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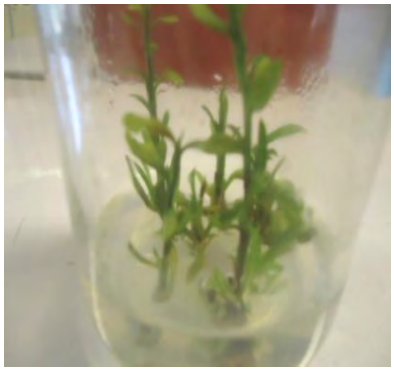
Micropropagation of *Ximenia americana* L. from shoot tip explants



A Thesis Submitted to the School of Graduate Studies, Addis Ababa University, in Partial Fulfilment of the Requirements for the Degree of Master of Science in Biotechnology

By

Keredin Mohamed

Addis Ababa, Ethiopia

June, 2017

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ABSTRACT: Ximenia americana *is a critically threatened multipurpose plant. It is among the most important medicinal plants, valued primarily for its fruit, roots, leaves and bark parts. This study aimed to develop micropropagation protocol for* Ximenia americana *using shoot tip explants. The study started with seed sterilization test using different clorox concentration and exposure time. Shoot tips from* in vitro *germinated seedlings were cultured on shoot initiation MS medium supplemented with 0.0, 0.5, 1.0, 1.5 and 2.0 mg/l BAP alone or KN alone. Explants were cultured on shoot proliferation media fortified with kinetin or BAP, each at 0.0, 0.25, 0.5, 0.75, 1.25, 1.75 and 2.0 mg/l in combination with 0.0, 0.1, 0.20 and 0.40 mg/l NAA on full salt strength MS medium. For rooting, half strength MS media supplemented with IBA and NAA alone each at 0.0, 0.1, 0.25, 0.5, 1.0, and 2 mg/l were used. Growth regulator free MS medium was used as control. Study results showed that 73.32% germination was recorded on 35% clorox concentration within 20 minutes. Highest mean shoot number per explants on initiation medium (2.06±0.15) and multiplication medium (4.16±0.17) were recorded on MS medium supplemented with 0.5mg/l BAP. Best rooting was obtained on half strength MS media supplemented with 0.5mg/l IBA with 3.36±0.69 roots per shoot and 2.21±0.40 cm root length on 2.0 mg/l IBA and established in greenhouse with 100% survival. The results show that, the study is important for mass propagation, rehabilitation in their natural habitat and conservation of this threatened multipurpose plant.*

Key words: Micropropagation, Plant Growth Regulator, shoot tip, *Ximenia americana*

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List of Abbreviations

BAP	6-Benzyl Amino Purine
IAA	Idol acetic acid
GA3	Gibberrellic Acid
IBA	Indol-3-Butyric Acid
KIN	Kinetin
MS	Murashige and Skoog basal media
NAA	Naphthalene acetic acid
PGR	Plant Growth Regulator

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1. INTRODUCTION

1.1. Background of the study

The use of traditional medicine throughout the world increases, especially in developing world rely on traditional medicinal products as a primary source of healthcare and traditional medical practice which involves the use of plants (WHO, 2004). *Ximenia americana* L. is very important plant uses for humans, livestock and wild life as medicine, food, habitat and environmental services. In different parts of Africa including Ethiopia, the different parts of this plant 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)). Ethno botanical 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, antrheumatic, antioxidant, analgesic, moluscicide, pesticidal, antipyretic and antifungal (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 and alkaloids glycosides, phenols, saponins and volatile compounds support traditional uses of this plant (Mevy *et al*., 2006).

Besides its medicinal uses, *Ximenia americana* uses as a food source for human and livestock forage has been reported in Ethiopian 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 and Maundu *et al*., 1999). Also, it is an apicultural plant uses for honey production in Morocco (Tchuenguem *et al*., 1997)

Ximenia americana L., belongs to family Olacaceae, commonly known as wild plum, blue sour plum and tallow nut. It named hudha in Afaan Oromo and Inkoy in Amharic language. It is scrambling spiny shrub or small tree up to 6 m. *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. 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 (0-2000 m.a.l) above sea level (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.

X. americana is critically threatened plant due to overexploitation for different purposes, especially using the roots and barks as a medicine may increase threatening of the plant and seed germination is very poor, possibly the lack of viable seeds may be due to insufficient pollination, caused by long distances between male and female trees (Sacande & Vautier., 2006). 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.

Therefore, micropropagation offers a great potential for large scale multiplication of such useful species for subsequent exploitation. The development of a rapid *in vitro* plant propagation method using the shoot tip explants of *Ximenia americana* promotes scientific activities including pharmacological studies and extraction of medicinally important compounds, commercial cultivation and sustainable use of the species. Reports in this area are so limited. Therefore, considerable efforts are still required to find out efficient *in vitro* methods for the propagation of this critically threatened medicinal plant. The objective of this study was to

develop optimal micropropagation protocol from *in vitro* germinated shoot tips of *X. americana* seed. This will have its own contribution to improve multiplication and conservation of this plant.

