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In vitro propagation of *Ximenia americana* L. from shoot tip explants: a multipurpose medicinal plant

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ABSTRACT: *Ximenia americana* L. is among the most important multipurpose medicinal plants.It is highly vulnerable plant in Ethiopia due to overexploitation for different purposes, especially using the roots and barks for medicinal purposes.Seed germination is very poor and identical clones of superior quality plants cannot be obtained in nature as it is dioecious species. Therefore, the objective of this study was to develop *in vitro* propagation protocol for *X. americana*. After seed sterilization and germination, shoot tips from *in vitro* germinated seeds were cultured on MS (Murashige and Skoog) medium supplemented with different concentrations of benzyl amino purine (BAP) or kinetin. For shoot multiplication, the initiated shoots were cultured on MS medium containing different concentrations of kinetin or BAP in combination with α-naphthalene acetic acid (NAA). Multiplied shoots were cultured on half strength MS medium supplemented with different concentrations of indole butyric acid (IBA) or NAA for rooting. The highest mean shoot number per explant (4.16 ± 0.17) was obtained on MSmedium supplemented with 0.5 mg/l BAP. The best rooting was obtained on half strength MS medium supplemented with 0.5 mg/l IBA with 3.36 ± 0.69 roots per shoot and 2.21 ± 0.40 cm root length on medium containing 2.0 mg/l IBA and established in greenhouse with 100% survival. The results of this study are important for mass propagation of superior genotypes, rehabilitation in natural habitat and conservation of this threatened multipurpose plant.

Key words/phrases: Conservation, dioecious plant, medicinal plant, multipurpose tree, plant growth regulator

INTRODUCTION

Between 65% and 80% of people in developing countries rely on traditional medicinal products as a primary source of healthcare and traditional medical practice (WHO, 2011). *Ximenia Americana* L. is very important plant for humans, livestock and wild life as medicine, food, habitat and environment. It belongs to family Olacaceae, commonly known as wild plum, blue sour plum and tallow nut. It is named Hudha in Afaan Oromo and Inkoy in Amharic language in Ethiopia. It is scrambling spiny shrub or small tree of up to 10 m (Vollesen, 1989). *X. Americana* is distributed throughout the tropics in Africa, India and South East Asia to Australia, New Zealand, Pacific Islands, West Indies, Central and South America (Maundu *et al*., 1999). It is a plant of diverse habitats in semi-arid bush land, in many types of dry woodland, sandy open woodland and dry hilly areas and coastal bush lands that grows at altitude range of sea level to 2000 m.a.s.l. (Debela Hunde *et al*., 2012). It is frequently found on coastal dunes, along water courses and on stony slopes. It grows on many soil types and on poor dry lands.

In different parts of Africa including Ethiopia, the different parts of this plant are used as a folk medicine for curing different diseases. Bark, roots and leaves are used to treat ailments such as leprosy, fever, headaches, ulcers and skin complaints (Watt and Breyer-Brandwijk, 1962). Ethnobotanical surveys also show that this plant has long been traditionally used to prepare medicines against malaria, ulcers, fever, edema, diarrhea and sexually transmitted diseases. Pharmacological studies seem to support some of these traditional medical uses. The crude extracts, especially aqueous and methanolic, showed several biological activities such as antimicrobial, antifungal, anticancer, antitrypanosomal, antirheumatic, antioxidant, analgesic, moluscicide, pesticidal, antipyretic and antifungal

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(Monte *et al*., 2012). Antimicrobial effects have been reported for water extracts of leaves, which could support the traditional use in treating infections and venereal diseases in Mali (Diallo *et al*., 2002). The presence of tannins, flavonoids, alkaloids, glycosides, phenols, saponins and volatile compounds in this plant supports its traditional uses (Mevy *et al*., 2006).

Besides its medicinal uses, *Ximenia Americana* uses as food for human and forage for livestock have been reported in Ethiopia and Kenya. The oil from the seeds was used as a cosmetic and skin ointment. The edible fruit was made into a type of beer, and the pulp is used as a preservative and to make jellies (Debela Hunde *et al*., 2012;Maundu *et al*., 1999).

