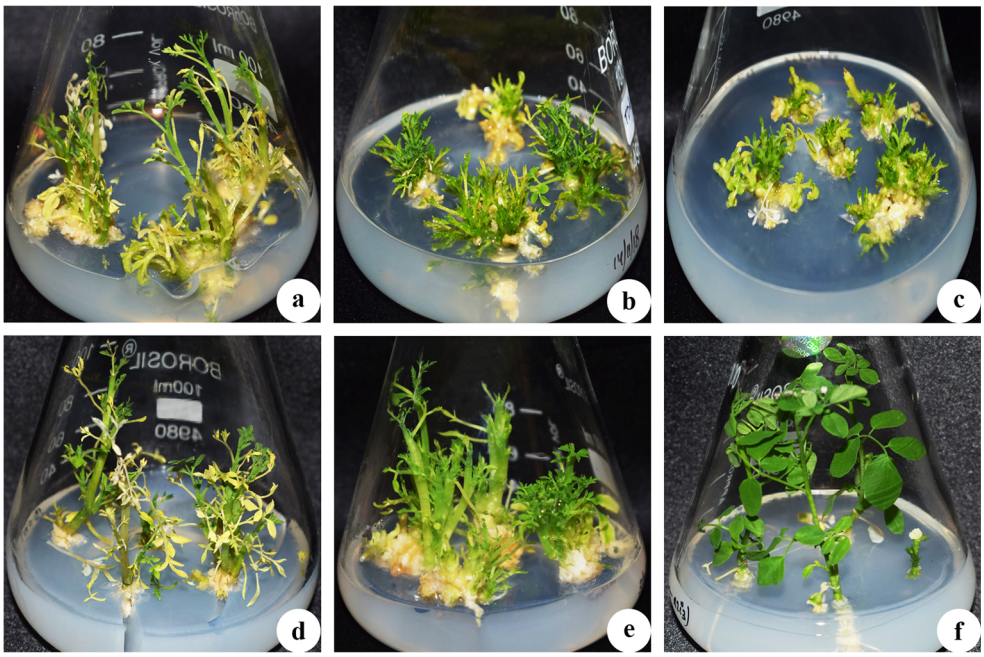

Figure 2. Response of *Moringa oleifera* Lam. nodal segments cultured on Murashige and Skoog medium supplemented with different combinations of plant growth regulators: (*a*) 6-benzyladenine (BA 0.5 mg L^{-1}) + Indole-3-acetic acid (IAA 1 mg L^{-1}); (*b*) BA (1 mg L^{-1}) + IAA (0.5 mg L^{-1}); (*c*) BA (3 mg L^{-1}) + IAA (1 mg L^{-1}); (*d*) BA (0.5 mg L^{-1}) + Kinetin (Kn 5 mg L^{-1}); (*e*) BA (1 mg L^{-1}) + Kn (5 mg L^{-1}); and (*f*) IAA (1 mg L^{-1}) + Kn (1 mg L^{-1}).

In contrast to the effect of BA + IAA and BA + Kn on shoot morphogenesis from nodal segments, combinatorial effects of Kn and IAA led to single shoot elongation, and the average length of the shoots obtained was significantly higher than those found on MS + IAA or Kn, which suggested synergy between Kn and IAA at all the various combinations.

When both cytokinins, BA and Kn, were used in combination, profuse shoot multiplication along with elongation at lower concentrations of BA was found (Fig. 2*d–e*). The shoot count was found to increase significantly on MS medium supplemented with 1 mg L^{-1} BA and varying Kn concentrations (0.5 to 5 mg L^{-1}), with a maximum shoot count of 17.36 ± 2.32 on MS + BA (1 mg L^{-1}) + Kn (1 mg L^{-1}). MS medium augmented with BA (0.5 mg L^{-1}) + Kn (0.5 to 5 mg L^{-1}) resulted in both shoot bud proliferation and elongation from the cultured nodal segments, while at higher BA concentrations, there was no significant shoot elongation (Table 3). These results suggested synergistic effects of both the hormones in combination that results in a cumulative response on the shoot multiplication and elongation, based on their individual effects when used alone.

These observations were similar to the results observed with combination of BA and IAA. In both scenarios, the concentration of BA governed the synergy in combination. It can also be deduced from the results that MS medium containing BA (0.5 mg L^{-1}), along with Kn (0.5 to 5 mg L^{-1}) or IAA (0.5 to 2 mg L^{-1}), can be further optimized to obtain the maximum synergy for shoot morphogenesis from nodal segments.