2. LITERATURE REVIEW

2.1. Description, ecology, taxonomy and distribution of *Ximenia americana*

Ximenia americana L., belongs to family *Olacaceae*, commonly known as wild plum, blue sour plum and tallow nut. It is spreading or, less often, scrambling spiny shrub or small tree up to 6 m, commonly less than 4 m. Branches normally arching down often armed with straight spines. Leaves are simple, alternate or clustered on spur shoots with rounded and slightly notched; broadly tapering base or rounded and occasionally softly haired. Small greenish, white fragrant flowers were born on short shoots (Sacande & Vautier, 2006). The flowers are unisexual and male and female flowers occur on different plants. Flowering and fruiting varies between localities, but flowering typically occurs in the dry season. In southern Africa, flowering occurs in September to December, with fruiting taking place in December to February. In many places it flowers and fruits throughout the year. On good sites trees may produce fruit after 3 years of growth. Fruits, up to 3 cm long, oval, shiny, light green, turning yellow, orange or red on ripening. The fruit is yellow-red edible drupe which is oval, approximately 2.5 cm in diameter and contains one large endospermic seed within its green pulp containing a small embryo near a thin testa. They have up to 60% oil content. The fruits are dispersed by animals. It grows on many soil types; however, they are often on poor and dry. This species is a root hemi parasite, i.e. it is able to take water and nutrients from other plants through the roots, but does not depend on this for survival.

Ximenia was named after a Spanish monk, Francisco ximenez. The specific name is the Latin form of ‘American. *Ximenia* is a tropical and subtropical genus, root-parasite which is composed of eight species *Ximenia americana, Ximenia roiigi, Ximenia aegyptiaca, Ximenia parviflora,*

Ximenia aculeata, Ximenia gabonensis, Ximenia caffra, and Ximenia coriaceae (Brasileiro *et al*., 2008).

Among those species *Ximenia americana* L. globally widespread throughout the tropics in Africa, India and South East Asia to Australia, New Zealand, Pacific Islands, West Indies, Central and South America. 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 (0-2000 m.a.s) (Maundu *et al*., 1999). It is frequently found on coastal dunes, along water courses and on stony slopes. It occurs at altitudes up to 2000 m.a.s.l. and where rainfall exceeds 500 mm per year. It grows on many soil types; however, often on poor and dry (Sacande & Vautier, 2006). Generally, it is grown in Ethiopia's Flora from 500-2100 m.a.s.l (Vollesen, 1989) which is relatively narrow altitudinal range.

2.2. Significance and uses of *Ximenia americana*

Medicinal uses

This species is widely used in folk medicine of different countries to treat several human ailments and livestock. Data from pharmacological studies seem to support some of these traditional medical uses. Fruits of *X. americana* are used in Burkinafaso as a remedy for constipation and as a natural source for astringent and tonic purposes (Meda *et al*., 2008). Bark and roots are used for healing skin diseases (ulcers or wounds) in Mali (Grønhaug *et al*., 2008). In western tropical Africa, the roots have been used to treat 'sleeping sickness', in combination with those of *Annona chrysophylla* Boj and febrile headache (Okigbo *et al*., 2009). The roots have also been used in different African countries as an antiseptic to treat fever, edema, diarrhea, sexually transmitted diseases and as an antidote or cure for poisons (Okigbo *et al*., 2009). A decoction of leafy twigs is given in Zimbabwe to heal febrile colds and cough and as a laxative

(Okigbo *et al*., 2009); in Nigeria, the same preparation is used as a mouth wash and to relieve toothache Leaves, barks, peeling and roots are used in different African countries for treating toothaches, mumps and conjunctivitis.

Food and other values of X. americana

Wild edible plants are valuable resources for improving food, nutritional security and income of households living in dry land areas, to supplement the staple food, to fill seasonal food shortages, and as emergency food during famine **(**Assegid and Tesfaye, 2011). The fruits, as well as being pleasant to eat raw, can be used to make juice, jams and jellies, or an intoxicating drink. The pulp of seed and fruit contains hydrocyanic acid, and it is advisable not to chew the seed. Kernel oil is used as a vegetable butter (oil) and as a ghee substitute. The oil from the seed has multiple uses; it is traditionally used to soften leather, as well as being used as a cosmetic and skin ointment. Young leaves are edible after thorough cooking. In leather tanning, bark is used for tanning and strengthens indigo dyes. It contains approximately 17% tannin, which gives leather a reddish colour. Roots are also used in tanning. The fruit yields up to 67.4% oil from the seed that has been used as a body and hair oil. The oil is not edible, and the presence of a rubberlike substance excludes it from many industrial uses. Heartwood contains an essential oil used for fumigation. The flowers have an essential oil that could be a good substitute for orange blossom. In South Africa, the fruits have been used to make a kind of beer (Orwa *et al.,* 2009) and in Morocco; flower is very highly nectariferous and polliniferous bee plant that used for honey production (Tchuenguem *et al.,* 2009). It provides environmental service in well adapted to poor, dry soil and also grows well in wet soils, drought resistant , tolerant to salinity and used as ornamental plant (Sacande & Vautier, 2006).

2.3. Ximenia americana propagation and its challenges

Ximenia americana is not cultivated. It regenerates naturally from the seed in the forested areas Seeds are normally dispersed by animal. It is propagated by seed. Seed germination is very poor, possibly the lack of viable seeds may be due to drying , low seed moisture content, temperature and insufficient pollination, caused by long distances between male and female trees. Seedling morphology is variable, when young the leaves are densely hairy, but become smooth and shiny with growth due to above problems (Maundu *et al*., 1999; Sacande & Vautier, 2006). The flowers are unisexual *i.e.* male and female flowers occur on different plants, Also flowering and fruiting varies between localities (Sacande & Vautier, 2006). Despite these problems, the local people destruct the whole plant as they use the root, bark and leaves for medicinal and remaining part for charcoal purpose. Propagation through seeds is time consuming to accomplish large scale production for conservation and cultivation of the species.