Ximenia Americana is highly vulnerable plant due to overexploitation for different purposes, especially using the roots and barks for medicinal purposes. Seed germination is very poor, possibly due to insufficient pollination caused by long distances between male and female trees (Sacande and Vautier, 2006). As the plant is dioecious with separate male and female plants, both plants must grow in close proximity for efficient pollination to get fertile seeds. Despite these problems, the local people destruct the whole plant as they use the root and bark part for medicinal purpose, fruits for food and the remaining part for charcoal. As it is dioecious plant, identical clones of superior genotype cannot be obtained and propagated in nature.

In vitro propagation offers a great potential for large scale multiplication of such useful species for subsequent exploitation. Moreover, it can be used to propagate identical clones of superior genotypes in large quantities and high quality, and this is not possible with *X. americana* in nature. In addition, the development of a rapid *in vitro* plant propagation method using the shoot tip explants of this plantpromotes scientific activities including pharmacological studies and extraction of medicinally important compounds, commercial cultivation and sustainable use of the species. Reports on *in vitro* propagation of this species are limited (Aloufa *et al*., 2003). Therefore, considerable efforts are still required to find out efficient *in vitro* methods for the propagation of this highlyvulnerable butmultipurpose medicinal plant. The objective of this study was to develop *in vitro* propagation protocol using shoot tip explants of *X. americana*.

MATERIALS AND METHODS

Plant material

Yellow red, mature fruits of *X. Americana* were collected from Boosat district, east Shewa zone of Oromia regional state and around Gambella town of Gambella regional state, Ethiopia. The ripen fruits were collected by hand from the branches of the tree. The fruits were kept at room temperature. The seeds were extracted by rubbing the fruit on a wire mesh to remove the pulp, and then washing the seeds in running tap water to remove the mucilage. The seeds were then cleaned by hand sorting, and dried in the shade. Seed quality was assessed visually whereby damaged, infested or deformed seeds were discarded.

Sterilization and in vitro seed germination

Seed coat was removed to break dormancy and washed under running tap water. The dehusked seeds were soaked in distilled water for 24 h. The imbibed seeds were washed with detergent using distilled water followed by rinsing in 70% ethanol for 30 seconds. The seeds were then washed three times with sterile distilled water followed by surface sterilization with three concentration levels (25%, 35%, and 40%) of Clorox for exposure times of 15, 20 and 25 minutes. The seeds were washed three times with sterile distilled water and cultured horizontally in culture vessels containing 50 ml full strength MS (Murashige and Skoog, 1962) medium containing 3% sucrose and 0.7% agar. The culture vessels were sealed with parafilm and maintained in culture room under 16 h photoperiod at light intensity of 22 µmol $m^{-2}s^{-1}$ and a temperature of 25 ± 2°C using cool-white fluorescent lamps. Six seeds per culture vessels were cultured in five replications. The number of germinated seeds was recorded starting from two weeks after culture until no germination occurred.

Shoot initiation

Shoots excised from the seedlings of germinated seeds were cultured in baby food jars containing 50 ml full strength MSmedium supplemented with 0.0, 0.5, 0.75, 1.0, 1.50 and 2.0 mg/l benzyl amino purine (BAP) alone and kinetin alone containing 30% (w/v) sucrose and 0.7% agar. The pH of the medium was adjusted to 5.8 before addition of agar and autoclaved at 121°C for 15 min at a pressure of 105 KPa. Six explants per culture vessel and five replications per treatment were used. All cultures were maintained in culture room under 16 h photoperiod at light intensity of 22 µmol $m^{-2}s^{-1}$ and a temperature of 25 ± 2°C using cool-white fluorescent lamps. Number of shoots per explant and shoot length was recorded after one month of culture.

Shoot multiplication

Shoots from initiation medium were transferred to 50 ml full strength MSmedium containing BAPor kinetin (0.0, 0.25, 0.5, 0.75, 1.0, 1.50 and 2.0 mg/l) in combination with 0.0, 0.1 0.20, and 0.4 mg/l α-naphthalene acetic acid (NAA). Six shoots were cultured in each culture vessel in five replications per treatment. The cultures were maintained under culture conditions as for shoot initiation.

Rooting and acclimatization

For root induction, half strength MSmedium supplemented with 0.0, 0.25, 0.5, 0.75, 1.0, 2.0 mg/l NAA and indole-3-butyric acid (IBA) were used separately. The pH was adjusted to 5.8 prior to autoclaving. Shoots from shoot multiplication media were transferred to rooting media. Six explants per culture vessel in five replications were used. The cultures were maintained at culture conditions as for shoot initiation. Number of roots and root length per explant were recorded after four weeks of culture.

