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RESEARCH ARTICLE

MICROPROPAGATION OF *MORINGA OLEIFERA* LAM. FROM SHOOT TIP EXPLANTS

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ABSTRACT: *Moringa oleifera* Lam. is one of the most important plants that serve as medicinal and functional food. It is found in very limited areas in Ethiopia and traditional propagation is by seeds where it is difficult to get uniform plants of superior genotypes. The objective of this study was to develop efficient *in vitro* propagation protocol of *M. oleifera* from shoot tip. Seeds of *M. oleifera* were sterilized with calcium hypochlorite. The sterilized seeds were germinated on growth regulators free Murashige and Skoog (MS) medium. Shoot tips of *M. oleifera* were excised from *in vitro* grown seedlings and cultured on MS medium supplemented with 0.5 mg/l kinetin or 6-benzylaminopurine (BAP). The initiated shoots were transferred to shoot multiplication medium containing different concentrations of BAP in combination with indole butyric acid (IBA) or kinetin. The multiplied shoots were transferred to half strength MS medium containing different concentrations of IBA in combination with α-naphthalene acetic acid (NAA) for rooting. Seeds sterilized with 10% calcium hypochlorite for 30 min showed 100% germination. All cultured shoots were initiated and the highest shoot number per explant (5.66±0.44) was obtained on MS medium supplemented with 1.0 mg/l BAP in combination with 0.5 mg/l IBA and 94% of shoots rooted on half strength MS supplemented with 0.25 mg/l NAA. After acclimatization, 90% of plants survived. This *in vitro* propagation protocol can be used for clonal propagation of superior genotypes of this tree plant in short period of time contributing to its conservation and genetic improvement.

Key words/phrases: Calcium hypochlorite, *In vitro* propagation, Plant Growth Regulators, Rooting, Shoot multiplication.

INTRODUCTION

Moringa oleifera Lam. commonly known as the drumstick or ben oil tree is a widely cultivated species of family Moringaceae. It is a deciduous tree that reaches a height of up to 10 m. The tree is growing in many parts of southern Ethiopia. Konso people plant it around their homesteads and also

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in the terraced fields (Padayachee and Bajnath, 2012). It originated from India and Arabia and was introduced to Ethiopia long ago.

M. oleifera is referred to as the miracle plant of life because of its uses, particularly as medicine and nutrition (Oyeyinka and Oyeyinka, 2018). It is valued as the 'natural nutrition' of the tropics. The fruits, leaves, flowers and immature pods of this tree are highly nutritious and are used as vegetable in many parts of the world, especially in Africa, India, Pakistan and the Philippines (Shahzad *et al*., 2014). The leaves of *M. oleifera* are rich in nutrients and the plant has maximum leaves at the end of the dry season when other foods are scarce. It is rich in vitamins A, B, C, D, E and K. As is the case with *Moringa stenoptela*, the vital minerals present in *M. oleifera* include calcium, copper, iron, potassium, magnesium, manganese and zinc. Its nutritional and medicinal uses are attributed to its roots, bark, leaves, flowers, fruits, and seeds (Anwar *et al*., 2007).

In addition to its use as food and medicine, *M. oleifera* is used as animal feed to increase milk production. There are tremendous potential opportunities with *M. oleifera* for sustainable agriculture, and the development of cash crops in semi-arid regions. However, it is underutilized even at its place of origin due to underutilization and consequently is depleting from these areas (Shahzad *et al*., 2014).

M. oleifera is not widely distributed in Ethiopia and sexual propagation is tedious and not even possible without having enough individual plants for cross-pollination. Moreover, it is difficult to produce uniform plants of superior genotypes by using seeds as propagation materials. This method of seed propagation results in one plant per seedling. The plants obtained from seeding vary depending on the genotype leading to variation in fruit production and nutritional values.