2.4. Plant tissue culture and its application

Tissue culture is the *in vitro* aseptic culture of cells, tissues, organs or whole plant under controlled nutritional and environmental conditions often to produce the clones of plants (Thorpe, 2007). Along with the totipotent potential of plant cell, the capacity of cells to alter their metabolism, growth and development is also equally important and crucial to regenerate the entire plant (Thorpe, 2007). Tissue culture techniques are being increasingly exploited for clonal multiplication and *in vitro* conservation of valuable indigenous germplasm threatened to extinction. Greater demand for plants especially for the purpose of food and medicine is one of the causes of their rapid depletion from primary habitats. Micropropagation offers a great potential for large scale multiplication of such useful species for subsequent exploitation (Boro *et al*., 1998**).**

In vitro cell and organ culture offers an alternative source for the conservation of endangered genotypes (Sengar *et al.,* 2010). Germplasm conservation worldwide is increasingly becoming an essential activity due to the high rate of disappearance of plant species and the increased need for safeguarding the floristic patrimony of the countries (Filho *et al.,* 2005). Tissue culture protocols can be used for preservation of vegetative tissues when the targets for conservation are clones instead of seeds, to keep the genetic background of a crop and to avoid the loss of the conserved patrimony due to natural disasters, biotic or abiotic stress (Tyagi *et al.,* 2007). The plant species which do not produce seeds (sterile plants) or which have 'recalcitrant' seeds that cannot be stored for long period of time can successfully be preserved via *in vitro* techniques for the maintenance of gene banks (Maara *etal*., 2006). Clonal propagation of plants from shoot tips, meristem tips, or nodal explants, usually has an accelerated proliferation of (axillary) shoots during subcultures. Development of these types of protocols for tropical tree species is important for the mass production of Clonal germplasm, that otherwise may be lost because of the inability to propagate this material or the incompatibility of plant material using conventional propagation methods (e.g. rooted cuttings or grafting)**.** Considerable work has been done in the last few years on *in vitro* propagation of woody species (Paula *et al.,* 2012). Micropropagation of *X. americana* using axillary buds from mature plants by (Aloufa *et al*., 2003) provides an alternative for its rapid clonal propagation of this plants. *In vitro* propagation has been accomplished for *Albizia lebbeck* using three different explants to developing a successful tissue culture protocol for this plant using cotyledons, *in vitro* seedling derived shoots, or nodal segments from juvenile trees. Cotyledonary segments responded preferentially producing shoots at a rate of 83%., however the highest shoot regeneration for nodal segments was reported when low BAP concentration was used (Mamun *et al*. 2004). Also successfully tissue culture protocol was developed for shoot

proliferation and plantlet formation of *Khaya senegalensis* using shoot tips on medium containing benzyl adenine (Hung and Stephen 2011).

2.5. Advantages of Micropropagation

Plant tissue culture is the science or art of growing plant cells, tissues or organs on artificial media by isolating them from the mother plant. It is based on the cell doctrine that states a cell is capable of autonomy and is potentially totipotent. The most important kinds of organ cultures used for Micropropagation are meristem cultures, shoot cultures, embryo cultures and isolated root cultures. Micropropagation is one form of tissue culture which allows the production of large number of plants from small pieces of the mother plant in relatively short period of time and limited space. It is an aseptic process which requires sophisticated laboratory procedure with unique facilities and special skills (Hartman *et al*., 2004). In the shoot proliferation stage, it consists of the *in vitro* establishment of suitable pieces of tissue, free from obvious contamination, where as in the multiplication stage, each explants has expanded in to a cluster of microshoots. *In vitro* propagation via organogenesis usually involves five stages including plant material selection, initiation of cultures, multiplication of shoots, rooting of shoots, and acclimatization of plants (Cardoza, 2008).

Although living cells are considered potentially totipotent, only some cells that are competent divide and give rise to complete plant in tissue culture. Furthermore, not all plant species are equally amenable to tissue culture. Production or improvement of perennial plants, both woody and herbaceous, using tissue culture especially for cloning and genetic engineering seem very attractive, the complex seasonal cycles and life cycles of those plants complicate the control of their growth in tissue culture. The early establishment of shoot cultures for these perennial plants is one of the important approaches. Stabilized shoot cultures are excellent sources of cells,

tissues and organs that can be used in further complex procedures such as protoplast generation. (Rasool, 2013) gene insertion and transclone recovery. If shoot cultures cannot be readily established, these advantages cannot be realized. Therefore, a major cause of recalcitrance in perennial plants is the inability to establish fully stabilized shoot cultures. Establishing shoot culture involves multiple steps. Murashige (1978) generalized these steps into three stages; aseptic culture establishment, multiplication of propagula and preparation for reestablishment of plants in soil. It is difficult to generate stabilized shoot cultures for plants that have seasonal growth dynamics dominated by strong episodic or determinant shoot growth. The relatively slow growth rate of perennials in culture also complicates the tissue culture procedures as many perennial tissues release high content of phenolic compounds into a culture medium. In some cases, some technical approaches can overcome those limitations in tissue culture. However, development of a deeper understanding of physiological bases of such genetically predetermined phenomena is important

Micropropagation confers distinct advantages not possible with conventional propagation method. It is possible to multiply single explants into several thousands in less than a year. Actively dividing cultures are continuous sources of plantlets without seasonal interruption. It has high commercial potential due to the speed of propagation, clonal propagation, germplasm conservation, genetic transformation and its high quality and ability to produce disease-free plants (Tileye Feyissa *et al*., 2005 and Hartman *et al*., 2004).