Well rooted plantlets were removed from the rooting media and the roots were washed under running tap water. The plantlets were planted in plastic pots containing a sterile garden soil. The pots were covered with aerated plastic bag and kept in the culture room for two weeks and watered at an interval of two-to-three days. After two weeks, the plants were shifted to greenhouse and covered with polyethylene bags. The polyethylene bags were gradually removed after two weeks. The number of survived plants was recorded after four weeks.

Data analysis

Data were subjected to analysis of variance (ANOVA) and least significant difference (LSD) test using statistical data analysis software SPSS20.0 version at 0.05 probability level.

RESULTS

Effect of different concentrations of sterilants and exposure time on X. americana in vitro seed germination

All seeds sterilized with 25%, 35% and 40% concentrations of commercial bleach (Clorox) and exposed to the sterilants for 25, 20 and 25 min, respectively were found to be free from any microbial contamination (Table 1). However, there were variable survival patterns under different seed sterilization treatments. Maximum germination (72.33%) of sterilized seeds was obtained from sterilization with 35% Clorox for 20 min exposure time.

Table 1. Effect of different concentrations of Clorox and exposure time on *in vitro* germination of *X. Americana* seeds.

Clorox (%)	Exposure time (min)	Clean explants (%)	Germination (%)
25	15	41.8	0.00
25	20	56.42	39.34
25	25	100.00	42.42
35	15	93.20	32.0
35	20	100.00	72.33
35	25	87.50	54..6
40	15	91.83	22.76
40	20	93.20	13.67
40	25	100.0	00

Shoot initiation

Shoots cultured on growth regulators free MS medium which was used as a control and shoots cultured on MSmedium supplemented with BAP or kinetin alone, showed a significant

variation ($p < 0.05$) in terms of number of shoots per explant and shoot length.Shoots cultured on MS medium supplemented with BAPor kinetin were able to initiate irrespective of their concentration (Fig. 1A). The highest mean shoot induction per explant (2.06±0.15) was obtained on MS medium supplemented with 0.5 mg/l BAPand lowest mean shoot induction per explant (1.20±0.07) was produced on MS medium supplemented with 2.0 mg/l kinetin (Table 2). ANOVA showed significant difference in number of shoots and shoot length among the treatments (Table 3).

Table 2. Effect of different concentrations of kinetin or BAP on *in vitro* shoot induction of *X. Americana.*

Kinetin (mg/l)	BAP (mg/l)	No. of shoots/ explant	Shoot length (cm)
0.0	0.0	1.43±0.04^{bcd}	1.26±0.34^{ab}
0.5	0.0	2.00±0.15^{a}	1.37±0.01^{a}
1.0	0.0	1.50±0. 09^{bc}	0.84±0.01^{bc}
1.5	0.0	1.63±0. 08^{ab}	0.78±0.02^{d}
2.0	0.0	1.20±0. 07^{cd}	0.98±0.02^{bcd}
0.0	0.5	2.06±0.15^{a}	0.89±0.01^{cd}
0.0	1.0	1.63±0.11^{abc}	1.26±0.014^{ab}
0.0	1.5	1.73±0.09^{ab}	0.96±0.02^{bcd}
0.0	2.0	1.26±0.08^{cd}	0.89±0.02^{bcd}

Means not connected by the same superscript in the same column are significantly different at 5% probability level. The values represent mean ± SE

Table 3. ANOVA of effect of kinetin and IBA on shoot initiation of *X. Americana.*

		Sum of Squares	Df	Mean Square	F	Sig.
Shoot number	Between Groups	20.163	7	2.880	7.785	.000
	Within Groups	85.833	232	.370		
	Total	105.996	239			
Shoot length	Between Groups	.608	7	.087	8.805	.000
	Within Groups	2.288	232	.010		
	Total	2.896	239			

Shoot multiplication

The responses of explants to BAP and kinetin alone or in combination with NAA on MS medium resulted in different rates of shoot multiplication. ANOVA revealed that the combinations and concentrations of BAP and kinetin with NAA showed highly significant ($p < 0.05$) effect on mean value of shoot number and shoot length (Tables 4, 5 and 6). There was no induction of multiple shoots on growth regulators free MSmedium. The shoots on multiplication medium resulted in different responses based on the different growth regulators composition.