The synergy between cytokinin and auxin has been widely reported for efficient shoot regeneration in various plants including *Sarcostemma brevistigma* (Thomas and Shankar 2009), *Artemisia amygdalina* (Rasool *et al* 2013), and *Lilium* (Takayama and Misawa 1982). Combinations of cytokinins and auxins for shoot proliferation and elongation from drumstick tree explants was recently reported (Shaaban and Maher 2016; Jun-jie *et al* 2017), but their synergistic effect has not been studied in detail. This effect can be attributed to the interaction between the hormones due to differences in their signaling pathways. Another explanation could be the ratio of endogenous and exogenous hormone levels, and the minimum threshold levels required for optimum proliferation and elongation (Takayama and Misawa 1982; Rasool *et al* 2013). In addition, BA has different physiological effects than Kn, due to different physiochemical properties. These differences have been identified as the potential reasons for the synergism between different hormone combinations at different concentrations (Takayama and Misawa 1982).

Effect of auxins on rooting of regenerated shoots: Healthy and elongated shoots (> 3 cm) were transferred to half-strength MS medium fortified with or without auxins. For rooting, IAA (0.1, 0.2 mg L^{-1}) and IBA (0.1, 0.2 mg L^{-1}), alone or in combination, were used in MS medium. Rooting initiated within 2 wk of culture from the shoots maintained on both with and without auxins. A 100% rooting response was observed from the shoots cultured on half-strength MS + IBA or IAA (0.1 mg L^{-1}) + IBA (0.1 mg L^{-1}) (Table 4). The percentage of shoots developing roots reduced on IAA (0.1 mg L^{-1}) + IBA (0.1 mg L^{-1}) (Table 4). The maximum number of roots (14.1 ± 2.9) developed from the shoots inoculated on half-strength MS supplemented with IBA (0.2 mg L^{-1}) and followed by IAA (0.1 mg L^{-1}) (12.8 ± 0.7) (Table 4). In contrast, some studies reported the maximum number of roots (4) and 100% rooting efficiency on MS

Table 4. Rooting response of *Moringa oleifera* Lam. regenerated shoots on half-strength Murashige and Skoog media supplemented with different combinations of auxins

IAA (mg L^{-1})	IBA (mg L^{-1})	% Response	Avg. no. of roots per shoot Mean ± S.E.*	Average root length Mean ± S.E.*	Average shoot length Mean ± S.E.*	Average number of nodes per shoot Mean ± S.E.*
0.1	–	80	12.8 ± 0.7^{ab}	6.4 ± 1^{abc}	11.6 ± 1.1^{a}	8.7 ± 0.9^{cdef}
0.2	–	90	8.8 ± 0.7^{bc}	5.6 ± 0.4^{abc}	9.4 ± 0.8^{abcd}	8.6 ± 0.8^{def}
–	0.1	100	10.4 ± 0.6^{abc}	7.3 ± 0.8^{a}	9.4 ± 0.8^{abcd}	7.6 ± 0.4^{f}
–	0.2	100	14.1 ± 2.9^{a}	5.2 ± 0.4^{bc}	8.8 ± 1.3^{bcd}	8.1 ± 0.9^{ef}
0.1	0.1	77.8	11.7 ± 1.9^{abc}	6 ± 0.5^{abc}	8 ± 0.9^{cd}	9.3 ± 0.4^{bsdef}
0.1	0.2	100	7.5 ± 1.2^{c}	4.4 ± 0.6^{c}	7 ± 0.2^{d}	11.6 ± 0.8^{a}

*Different *letters* in *superscript* indicate significantly different values at $P < 0.05$