2.6. Source of Explants and Aseptic Techniques

Tissue culture success mainly depends on the age, types and position of explants because not all plant cells have the same ability to express tot potency (Sasikumar *etal.*, 2009; Murashige and Skoog, 1962). The most commonly used explants are shoot tips, nodal buds and root tips. Large

explants can increase chances of contamination and small explants like meristem can sometimes show less growth (Fowler, 1993).

Most surface contaminants such as bacteria and fungi can be eliminated by surface sterilizing the plant material with a suitable sterilizing agent. Surface sterilizing agents are normally applied for 10-15 minutes. Under aseptic conditions, the sterilizing solutions are then removed and the plant material washed 3 or 4 times for 5 minutes each time by agitation in sterile distilled water. Washing is important to remove excess sterilizing agent which inhibits plant growth (Rolando *et al*., 1986). Explants may also be surface sterilized with an aqueous solution of sodium or calcium hypochlorite. The calcium salt is preferred as it is less phytotoxic. Many laboratories use a household's bleach such as clorox. These commercial products usually contain 5.25% NaOCl as the active agent, when diluted with water (1part bleach: 9 parts water), the final sterilizing solution contains not less than 0.5 % NaOCl (Rolando *et al*., 1986). Sterilization of laboratory instruments is carried out by autoclaving, alcohol washing, baking, radiations, flaming and fumigation (Rayns and Hunter, 1993).

2.7. **Composition of Culture Medium requirements for optimal growth of a tissue**

Tissues from different parts of a plant may have different requirements for satisfactory growth. The composition of culture medium is a major determinant of *in vitro* growth of plants. Plant tissue culture medium contains all the nutrients required for the normal growth and development of plants. It is mainly composed of macronutrients, micronutrients, vitamins, other organic components, plant growth regulators, carbon source and some gelling agents in case of solid medium.

According to the recommendations of the International Association for Plant Physiologists, the elements required by plants in concentrations greater than 0.5 mmol 1^{-1} are referred to as macro elements, relatively large amounts of some inorganic elements (the so-called major plant nutrients): ions of nitrogen (N), potassium (K), calcium (Ca), phosphorus (P), magnesium (Mg) and sulphur (S); and those in concentrations less than 0.5 mmol 1^{-1} are microelements, small quantities of other elements (minor plant nutrients or trace elements): iron (Fe), nickel (Ni), chlorine (Cl), manganese (Mn), zinc (Zn), boron (B), copper (Cu), and molybdenum (Mo). (De Fo ssard, 1976).

Carbohydrates play an important role in *in vitro* cultures as an energy and carbon source, as well as an osmotic agent. In addition, carbohydrate-modulated gene expression in plants is known (Koch, 1996). Sugar is an important component in medium and its addition is essential for *in vitro* growth and development of plants because photosynthesis is insufficient, due to the growth taking place in conditions unsuitable for photosynthesis or without photosynthesis. The sugar concentration chosen is dependent on the type and age of growth material; very young embryos require a relatively high sugar concentration. Generally growth and development increases with sugar concentration, until an optimum is reached and then decreases at high concentrations. The most commonly used source of carbon is sucrose at a concentration of 2- 5%. However, glucose and fructose are also known to support good growth of some tissues. Variation in shoot response was observed in different sugars and lower concentration of dextrose is found to enhance the root and shoot growth in comparison to sucrose and maltose. Sucrose has been replaced by dextrose in rice grain culture and found to be more efficient and can be used for further tissue culture experiments (Bhojwani, 1996).

Four vitamins; *myo*inositol, thiamine, nicotinic acid, and pyridoxine are ingredients of Murashige and Skoog (1962) medium and have been used in varying proportions for the culture of tissues of many plant species . The requirements of cells for added vitamins vary according to the nature of the plant and the type of culture.

2.8. Plant Growth Regulators

The highest rate of micropropagation often depends not only on the selection of the most suitable explants, but also on the discovery of the correct combination of growth regulators, and/or the best nutritional composition of the medium for particular explants. The growth regulators are required in very minute quantities. There are many synthetic substances having growth regulatory activity, with differences in activity and species specificity. It often requires testing of various types, concentrations and mixtures of growth substances during the development of a tissue culture protocol for a new plant species (Bhojwani, 1996).

Auxins (IAA, IBA, NAA and 2, 4-D) are involved in the regulation of several physiological processes. These growth regulators generally cause cell elongation and swelling of tissues, cell division, callus formation and the formation of adventitious roots as well as the inhibition of adventitious and axillary shoot formation (Pierik, 1997). Also auxins are often added to the culture medium to promote the growth of callus, cell suspensions or organs, and to regulate morphogenesis, especially in combination with cytokinins (George, 1993). IBA and IAA are widely used for rooting and, in interaction with cytokinins, for shoot proliferation. 2, 4-D and 2, 4, 5-T are very effective for the induction and growth of callus. 2, 4-D is also an important factor for the induction of somatic embryogenesis and usually used after dissolved in ethanol or dilute NaOH (Bhojwani, 1996).