Effect of BAP, kinetin and NAA on shoot multiplication

The highest mean shoot number per explant (4.16±0.17) was obtained on MS medium supplemented with 0.5 mg/l BAP (Table 4 and Fig. 1B). The lowest shoot number per explant (1.33±0. 09) was obtained on MS medium supplemented with 2.0 mg/l kinetin. Among different concentrations of BAP in combination with NAA, the highest mean shoot number per explant (3.32±0.05) was obtained on MS medium supplemented with 0.75 mg/l BAP in combination with 0.1 mg/l NAA (Table 5). ANOVA also showed significant difference in shoot number and length (Table 6). Similarly, different concentrations of kinetin in combination with NAA showed significant difference in shoot number and length. The highest mean shoot number per explant (2.64±0.05) was recorded on MS medium supplemented with 0.75 mg/l kinetin in combination with 0.1 mg/l NAA (Tables7 and 8).

Table 4. Effect of different concentrations of BAP or kinetin on shoot multiplication of *X. Americana.*

Kinetin (mg/l)	BAP (mg/l)	No. of shoots/ explants	Shoot length (cm)
0.0	0.0	1.0±0.011^{f}	4.42±0.12^{bcd}
0.0	0.25	2.86±0.19^{b}	4.49±0.08^{bc}
0.0	0.5	4.16±0.17^{a}	4.46±0.18^{bc}
0.0	0.75	2.56±0.15^{bc}	5.72±0.21^{a}
0.0	1.25	2.06±0.14^{cd}	3.14±0. 14de
0.0	1.75	1.93±0.15^{de}	2.27±0.09^{cde}
0.0	2.0	1.83±0.16^{def}	2.11±0.11^{f}
0.25	0.0	2.00±0.13^{cdef}	2.58±0.10^{def}
0.5	0.0	2.83±0.36^{bc}	3.96±0.09^{cd}
0.75	0.0	2.70±0.15^{bcd}	4.89±0.16^{b}
1.25	0.0	1.31±0.08^{f}	2.54±0.10^{def}
1.75	0.0	2.16±0. 15^{bcde}	3.31±0.09^{de}
2 .0	0.0	1.80±0. 10^{ef}	2.12±0.08^{ef}

Means not connected by the same superscript in the same column are significantly different at 5% probability level. The values represent mean ± SE

Table 5. Effect of different concentrations and combinations of BAP and NAA on shoot multiplication of *X. Americana.*

BAP (mg/l)	NAA (mg/l)	No. of shoots/explants	Shoot length (cm)
0.25	0.1	1.73±0.20^{bc}	3.91±0.25^{ab}
0.5	0.1	1.77±0.06^{bc}	3.76±0.22^{ab}
0.75	0.1	3.32±0.05^{a}	4.09±0.25^{a}
0.25	0.2	1.46±0.17^{bcd}	3.98±0.28^{ab}
0.5	0.2	1.53±0.05^{bcd}	2.80±0.29^{bc}
0.75	0.2	2.42±0.00^{b}	3.01±0.72^{bc}
0.25	0.4	1.13±0.14^{cd}	2.30±0.32^{bcd}
0.5	0.4	1.23±0.12^{cd}	2.02±0.31^{cd}
0.75	0.4	1.01±0.00^{d}	1.83±0.27^{d}

Means not connected by the same superscript in the same column are significantly different at 5% probability level. The values represent mean ± SE

Table 6. ANOVA showing effect of BAP in combination with NAA on shoot multiplication of *X. Americana.*

		Sum of Squares	df	Mean Square	F	Sig.
Shoot number	Between Groups	323.524	14	23.109	45.873	.000
	Within Groups	219.133	435	.504		
	Total	542.658	449			
Shoot length	Between Groups	130.056	14	9.290	12.245	.000
	Within Groups	330.019	435	.759		
	Total	460.075	449			

Table 7. Effect of different concentrations and combinations of kinetin and NAA on shoot multiplication of *X. Americana.*