Selected characteristics of plants may be maintained via *in vitro* propagation. As a widely used method for the rapid clonal propagation of many economically important crops, it could satisfy the demand for *M. oleifera* planting materials. Mass propagation of these rare individuals by *in vitro* propagation would make them more widely available. Micropropagation is used to propagate plants within short period of time and for *in vitro* conservation of genetic resource, understanding of gene structure, function in molecular biology and plant improvement through transgenic technology. There are reports of different tissue culture research results on *M. oleifera* in different countries (Islam *et al*., 2005; Marfori, 2010; Riyathong *et al*., 2010; Saini *et al*., 2013; Shahzad *et al*., 2014).

However, the response of different plant genotypes differs under *in vitro* culture conditions. Therefore, each genotype of a plant species requires its own micropropagation protocol and there is no report of micropropagation of any of the genotypes of *M. oleifera* in Ethiopia. The present study aimed at developing micropropagation protocol of this tree plant in Ethiopia.

MATERIALS AND METHODS

In vitro seed germination

M. oleifera seeds were collected from Konso, Southern Nations, Nationalities and Peoples (SNNP), Ethiopia. The seed coats were removed and the de-coated seeds were stored at room temperature. These seeds were washed under running tap water and surface sterilized with 70% ethanol for 9 min followed by sterilization with 5%, 10% and 15% calcium hypochlorite for 20, 25 or 30, min and rinsed 3 to 4 times with sterile distilled water. The sterilized seeds were cultured on growth regulators free MS (Murashige and Skoog, 1962) medium containing 3% sucrose (w/v) and 7.0 g/l agar. The pH was adjusted to 5.8 before addition of agar and autoclaved at 121°C for 15 min. The medium (20 ml each) was then dispensed in culture vessels (10 x 6 cm, height, width, respectively). Five seeds per culture vessel were cultured in six replications per treatment. The cultures were maintained at 25±2°C under 16 h photoperiod at light intensity of 22 μmol m^{-2} s^{-1} using cool white fluorescent. Seed germination indicated by at least radicle emergence was recorded on 16, 20, and 24 days.

Shoot initiation

The shoot tips of the germinated seedlings were excised and cultured on shoot initiation medium. The shoot initiation medium was full strength MS medium fortified with 3% sucrose (w/v), BAP (0.0, 0.25 0.5, 0.75, 1.0 mg/l) and kinetin (0.0, 0.25 0.5, 0.75, 1.0 mg/l) separately. The pH of the medium was adjusted to 5.8 and then 0.7% agar was added and autoclaved. The explants were cultured in Magenta GA-7 culture vessels containing 50 ml medium. All cultures were maintained at 25±2°C under 16 h photoperiod at light intensity of 23 μmol $m^{-2}s^{-1}$ using cool white fluorescent light. Five shoots were cultured in each culture vessel in six replications. The number of initiated explants was recorded after four weeks of culture.

Shoot multiplication

Shoots initiated on initiation medium were cultured on 50 ml full strength MS medium in Magenta GA-7 culture vessels supplemented with different concentrations of BAP (0.0, 0.25, 0.5, 1.0, 1.5, 2.0 mg/l), in combination

with IBA (0.0, 0.1, 0.5, 1.0, mg/l) and kinetin (0.0, 0.1, 0.5, 1.0, mg/l). Five shoots were cultured per culture vessels in six replications. The cultures were maintained under culture conditions as of that of shoot initiation. Shoot number per explant was recorded after four weeks of culture.

Rooting

For root induction, half strength MS medium supplemented with IBA (0.0, 0.1, 0.25, 0.5 mg/l) in combination with NAA (0.0, 0.1, 0.25, 0.5 mg/l), 1.5% sucrose (w/v) and 0.6% agar was used. The pH of the medium was adjusted to 5.8 before addition of agar prior to autoclaving. About 2.5 to 3.5 cm long shoots were excised and transferred to the rooting media. Five shoots per culture vessel in six replications were used. The cultures were maintained at culture conditions as of shoot initiation. The number of shoots that produced roots, root number and length were recorded after four weeks of culture.