Cytokinins are derived from adenine (aminopurine) and play an important role in the *in vitro* manipulation of plant cells and tissues. The most common cytokinins used are kinetin, 6-BenzylAminoPurine (BAP), thidiazuron (TDZ), zeatin and 2iP (Pierik, 1997). These hormones are concerned with cell division, modification of apical dominance, shoot differentiation, etc. In tissue culture media, cytokinins are incorporated mainly to initiate cell division and differentiation of adventitious shoots from callus and organs. These compounds are also used for shoot proliferation by the release of axillary buds from apical dominance (Bhojwani, 1996).

Gibberellins are a group of compounds that are not necessarily used in the *in vitro* culture of higher plants. In some species, these growth regulators are required to enhance and in others to inhibit growth Gibberrellic acid (GA3) is the most common gibberellins used in tissue culture. It induces the elongation of internodes and the growth of meristem or buds *in vitro* (Razdan, 1993).

All kinds of plant tissue cultures produce ethylene, and the rate of production increases under stress conditions. In cultures, ethylene is also produced when the organic constituents of the medium are subjected to heat, oxidation, sunlight or ionizing radiation. Abscisic acid is most often required for normal growth and development of somatic embryos and only in its presence do they closely resemble zygotic embryos (Ammirato, 1988). It is also known to promote morphogenesis in *Begonia* cultures. There has been some interest in the application of growth retardants, such as paclobutrazol, during the acclimatization stage of micropropagation to reduce hyperhydricity and regulate leaf growth and function in relation to control of water stress (Smith and Krikorian, 1990)

3. OBJECTIVE

3.1. General objective

To develop efficient *in vitro* propagation protocol for *Ximenia americana* from shoot tip explants

3.2. Specific objectives

- ✓ To determine and establish seed sterilization protocol for *in vitro* seed germination
- ✓ To develop and determine optimum concentration of growth regulators for shoot induction and multiplication from shoot tip explants and
- ✓ To determine optimum concentration of auxins for rooting and to acclimatize *in vitro* rooted *X. americana* plantlets in greenhouse

4. MATERIALS AND METHODS

The study was conducted at Plant Tissue Culture and Molecular Biology laboratory, Institute of Biotechnology, Addis Ababa University.

4.1. Plant material

Yellow red, mature fruits of *X. americana* were collected from Boosat district, east Shewa zone of Oromia regional state and around Gambela town of Gambela regional state. 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.

4.2. Experimental Procedures

4.2.1. Preparation of stock solutions

Macronutrients, micronutrients, and vitamins of Murashige and Skoog (1962) nutrient media components were used. Stock solutions of major and minor salts, vitamins and plant growth regulators (PGRs) were prepared by dissolving the required quantity of chemicals in distilled water. The stock solutions of nutrients were prepared freshly every month while vitamins and PGRs every two weeks. All of the MS stocks were stored in refrigerator at +4 $^{\circ}$C.

4.2.2. Plant growth regulators stock solution preparation

Plant growth regulators (PGRs) were prepared in 1mg/ml concentration. The cytokinins used for the study were the 6- benzyl amimopurine (BAP) and kinetin (KIN) whereas the auxins were indol-3- butyric acid (IBA) and Naphthalene acetic acid (NAA). The powdered crystal of the PGRs was first weighed and dissolved in 3-4 drops of 1N NaOH. Upon complete dissolution,

the solution of each PGR was poured into labelled 50 ml plastic bottles and filled with double distilled water to the required volume. Then gently stirred and stored at a temperature of +4 °C for short term use.

4.2.3. Culture media preparation

In this studies, the culture media for seed germination, shoot initiation and shoot multiplication contained full strength MS basal medium (50 ml macronutrient, 5 ml micronutrient, and 5 ml vitamin per litter), 30 g/l sucrose and with or without (for control) PGRs. In the second group of experiments, half strength MS basal medium was used with different concentrations of IBA or NAA for rooting. Seven gram per litter plant tissue culture agar was used as a solidifying agent throughout the experiment. Finally, the pH of all media was adjusted to 5.8 by using 1N HCl and/or NaOH before addition of agar.

The medium was boiled on a stove until the agar melted. Then, 40 ml of the prepared medium was dispensed into baby food jar. The culture vessels were covered with caps immediately after dispensing the medium and autoclaved by steam sterilization at a temperature of 121°C and 105 KPa pressure for 15 minutes. Immediately after autoclaving, the medium was taken and kept in lamina-air-flow-cabinet bench until used.