Kinetin (mg/l)	NAA (mg/l)	No. of shoots/explants	Shoot length (cm)
0.25	0.1	1.53±0.20^{cd}	2.81±0.25^{abc}
0.5	0.1	1.87±0.06^{bc}	2.66±0.22^{bc}
0.75	0.1	2.64±0.05^{a}	1.88±0.28^{d}
0.25	0.2	1.56±0.17^{bcd}	3.10±0.25^{a}
0.5	0.2	1.93±0.05^{bc}	2.20±0.29^{cd}
0.75	0.2	1.82±0.00^{bc}	3.01±0.27^{a}
0.25	0.4	1.13±0.14^{d}	2.60±0.32^{bcd}
0.5	0.4	1.23±0.12^{cd}	2.92±0.31^{ab}
0.75	0.4	1.31±0.00^{cd}	1.63±0.27^{d}

Means not connected by the same superscript in the same column are significantly different at 5% probability level. The values represent mean ± SE

Table 8. ANOVA showing effect of kinetin in combination with NAA on shoot multiplication of *X. Americana.*

				Sum of Squares	Df	Mean Square	F	Sig.
Shoot number	Between Groups	(Combined)		142.333	11	12.939	66.154	.000
		Linear Term	Contrast	14.803	1	14.803	75.681	.000
			Deviation	127.531	10	12.753	65.202	.000
	Within Groups			68.067	348	.196		
	Total			210.400	359			
Shoot length	Between Groups	(Combined)		3.503	11	.318	33.791	.000
		Linear Term	Contrast	.079	1	.079	8.359	.004
			Deviation	3.425	10	.342	36.334	.000
	Within Groups			3.280	348	.009		
	Total			6.783	359			

Rooting and acclimatization

The shoots cultured on half strength MSbasal medium supplemented with different concentrations of IBA and NAA resulted in different rooting responses. The highest mean number of roots per shoot (3.36±0.69) was obtained on half strength MSmedium containing 0.5 mg/l IBA (Tables 9, 10 and Fig. 1C). The highest mean length of roots per explant (2.44±0.04 cm) was obtained on growth regulators free half strength MSmedium which was used as control. After one month of acclimatization (Fig. 1D), the survival rate of plants transferred to greenhouse was 100% and no aberrant plants were observed.

Table 9. Percentage of rooting, mean number of roots and mean root length obtained on half strength MS medium containing different concentrations of IBA and NAA.

PGR(mg/l)	Rooting (%)	No. of roots/ explant	Root length (cm)
0.0	0.0	0.0±0.0^{c}	0.0±0.0^{c}
IBA			
0.1	55	1.11±0.12^{b}	1.23±014^{b}
0.25	65.94	1.94±0.5^{b}	1.87±0.56^{b}
0.5	73.34	3.36±0. 69^{a}	1.74±0.88^{b}
1.0	70.33	3.08±0. 36^{ab}	2.11±0.40^{a}
2.0	67	2.14±0. 44^{b}	2.21±0.40^{a}
NAA			
0.1	60	1.12±0.18^{b}	1.51±0.11^{b}
0.25	63	1.46±0. 84^{b}	1.48±0.32^{b}
0.5	68.52	2.85±0.73^{ab}	1.53±0.41^{b}
1.0	64.7	1.7±0. 62^{b}	1.94±0.32^{b}
2.0	62	1.38±0.23^{b}	1.68±0.43^{b}

Means not connected by the same superscript in the same column are significantly different at 5% probability level. The values represent mean ± SE
PGR = Plant growth regulator

Table 10. ANOVA of effect of IAA and IBA on root number and root length of *X. Americana.*

		Sum of Squares	df	Mean Square	F	Sig.
Number of roots	Between Groups	70.003	9	7.778	1.148	.377
	Within Groups	135.515	20	6.776		
	Total	205.518	29			
Root length	Between Groups	2.719	9	.302	20.835	.000
	Within Groups	.290	20	.014		
	Total	3.009	29			