Acclimatization

Well rooted plantlets were taken out of the medium without damaging the roots and the roots were washed thoroughly in running tap water to remove all the residues of culture medium followed by planting the plantlets in plastic pots containing a sterile (1:2:1 v/v) mixture of sand, local soil and compost, respectively. The pots were covered with transparent polyethylene bags and kept in the culture room for two weeks and given adequate water to the plantlets at an interval of two-to- three days. After two weeks, the plants were shifted to a greenhouse. The plastic covers were gradually removed after two weeks. Finally, the plants were fully exposed to the normal growth conditions and the survival rate was evaluated after a month.

Data collection and analysis

Data were subjected to one-way analysis of variance (ANOVA) to detect the significance of differences among treatments. Mean separation of different treatments were performed by Tukey's test using statistical data analysis software SPSS 20.0 version at 0.05 probability level.

RESULTS

In vitro seed germination

Among the different concentrations of calcium hypochlorite, 10% concentration and 30 min exposure time resulted in the highest (100%) clean explants. All seeds were contaminated at 5% calcium hypochlorite concentration and exposure time of 20, 25 and 30 min (Table 1). There was

no germination of seeds at 5% calcium hypochlorite and exposure time of 20 min as well as 15% calcium hypochlorite and exposure time of 30 min. The data also revealed that the contamination percentage dramatically decreased as the exposure time increased at the same concentration of calcium hypochlorite. However, disinfection with 15% calcium hypochlorite for 30 min also resulted in least contamination and highest clean explants but no seed germinated. *M. oleifera* seeds sterilized with 10% calcium hypochlorite for 30 min showed the highest contamination-free germination percentage (100%).

Table 1. Effect of calcium hypochlorite concentration and exposure time on seed sterilization and *in vitro* germination of *Moringa oleifera* seeds.

Calcium hypochlorite (%)	Exposure time (min)	Clean explants (%)	Contamination (%)	Germination (%)
5	20	00	100	00
5	25	00	100	13.2
5	30	00	100	23.4
10	20	20	80	56.5
10	25	70	30	87.4
10	30	100	00	100.0
15	20	83	17	44.7
15	25	85	15	27.6
15	30	100	00	00

Shoot initiation

Shoot tips cultured on the control and on MS medium supplemented with BAP and kinetin alone showed significant variation ($p=0.05$) in terms of percentage of shoot initiation. All shoot tips produced proliferated shoots on MS medium supplemented with 0.5 mg/l kinetin and BAP. On the other hand, the lowest shoot initiation percentage (21.8%) was obtained at 1.0 mg/l kinetin.

Table 2. Percentage of shoot initiation on MS medium containing different concentrations of BAP and kinetin.

BAP (mg/l)	Kinetin (mg/l)	Shoot initiation (%)
00	00	42.46
0.25	00	76.63
0.5	00	100
0.75	00	67.23
1.0	00	37.33
00	0.25	81.24
00	0.5	100
00	0.75	68.54
00	1.0	21.8

Shoot multiplication

Based on the composition of growth regulators in the medium, there was variation in the number of proliferated shoots (Table 3 and 4). MS medium supplemented with BAP in combination with IBA showed better result than BAP alone and BAP in combination with kinetin. The highest mean shoot number per explant (5.66±0.44) was obtained on MS medium containing 1.0 mg/l BAP in combination with 0.5 mg/l IBA followed by MS medium containing 0.25 mg/l BAP in combination with 0.1 mg/l IBA which resulted in 4.63±0.33 shoots per explant. The maximum mean shoot length of 4.51± 0.21 cm was obtained on MS medium supplemented with 0.25 mg/l BAP in combination with 0.1 mg/l IBA followed by 4.30±0.11 cm on MS medium containing 1.0 mg/l BAP in combination with 0.5 mg/l IBA (Table 4).