4.2.4. Explants surface sterilization and in vitro seed germination

Seed coat was removed to break dormancy and washed with tap water to remove debris. The dehusked seeds were soaked in distilled water for 24 h. Then the imbibed seeds were washed by distilled water with powder soap. Then, the seeds were treated with 70% ethanol for 30 seconds under laminar air flow cabinet. After pre-treatment with ethanol, the seed were rinsed with autoclaved distilled water three times, to lower the toxic effect of ethanol. Then they were treated with three concentration levels (25%, 35%, and 40%) of clorox for varying exposure times (15,

20 and 25 minutes). After decanting the sterilizing solutions under safe condition, the seeds were washed three times each for five minutes with autoclaved distilled water to remove traces of clorox. The sterilized seeds were cultured horizontally in culture vessels containing 50 ml of MS medium fortified with 3% sucrose and 7g/l agar. The culture vessels containing the cultured seeds were properly sealed with parafilm and labelled. Thereafter, the cultures were transferred and randomly placed on the growth room shelf with a photoperiod of 16/8h light/dark using cool-white fluorescent lamps and light intensity of 22 µmol m^{-2} s^{-1} at 25 ± 2°C and relative humidity of 70-80%. For each sterilization treatments five replication each with six seeds per explants were used. Data recorded from the sterilization experiment included the number of germinated and survived (clean) cultures after 15 days of culturing till no further germination occurred. Then the data were converted into percentage.

4.2.5. Shoot initiation

The germinated shoots were cultured in baby food jar containing 50 ml full strength MS medium supplemented with 0.0, 0.5, 0.75, 1.0, 1.50 and 2.0 mg/l BAP alone and kinetin alone containing 30g/l sucrose and 7g/l agar. Six explants per culture vessel and 5 replications per treatment were used. All cultures were maintained under white fluorescent light at light intensity of 22 µmol m^{-2} s^{-1} with 16 h photoperiod at 25±2°C temperature. Number of shoots per explants and shoot length were recorded after one month of culture.

4.2.6. Shoot multiplication

Proliferated shoots were used as explants in shoot multiplication. Shoots from initiation medium were transferred to 50 ml full strength MS medium containing BAP or KIN (0.0, 0.25, 0.5, 0.75, 1.0, 1.50 and 2.0g/l) or in combination with 0.0, 0.1 0.20, and 0.4 mg/l NAA in baby food jar

Each culture vessels contained six explants in five replications. The cultures were maintained at culture conditions as for shoot initiation.

4.2.7. Rooting

For root induction, half strength MS medium supplemented with 0.0, 0.25, 0.5, 0.75, 1.0, 2.0 mg/l naphthalene acetic acid (NAA) alone indole-3-butyric acid (IBA) alone were used. The pH adjusted to 5.8 prior to autoclaving. Shoots were transferred to the rooting media. Six explants per baby food jar were used. The cultures were maintained at culture conditions as for shoot initiation.

4.2.8. Acclimatization

Well rooted plantlets were removed from the rooting media carefully without damaging the roots and the roots were washed thoroughly in running tap water to remove all the residues of culture medium. 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 given adequate water to the plantlets at an interval of two-to- three days. After two weeks, the plants were shifted to greenhouse and covered with light polyethylene bags. The polyethylene bags were gradually removed after two weeks. The number of survived plants was recorded after a two weeks.

4.3. Experimental design

A completely randomized design (CRD) was used for all experiments. Data were subjected to analysis of variance (ANOVA) and least significant difference (LSD) test using statistical data analysis software SPSS 20.0 version at 0.05 probability level

5. RESULTS

5.1. Effect of different concentration of sterilants and exposure time on *X. americana* seed explants and *in vitro* germination

According table 1, 100% was recorded for 25%, 35% and 40% concentration of commercial bleach (clorox) and for 20 and 25 min exposure time, respectively. There were variable survival patterns under different seed sterilization treatment. Maximum germinated clean seed per explants was (72.33%) recorded upon using 35% concentration of commercial bleach (clorox) for 20 min exposure time.

5.2. Shoot initiation

Shoot tips cultured on the control and on MS medium supplemented with BAP or KN alone, showed a significant variation ($P = 0.05$) in terms of number of shoots per explants and length. Explants cultured on MS medium supplemented with cytokinins (BAP or KN) were able to initiate irrespective of their concentration. Among MS media containing different concentrations of cytokinins, maximum shoot induction per explants (2.06±0.15) was recorded on MS medium supplemented with 0.5 mg/l BAP and lower shoot induction per explants (1.20±0.07) was produced on MS medium supplemented with 2.0 mg/l KIN.

Table 1: Effect of different concentration of clorox and exposure time on *in vitro* germination of *Ximenia americana* seed

Treatment code	Clorox %	Time of exposure (min)	Clean explants (%)	Germination (%)
T01	25	15	41.8	00
T02	25	20	56.42	39.34
T03	25	25	100.00	42.42
T04	35	15	93.20	32.0
T05	35	20	100.00	72.33
T06	35	25	87.50	54..6
T07	40	15	91.83	22.76
T08	40	20	93.20	13.67
T09	40	25	100.0	00

(In this study 270 seed of *Ximenia americana* were used for sterilization and *in vitro* germination)

Table 2: Effect of different concentration of KIN or BAP on shoot induction from shoot tip *of X. americana*

PGR(mg/l)		No. of shoots/ explantsMean±SE	Shoot length(cm) Mean±SE
KIN	BAP		
0.0	0	1.43 ± 0.04^{bcd}	1.26 ± 0.34^{ab}
0.5	0	2.00 ± 0.15^{a}	1.37 ± 0.01^{a}
1.0	0	1.50 ± 0.09^{bc}	0.84 ± 0.01^{bc}
1.5	0	1.63 ± 0.08^{ab}	0.78 ± 0.02^{d}
2.0	0	1.20 ± 0.07^{cd}	0.98 ± 0.02^{bcd}
0	0.5	2.06 ± 0.15^{a}	0.89 ± 0.01^{cd}
0	1.0	1.63 ± 0.11^{abc}	1.26 ± 0.014^{ab}
0	1.5	1.73 ± 0.09^{ab}	0.96 ± 0.02^{bcd}
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 ± standard error (S.E).