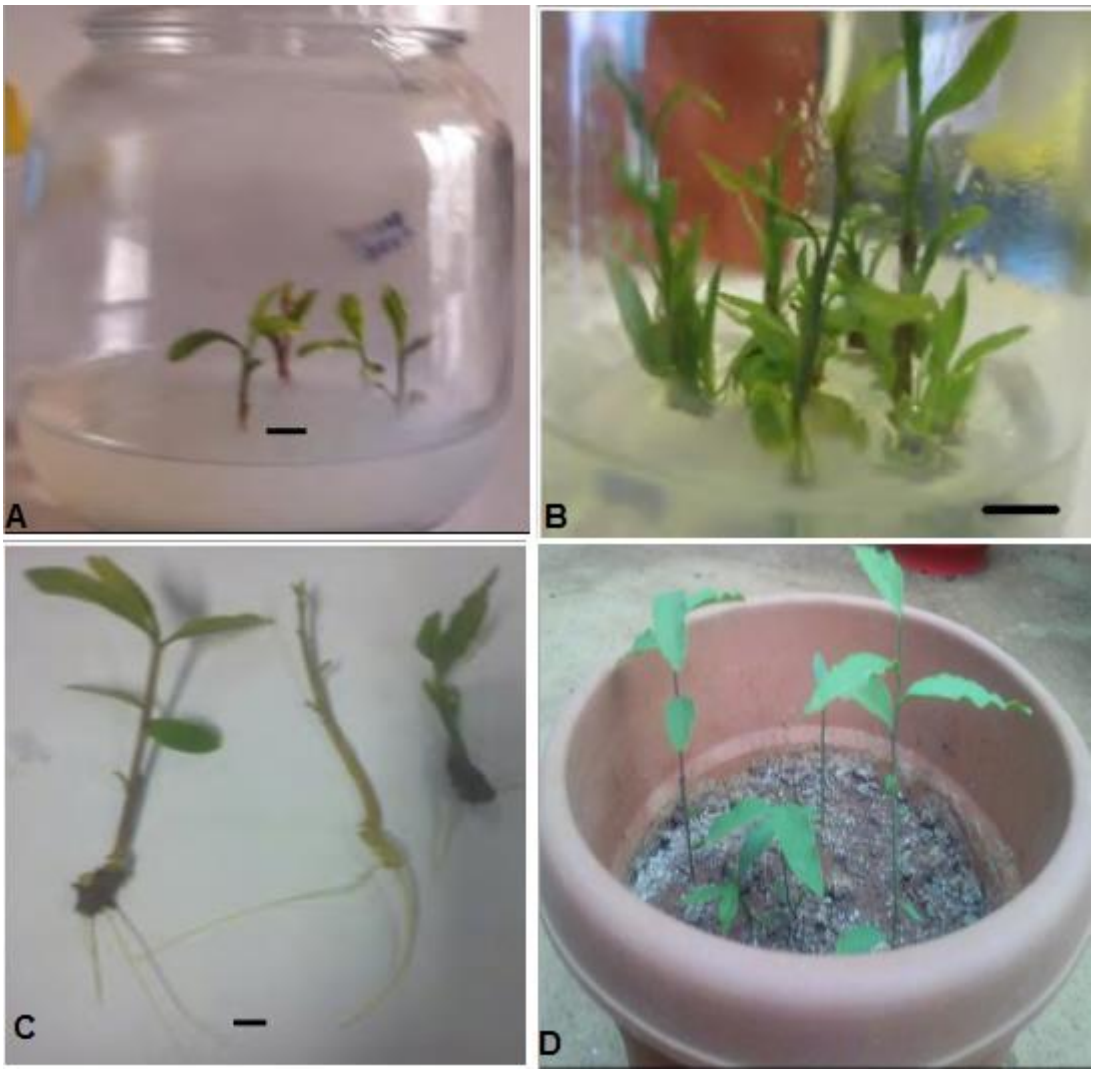

Figure1. ***In vitro*** **propagation of** ***Ximenia americana*****. (A) Shoot initiation on MS medium containing 0.5 mg/l kinetin. (B) Shoot multiplication on MS medium containing 0.5 mg/l BAP. (C) Rooting on ½ strength MS medium containing 0.5 mg/l IBA. (D) Acclimatization after three months. Scale bar: A = 0.4 cm; B,C,D = 1.5 cm.**