Among the different concentrations of BAP, the medium containing 0.25 mg/l BAP resulted in maximum mean shoot number per explant (3.63± 0.35) followed by 1.0 mg/l BAP that produced 3.56±0.37 shoots per explant. The maximum mean shoot length (3.98±0.18) was also obtained on MS medium supplemented with 0.25 mg/l BAP. The response of explants cultured on MS medium supplemented with different concentrations of BAP in combination with kinetin is presented in Table 3. The medium containing 0.25 mg/l BAP in combination with 0.5 mg/l kinetin resulted in maximum mean shoot number per explant (2.36±0.29) followed by the medium containing 0.25 mg/l BAP in combination with 0.10 mg/l kinetin that produced 1.93±0.15 shoots per explant. The highest mean shoot length (2.73±0.17 cm) was achieved on MS medium supplemented with 0.25 mg/l BAP in combination with 0.10 mg/l kinetin (Table 3).

Table 3. Effect of different concentrations of BAP alone and in combination with kinetin on shoot multiplication. Mean values are indicated as ±SE.

BAP (mg/l)	Kinetin (mg/l)	No. of shoots/explant	Shoot length (cm)
0.0	0.0	1.71 ±0.17^{cd}	2.77±0.14^{b}
0.25	0.0	3.63±0.35^{a}	3.98±0. 18^{a}
0.5	0.0	3.10±0.25^{ab}	3.45±0. 20^{a}
1.0	0.0	3.56±0.37^{a}	3.43±0. 17^{a}
1.5	0.0	1.80±0.17^{cd}	2.16±0. 11^{bc}
2.0	0.0	1.73±0.23^{cd}	2.58±0. 21^{b}
0.25	0.1	1.93±0.15^{cd}	2.73±0. 17^{b}
0.5	0.1	1.16±0.06^{d}	2.47±0. 12^{b}
1.0	0.1	1.73±0.15^{cd}	2.30±0. 11^{bc}
1.5	0.1	1.47±0.14^{cd}	2.28±0. 11^{bc}
0.25	0.5	2.36± 0.29^{bc}	2.59±0.14^{b}
1.0	0.5	1.40±0.14^{cd}	2.40±0.11^{bc}
1.5	0.5	1.50±0.16^{cd}	2.22±0. 10^{bc}
0.5	1.0	1.67±0.13^{cd}	2.05±0. 08^{bc}

BAP (mg/l)	Kinetin (mg/l)	No. of shoots/explant	Shoot length (cm)
1.0	1.0	1.20±0.07^{d}	2.06±0. 08^{bc}
1.5	1.0	1.43±0.11^{cd}	1.78±0. 07^{c}

Means with the same letter within a column are not significantly different at p<0.05.

The response of explants cultured on MS medium supplemented with different concentrations of BAP in combination with IBA is presented in Table 4. After two weeks of culture on MS medium containing different concentrations of BAP in combination with IBA, multiple shoots emerged from the explants (Fig. 1). The highest mean shoot number per explant (5.66±0.44 and 4.63±0.33) were induced on MS medium supplemented with 1.0 mg/l BAP in combination with 0.5 mg/l IBA and 0.25 mg/l BAP in combination with 0.1 mg/l IBA, respectively. Maximum mean shoot length per explant (4.51±0.21 cm) was obtained on MS medium supplemented with 0.25 mg/l BAP in combination with 0.1 mg/l IBA. The lowest mean number of shoots per explant (1.23±0.09) was recorded on MS medium supplemented with 2.0 mg/l BAP in combination with 0.1 mg/l IBA.

Table 4. Effect of different concentrations of BAP in combination with IBA on shoot multiplication. Mean values are indicated as ±SE.