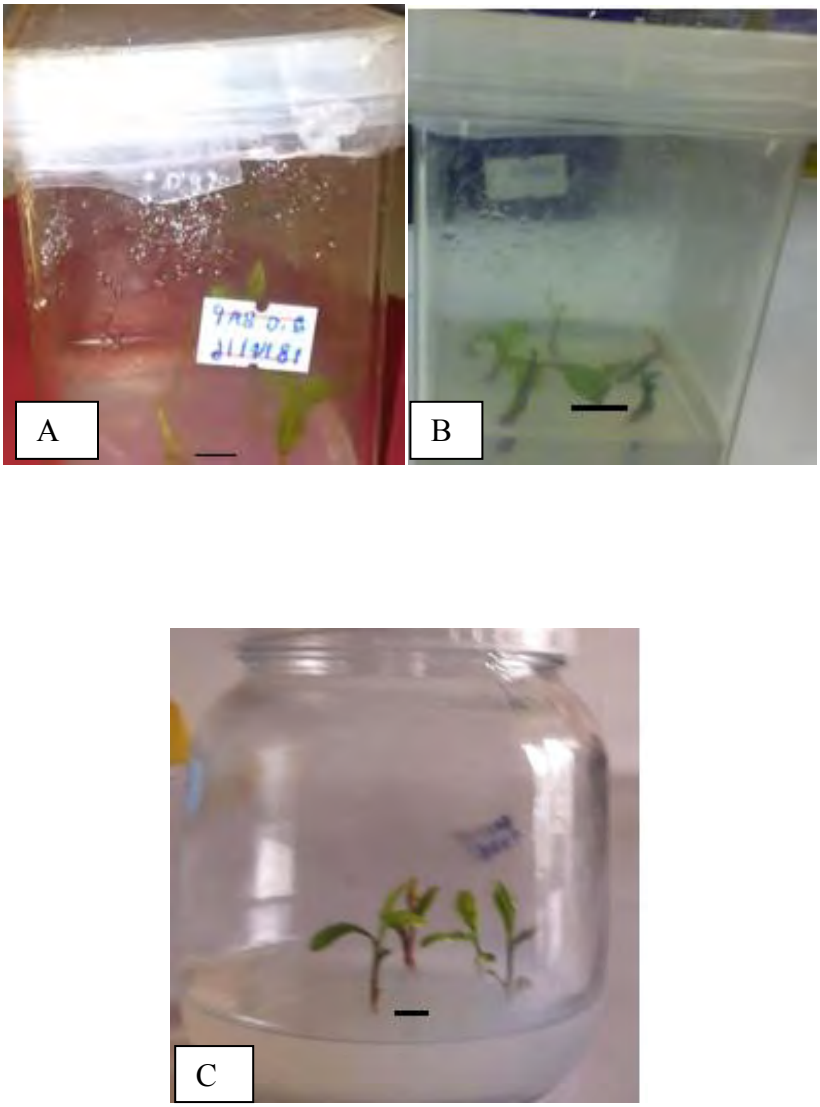

Figure 1: Effect of different concentrations of KN and BAP on shoot induction of shoot tip explants of *X. americana:* A) 0.5 mg/l BAP, B) 2mg/l BAP and C) 0.5 mg/l KIN. Bar represents 0.4 cm

5.3. Shoot multiplication

The responses of explants to BAP and KN alone or in combination with NAA on MS media resulted in varying degree of shoot proliferation. The analysis of variance (ANOVA) revealed that the combination of PGR type and concentration had highly significant ($p < 0.05$) effect on mean value of shoot number and shoot length of the shoots. There was no induction of multiple shoot on growth regulators free MS medium. The shoots on multiplication medium gave different responses based on the different hormonal composition presented in Tables, 3, 4 and 5 below

Effect of different concentrations and combination of BAP, kinetin and NAA on shoot multiplication

The response of explants cultured on MS medium supplemented with different concentrations of BAP or KIN alone was presented in Table 3. MS medium supplemented with BAP at different concentrations (0.25 - 2.00 mg/l) showed significant differences in shoot number and shoot length. Highest number of shoots per explants (4.16±0. 17) was recorded on MS medium supplemented with 0.5 mg/l BAP. Minimum number of shoots per explants (1.33±0. 09) was obtained on MS medium supplemented with 2.0 mg/l KIN. Different concentrations of BAP in combination with NAA also resulted in (3.32±0.05) shoots per explants on MS medium supplemented with 0.75 mg/l BAP combining with 0.1 mg/l NAA. Also different concentrations of KIN along with NAA showed significant difference in shoot number and length. The highest mean shoot number per explants (2.64±0.05) was recorded on MS medium supplemented with 0.75 mg/l KIN along 0.1 mg/l NAA

Table 3: Effect of different concentrations of BAP or KIN on shoot multiplication of *X. americana*.