DISCUSSION

Sterilization and in vitro seed germination of X. americana

Concentration of Clorox, exposure time, and their interaction showed significant difference ($p < 0.05$) on overcoming contamination of growth media and improving survival and germination level of *X. americana in vitro* seed culture. This study showed the seeds responded differently to different concentrations of Clorox and exposure time. The highest mean percentage of clean surviving explants (72.33%) was obtained from seeds that were surface sterilized using 35% Clorox and 20 min exposure time. Further increase in exposure time led to a significant decline in percentage of surviving explants. The exposure time of sterilization is dependent on the type of tissue; i.e. seed tissue requires longer sterilization time than leaf tissue (Sharma and Nautiyal, 2009). In the present study, commercial bleach is effective to remove surface contaminants from *X. Americana* seed explants. The result is in agreement with report of Samani *et al*. (2014) who reported disinfection of field grown nodal explants of *Lantana camara* L. using 20% Clorox for 20 min exposure time and Dahab *et al*. (2010) who reported that the best results in sterilization of nodal explants for *Taxodium distichum* (L.) Richard

and *Taxodium distichum* (L.) Richardvar. *distichum* using 20% Clorox for 5 min followed by 0.2% mercuric chloride for 5 min.

Germination started on 20th day after culture and continued till 60 days. In previous report germination of *X. Americana* seeds started on day 16 and continued till day 38 in Kenya, which suggests the existence of some form of physiological dormancy (Maara *et al*., 2006). The difference between this report and our study in number of days of germination may be due to the difference in genotype and type of germination method. These authors suggested rapid germination may be an adaptation to produce seedlings to escape the adverse effects of unfavorable moisture conditions found in the area.

Shoot initiation

Different responses in shoot initiation were observed in all media containing different concentrations of growth regulators. Generally, media containing lower concentrations of BAPinduced more shoots than media containing comparative concentrations of knetin. The highest mean shoot number per explant (2.06±0.15) was recorded on MSmedium supplemented with 0.5 mg/l BAP. These results are similar to those of Hung and Stephen (2011) who obtained highest initiation (2.6 ± 0.2 shoots per explant) on full-strength MS medium containing 2.0 mg/l BAPalone. These authors also cultured node explants of African mahogany (*Khayasene galensis* (Desr.) A. Juss.) on medium containing1.0 mg/l BAPand obtained longer shoots with more nodes than media containing 4.0 mg/l and 8.0 mg/l BAP. The marked effects of BAPon shoot formation compared to kinetin as observed in this study may be due to high stability of BAP *in vitro* cultures. BAPis not easily broken down and therefore persists in the medium. Then larger amount of BAPexisting in free or ionized forms in the medium are readily available to plant tissues.

Shoot multiplication

Among all the treatments, the maximum number of shoots per explant was recorded on medium containing low concentrations of cytokinins. The most rapid and earliest proliferation was observed on mediacontaining lower concentrations of BAP alone. Proliferation of multiple shoots in different tree plants had been previously investigated with different growth regulators. The report by Aloufa *et al*. (2003) on *X. Americana* showed that MS medium supplemented with 2.5 mg/l BAP resulted in 6.6 mean number of shoots per explant from nodal explants. In the present study, the highest mean number of shoots per explant (4.16 ± 0.17) was recorded on MS medium supplemented with 0.5 mg/l BAP. The result reported by Aloufa *et al*. (2003) is slightly higher than the present study. This difference may occur due to the genotype difference and type and source of explants. These authors cultured nodal explants of *X. Americana* on MS medium containing 0.17 to 3.38 mg/l BAPand found that the number of shoots per explant increased with the increase in the level of cytokinin and then declined. The present study also showed this trend. For *X. americana*, the highest concentration (2.0 mg/l) BAPused in the present study inhibited shoot proliferation resulting in 1.83 ± 0.16 shoots per explant. As Arab *et al*. (2014) explained the high proliferation rate in MS medium containing 1.0 mg/l BAPcompared to the low proliferation rate in MS medium containing 1.25 mg/l BAP caused a reduction in shoot length. Higher concentrations of BAP had an inhibitory effect on proliferation. Borthakur *et al*. (2011) investigated different concentrations of BAP alone or in combination with NAA in *Albiziaodor atissima* (Bansa) and found that MS medium supplemented with 0.74 mg/l BAP resulted in the highest number of shoots per explant from apical buds of seven-day-old *in vitro* seedlings. In both studies best result was obtained relatively at low concentration of BAP. On the other hand, the highest shoot number per explant (2.83 ± 0.36) was obtained on MS medium supplemented with 0.5 mg/l kinetin. However, further increase in the concentration of kinetin from 0.5 to 2.0 mg/l significantly decreased mean shoot number per explant from 2.83 ± 0.36 to 1.80 ± 0.10. This also shows the same trend with BAP in inhibiting shoot proliferation as concentration increased.