BAP (mg/l)	IBA(mg/l)	No. of shoots/explant	Shoot length (cm)
0.0	0.0	1.71 ±0.17^{def}	2.77±0.14^{b}
0.25	0.1	4.63±0.33^{ab}	4.51±0.21^{a}
0.5	0.1	1.36±0.10^{ef}	2.34±0.12^{c}
1.0	0.1	3.10±0.32^{cd}	3.32±0.14^{b}
1.5	0.1	2.63±0.35^{cde}	2.19±0.14^{c}
2.0	0.1	1.23±0.09^{f}	1.92±0.08^{c}
0.25	0.5	3.73±0.36^{bc}	3.07±0.11^{b}
0.5	0.5	3.10±0.32^{cd}	3.15±0.14^{b}
1.0	0.5	5.66±0.44^{a}	4.30±0.11^{a}
1.5	0.5	1.50±0.11^{ef}	2.15±0.14^{c}
2.0	0.5	1.33±0.09^{ef}	1.88±0.05^{c}
0.25	1.0	3.60±0.35^{bc}	3.16±0.14^{b}
0.5	1.0	1.40±0.14^{ef}	2.30±0.11^{c}
1.0	1.0	1.90±0.16^{def}	2.18±0.08^{c}
1.5	1.0	1.93±0.30^{def}	2.03±0.11^{c}
2.0	1.0	1.40±0.13^{ef}	2.09±0.11^{c}

Means with the same letter within a column are not significantly different at p<0.05.

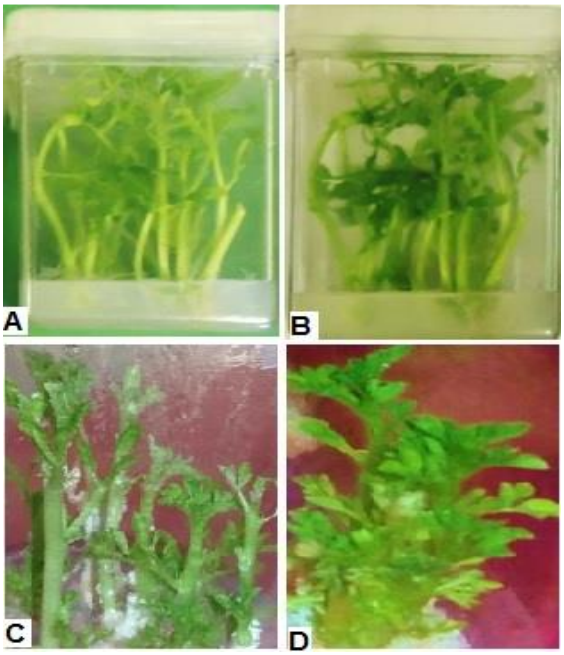

Fig. 1. *In vitro* shoot multiplication from shoot explants of *Moringa oleifera* on MS medium containing BAP alone and in combination with IBA. (A) 1.0 mg/l BAP in combination with 0.5 mg/l IBA; (B) 0.25 mg/l BAP in combination with 0.1 mg/l IBA; (C) 1.0 mg/l BAP; (D) 0.25 mg/l BAP.

Rooting and acclimatization

The shoots cultured on half strength MS basal medium supplemented with different concentrations of IBA, NAA and combination of IBA and NAA resulted in different rooting responses (Table 5 and Fig. 2). The highest mean number of roots per shoot (7.96±0.69) was obtained on half strength MS medium containing 0.25 mg/l NAA followed by 0.5 mg/l NAA. The highest mean root length per explant (2.44±0.04 cm) was obtained on growth regulators free half strength MS medium which was used as control. After one month of acclimatization, 90% of plants survived in greenhouse (Fig. 2).

Table 5. Rooting percentage, mean number and length of roots obtained on half strength MS medium containing different concentrations of IBA, NAA and combinations of IBA and NAA. Mean values are indicated as ±SE.