IAA indole-3-acetic acid; *IBA* indole-3-butyric acid

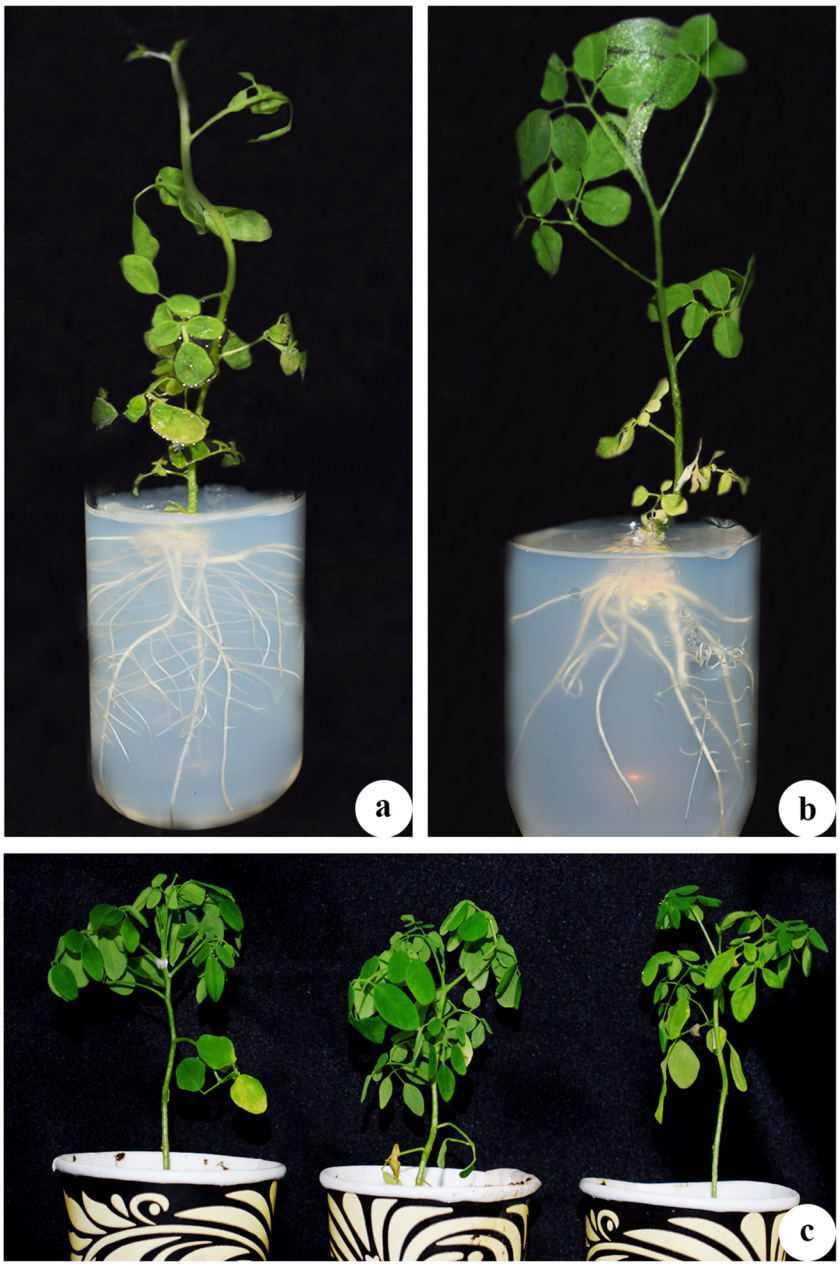

Figure 3. Rooting response of *in vitro* regenerated *Moringa oleifera* Lam. shoots on half-strength Murashige and Skoog medium supplemented with (*a*) Indole-3-acetic acid (IAA 0.1 mg L^{-1}) + Indole-3-butyric acid (IBA 0.2 mg L^{-1}), (*b*) IAA (0.1 mg L^{-1}), and (*c*) pot transferred plants after 1 mo.