Shoot explants cultured on MS medium supplemented with 0.75 mg/l BAPin combination with 0.1 mg/l NAA exhibited both the highest mean number of shoot (3.23±0.9) and mean length of shoots (3.98± 0.28 cm) per explant. The result from combined effect of NAA and BAP was found to vary with the concentration of NAA and BAP in two parameters of shoot growth (mean number of shoot and shoot length). This is due to the effect of cytokinin as it promotes the axillary branching or axillary bud proliferation (Vieitez and Vieitez, 1980). Combining BAP with NAA on MS medium

was comparatively more effective than kinetin with NAA for efficient shoot proliferation from shoot explants of *X. americana*. Previous report on *in vitro* propagation of *Melia azedarach* L. showed that BAP in combination with kinetin is more effective than kinetin with NAA for shoot proliferation (Husain and Anis, 2009). BAP was found to be the most suitable cytokinin for mass micropropagation of *Sature japunctate* (Benth.) Briq. (Indrias Teshome *et al.*, 2016). Among explants cultured on MS medium supplemented with different concentrations of kinetin along with NAA, the highest mean shoot number per explant (2.64±0.05) was obtained on 0.75 mg/l kinetin in combination with 0.1 mg/l NAA. Increase in the concentration of NAA from 0.1 to 0.4 mg/l showed a reduction of mean shoot number per explant from 2.64 ± 0.05 to 1.31 ± 0.00.

Rooting and acclimatization

Auxins are mainly used in root induction and their effect varies with type and concentrations used in different plant species (Swamy *et al*., 2002). In the present study, ANOVA revealed that root number and root length varied significantly with half strength MS medium supplemented with NAA and IBA. Applications of IBA alone exhibited the highest mean root number per shoot as compared to NAA. The highest mean root number per shoot (3.36± 0.69) was recorded on 0.5 mg/l IBA and mean root length (2.21±0.40 cm) was recorded on 2.0 mg/l IBA. This is similar to the results of Dahab *et al*. (2010) and Aloufa *et al*. (2003). The number of roots produced per shoot increased when concentrations of IBA increased from 0.1 mg/l to 0.50 mg/l. However, further increase in the concentration of IBA to 2.0 mg/l showed a reduction in the mean root number per shoot which is in *al*. (2005). As the present data show using lower concentrations of auxin is significantly better than higher concentrations for root induction and elongation. Ethylene deposition inhibits root elongation at higher concentration of growth regulators (Weiler, 1984). Auxins of all types stimulate plant cell to produce ethylene, especially when high amount of synthetic auxins are used. Ethylene retards root elongation. According to this author, the other reason for reduced response of root number and root length at higher concentration of auxin may be poor vascular connection of the root with the stem because of the interventions of callus. Moreover, the optimum concentration may be between 0.1 and 0.5 mg/l as the present result indicated. In the present study, IBA was found to be more effective in increasing root number and length than NAA.

Plantlets were transferred to polyethylene bags containing sterile garden soil and placed in the growth chamber for two weeks. After two weeks, the polyethylene bag covers were removed and transferred to greenhouse where 100% plants survived. This result showed much higher survival percentage than previous report by Aloufa *et al*. (2003) where 80% of the plantlets taken from auxin supplemented media survived versus 15% of those taken from auxin free media.

CONCLUSIONS

Ximenia Americana propagates by seed which has low percentage of germination as it is dioecious plant. There is efficient pollination only when both male and female plants grow in close proximity. Moreover, as identical clones cannot be naturally produced from this plant, superior genotypes can be clonally propagated by *in vitro* propagation using explants from mature trees. Thus, this protocol can be suitably exploited for the large scale production of cloned plants for their rehabilitation in natural habitat, sustainable utilization of this valuable medicinal plant, propagation of genetically uniform plant for commercial purpose and conservation. BAPis the most important cytokinin for initiation and multiplication of *X. americana*. The highest mean number of shoots per explant was 4.16 ± 0. 17 on full strength MSmedium supplemented with 0.5 mg/l BAP alone for shoot multiplication and half MSstrength supplemented with 0.5 mg/l IBA for root induction.

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