IBA (mg/l)	NAA (mg/l)	Rooting (%)	No. of roots/explant	Root length (cm)
00	00	73.33	3.04±0.36^{b}	2.44±0.04^{a}
0.25	00	33.33	3.30±0.85^{b}	1.42±0.07^{c}
0.5	00	63.33	2.94±0.44^{b}	1.57±0.06^{c}
00	0.1	73.33	3.08±0.36^{b}	1.44±0.05^{c}
00	0.25	94.00	7.96±0.69^{a}	2.11±0.08^{b}
00	0.5	86.67	6.16±0.84^{ab}	1.55±0.04^{c}

IBA (mg/l)	NAA (mg/l)	Rooting (%)	No. of roots/explant	Root length (cm)
0.1	0.1	70.00	4.23 ± 0.62^{b}	1.48 ± 0.03^{c}
0.25	0.1	66.67	4.85 ± 0.73^{ab}	1.53 ± 0.04^{c}
0.5	0.1	60.00	3.55 ± 0.42^{b}	1.44 ± 0.03^{c}
0.1	0.25	93.33	4.21 ± 0.43^{b}	1.52 ± 0.05^{c}
0.25	0.25	60.00	5.76 ± 0.88^{ab}	1.57 ± 0.06^{c}
0.5	0.25	20.00	$3.00\pm0.1.0^{b}$	1.51 ± 0.06^{c}

Means with the same letter within a column are not significantly different at $p<0.05$.

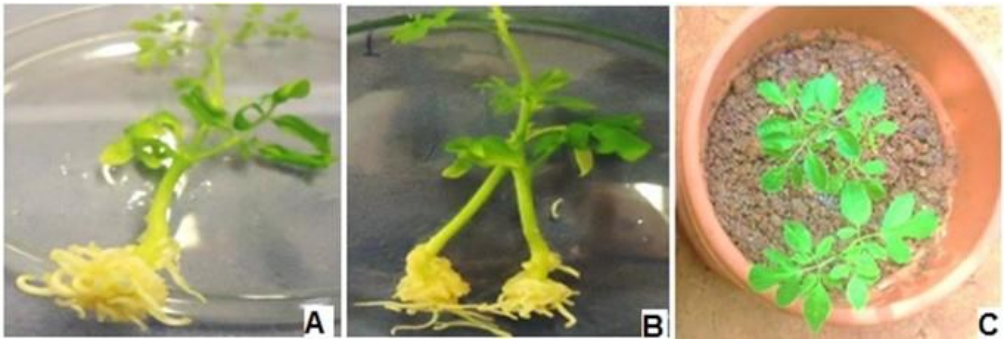

Fig. 2. *In vitro* rooting and acclimatization of *Moringa oleifera*. (A) Rooted shoots on half strength MS medium containing 0.25 mg/l NAA; (B) On 0.5 mg/l NAA; (C) Plants after 30 days of acclimatization.

DISCUSSION

Sterilization of the seeds

All seeds were free from any microbial contamination with 100% germination at 10% calcium hypochlorite and 30 min exposure. All seeds were contaminated at 5% calcium hypochlorite concentration and exposure time of 20, 25 and 30 min. These results indicate that the chemical concentration and the duration at which *M. oleifera* seeds exposed to the disinfectant significantly affected the seed germination percentage. This is similar to the finding of Saini *et al*. (2013) who reported the best surface sterilization for the seeds of *M. oleifera* were 30% Clorox solution for 30 minutes of exposure time. Saini *et al*. (2012) reported that surface sterilization of *M. oleifera* seeds using 0.1% mercury chloride for 2 min and 20% sodium hypochlorite for 10 min, then rinsing three times in sterile distilled water followed by removing seed coats and again surface sterilization by immersion in 20% sodium hypochlorite for 5 min was found to be effective. Calcium hypochlorite is effective against bacteria, viruses, and fungi depending on the concentration and exposure time. Generally, although the mechanism of action of chlorine compounds is not completely understood, they are believed to have the ability to oxidize proteins, inhibit enzyme activity, and react with nucleic acids (Fu *et al*., 2007).