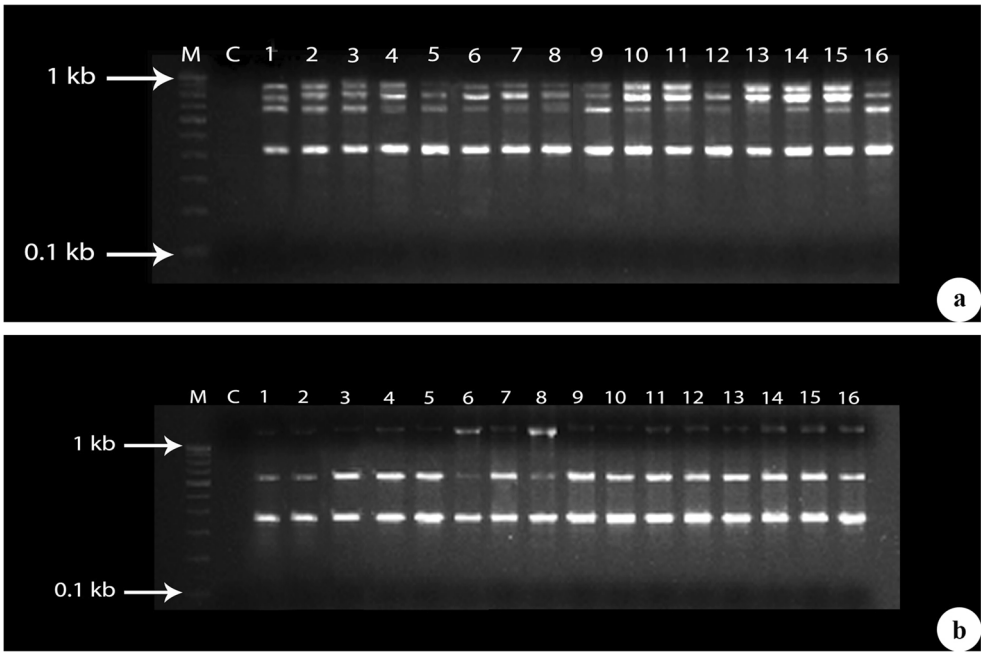

Figure 4. Agarose gel electrophoresis of randomly amplified polymorphic DNA (RAPD) fragments showing banding pattern amplified by (*a*) OPT-6 primer and (*b*) OPF-20 primer. *M* = Molecular marker and *C* = Control.

medium without any PGR (Islam *et al* 2005; Förster *et al* 2013). The longest roots with an average root length of 7.3 ± 0.8 cm developed on half-strength MS + IBA (0.1 mg L^{-1}). The length of roots formed on other types of rooting media ranged from 4.4 to 6.4 cm, which indicated that there was no significant effect of the media on the length of the roots formed. Slight callus formation was observed when IAA and IBA were used alone, while direct root initiation was observed on IAA + IBA (Fig. 3*a–b*). The internodal length was also observed to be significantly reduced on MS + IAA (0.1 or 0.2 mg L^{-1}) + IBA (0.1 or 0.2 mg L^{-1}), which indicated the presence of potential synergy between IAA and IBA (Table 4). Saini *et al* (2012) also reported that combinations of IAA and IBA induced the longest and maximum number of roots per shoot in drumstick tree.

Response of rooted plantlets to hardening and acclimatization Transfer of rooted plants to small pots containing a mixture of garden soil-organic mixture (2:1, *w/w*) for primary hardening was done by carefully removing the media remnants with minimal damage to the roots. The plants were successfully established during primary hardening in the greenhouse. About 80% of the plants survived and were transferred to a natural environment for secondary hardening. About 60% of the plants were able to establish well in secondary hardening. All the established micropropagated plants were healthy and followed a uniform growth pattern (Fig. 3*c*).

RAPD analysis: Out of 15 random primers (OPT 1–11 and OPF 16, 18–20) that were used, eight (OPT 1, 5, 6, 7, 11, and OPF 16, 19, 20) showed significant amplification with distinct and reproducible bands. A total of 480 amplicons of length ranging from 400 to 900 bp, with an average of 3.75 bands per primer set, was produced. Among all the primers that resulted in amplification, OPT 6 and OPF 20 showed the maximum reproducibility in the banding pattern (Fig. 4*a–b*). The banding pattern for a set of random primers was found to be identical, which confirmed clonality of all of the micropropagated plants. In a similar report, genetic stability of the micropropagated plants of drumstick tree was demonstrated using randomly amplified microsatellite polymorphism (RAMP) markers (Avila-Treviño *et al* 2017), which indicated that shoot proliferation is an efficient method for clonal propagation of *M. oleifera* with minimal risk of somaclonal variation. Molecular markers, RAPD, and inter-simple sequence repeats (ISSR), in particular, have been widely used to demonstrate the clonal fidelity of *in vitro* raised plantlets and validate their true-to-type nature (Arora *et al* 2004; Jain *et al* 2011; Behera *et al* 2019).

Conclusions

The synergistic effect of different combinations of PGRs on axillary multiplication from nodal explants of an economically and medicinally important tree species *M. oleifera* has been reported in the present investigation. The study also reports an efficient and reproducible micropropagation protocol for drumstick, with a maximum number of shoots per explant (17.62 ± 2.35), using axillary bud proliferation for the first time. The clonality of the regenerated plants was also ascertained through RAPD analysis. The species is prone to the infections that could affect the yield. Therefore, this protocol can be used for *ex situ* conservation of wild types and scaling-up to regenerate hundreds of plantlets to meet the huge

market demand. This micropropagation study can be extended to study the effect of various biotic and abiotic stresses on biosynthesis of industrially important metabolites and gum exudates. In addition, strategies will be developed for enhanced secretion of gum and biosynthesis of important metabolites in *in vitro* raised cultures of *M. oleifera*.

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