Shoot initiation

In culture initiation experiment, shoot tips cultured on control and MS medium supplemented with 0.25, 0.5, 0.75, 1.0 mg/l BAP and kinetin separately showed significant variation ($p>0.05$). MS medium containing 0.5 mg/l kinetin and BAP at 0.5 mg/l resulted in 100% shoot initiation. This is similar to the finding of Islam *et al*. (2005) who got 100% initiation of shoots from stem segment explants taken from 10-day-old seedlings of *M. oleifera*. The lowest shoot initiation percentage (23.33%) was obtained on MS medium containing 1.0 mg/l kinetin. This shows that shoot initiation percentage was greatly influenced by type and concentrations of cytokinin. Cytokinins are key factors in enhancing shoot initiation and multiple shoot proliferation (Riyathong *et al*., 2010). Previous reports on shoot multiplication of *M. oleifera* showed different results regarding the best growth regulators concentrations for induction of multiple shoots. Stephenson and Fahey (2004) reported 1.0 mg/l BAP to be the best for shoot initiation from nodal explants. Islam *et al*. (2005) also pointed out 1.0–1.5 mg/l BAP to be the best for initiation of shoots from shoot explants obtained from field-grown plants. These variable responses may be attributed to differences in the plant source and the type of explants. The present result is in agreement with the findings of other authors who have noted the effectiveness of BAP for the shoot initiation of *M. oleifera* cultured on MS medium supplemented with different concentrations of BAP, and it was found that all the media resulted in 100% shoot initiation (Riyathong *et al.*, 2010).

Shoot multiplication

There was significant difference ($p>0.05$) among the different treatments both in terms of shoot number per explant and shoot length. In this study, 31 different concentrations of kinetin in combination with BAP, and BAP in combination with IBA were used for shoot multiplication. BAP alone or in combination with IBA were found to be the most suitable plant growth regulators for micropropagation of *M. oleifera* rather than combinations of kinetin and BAP.

Al Khateeb *et al*. (2013) reported that MS medium supplemented with 1.0 mg/l kinetin resulted in 2.6 mean number of shoots per explant of *M. peregrina* (Forsk). Higher concentration of kinetin (2.0 mg/l) significantly reduced the number of leaves per shoot explant. Maximum mean shoot number per explant (5.66±0.44) was obtained on MS medium supplemented with 1.0 mg/l BAP in combination with 0.5 mg/l IBA. The result from

combined effect of BAP and IBA was found to vary with their concentration in two parameters of shoot growth, mean number of shoot and shoot length. Cytokinin and auxin stimulate the activity of cambium and phloem formation, facilitating delivery of different kinds of nutrients to all parts of a plant (Reinhardt *et al*., 2000; Friml, 2003). The present result showed that exogenous applications of cytokinins are essential for shoot proliferation of shoot explants of *M. oleifera* by promoting protein synthesis, cell division and consequently inhibiting apical dominance. Fatima *et al*. (2016) obtained the highest shoot number per explant (19.66) from nodal explants of *Moringa concanensis* on MS medium containing combination of 0.1 mg/l BAP and 0.5 mg/l NAA. This result differed from the present result because different plant species require different concentrations of cytokinin and auxin for production of optimum number of shoots per explant as the endogenous growth regulators concentration varies from species to species.

In the present study, when the concentration of BAP was raised from 0.25 mg/l to 2.0 mg/l, lower number of shoots per explant was obtained due to supra-optimal concentration of BAP. In contrast to the present study, Saini *et al*. (2013) reported MS medium containing 2.0 mg/l BAP produced the highest mean shoot number per explant (10.8) of *M. oleifera*. In the present study, using different concentrations of BAP alone resulted in formation of callus at the cut ends of the shoots. This is in agreement with the findings of Islam *et al*. (2005) who reported that higher concentration of the cytokinin increases the callus initiation at the cut ends of the shoots.

When the concentration of BAP was greater than that of IBA, relatively better result was recorded. This is due to the effect of cytokinin as it promotes the axillary branching or axillary bud proliferation. Fatima *et al*. (2016) obtained 11.00±1.15 mean shoot number with 5.00±1.95 cm mean length of *Moringa concanensis* on MS medium supplemented with 0.1 mg/l kinetin along with 0.05 mg/l NAA. On the other hand, BAP alone resulted in maximum mean shoot multiplication (3.63±0.35) on MS medium supplemented with 0.25 mg/l BAP when compared to BAP and kinetin combinations.

In general, the present study indicated that in shoot multiplication from *M. oleifera* shoot explants was found highly dependent on concentration and type of growth regulators. All growth regulators showed shoot multiplication and varied in their potential and efficiency. Islam *et al*. (2005) used BAP for in vitro shoot multiplication of *M. oleifera* and found that BAP at 1.0 and 1.5 mg/l concentration resulted in best shoot

multiplication.

Rooting and acclimatization

Roots were not spontaneously induced during culture initiation and shoot multiplication. The analysis of variance revealed that root number and root length varied significantly on half strength MS medium supplemented with NAA, IBA and the combination of both. Application of NAA alone exhibited the highest mean root number per shoot as compared to IBA alone and IBA in combination with NAA. The highest mean root number per shoot (7.96±0.69) was obtained on half strength MS medium containing 0.25 mg/l NAA and mean root length (2.44±0.04 cm) was obtained on growth regulators free medium. Stephenson and Fahey (2004) obtained 4.7 roots per explant of *M. oleifera* on half strength MS medium supplemented with 0.5 mg/l NAA. Riyathong *et al*. (2010) reported shoots cultured on growth regulators free MS medium were elongated and grew to be new *M. oleifera* tree plantlets with healthy roots. Islam *et al*. (2005) also found that growth regulators-free medium was found to be the best rooting medium indicating that endogenous concentration of auxin is sufficient for rooting. In the present study, lowest mean root number per shoot (2.94±0.44) was obtained on half strength MS medium containing 0.5 mg/l IBA and lowest shoot length (1.42±0.07 cm) was obtained on medium containing 0.25 mg/l IBA.

The number of roots produced per shoot increased when higher concentrations of NAA were applied. However, further increase in the concentration of NAA showed a reduction in the mean root number per shoot. The same trend was observed in both treatments, the effect of different concentrations of IBA alone, and IBA in combination with NAA. Auxins increase root number and length resulting in increase of water and nutrient intake. Auxin is the key factor since it functions as a signal informing about physiological processes in cells and their growing demand for nutrients (Reinhardt *et al*., 2000; Friml, 2003).

ANOVA indicated that NAA significantly affected length and number of roots, but not IBA, at 5% probability level. These variable responses could be due to different factors including genetic differences, differences in the explant source, the concentration difference of growth regulators and the type and/or age of explants used to establish the cultures (Marfori, 2010).

Acclimatization of *in vitro* rooted plantlets was successful, where 90% plants survived and established as healthy plants. This result showed better survival percentage than previous report by Saini *et al*. (2012) and Marfori (2010) who reported survival of 80% of the plants.

CONCLUSION

The highest contamination free and germination percentage of the seeds of *M. oleifera* were obtained by sterilization with 10% calcium hypochlorite and 30 min exposure time. The highest percentage of shoot initiation was obtained on MS medium containing 0.5 mg/l kinetin, and 0.5 mg/l BAP alone. BAP in combination with IBA for shoot multiplication was better than kinetin in combination with BAP. The highest shoot number per explant was obtained on MS medium containing 1.0 mg/l BAP combined with 0.1 mg/l IBA. Half strength MS medium containing 0.25 mg/l NAA was found to be effective for root induction. The protocol developed in this study enables mass propagation of *M. oleifera* from shoot tip explant. The Ethiopian Forestry Research Institute can use this protocol for propagation of superior genotypes as well as commercial and smallholder farmers can benefit from this in the future.

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