PGR(mg/l)		No. of shoots/ explants Mean±SE	Shoot length(cm) Mean±SE
KIN	BAP		
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. 15de	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. 09de
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 ± standard error (S.E).

Table 4: Effect of different concentrations and combinations of KN and NAA on Shoot multiplication of *X. americana*

PGR (mg/l)		No. of shoots/explants Mean±SE	Shoot length (cm) Mean±SE
KIN	NAA		
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 ± standard error (S.E).

Table 5: Effect of different concentrations and combinations of BAP and NAA on Shoot multiplication of *X. americana*

PGR (mg/l)		No. of shoots/ explants Mean±SE	Shoot length (cm) Mean±SE
BAP	NAA		
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 ± standard error (S.E).

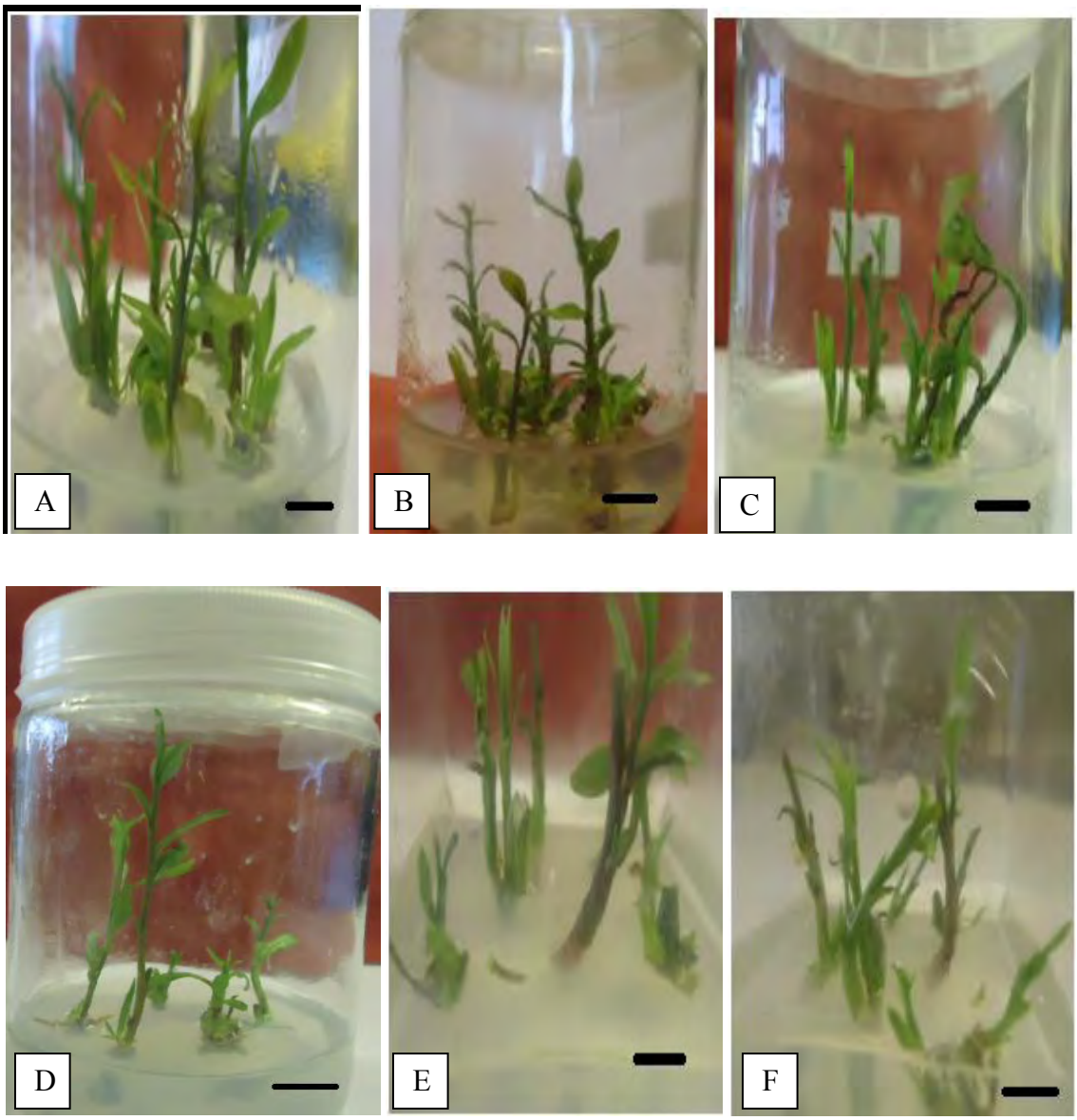

Figure 2: Shoot multiplication from shoot tip explants of *X. americana* on MS medium containing different concentrations of growth regulators: A) 0.5 mg/l BAP, B) 0.75 mg/l BAP + 0.1 mg/l NAA, C) 0.5 mg/l KIN, D) 0.75 mg/l KIN + 0.1 mg/l NAA, E) 0.25 mg/l BAP and F) 0.75 mg/l KIN. Bar represents 1.5cm

5.4. Rooting and acclimatization

Effect of NAA and IBA on rooting

The shoots cultured on half strength MS basal media 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 1/2 strength MS medium containing 0.5mg/l IBA. The highest mean length of roots per explants (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, the survival rate of total plants transferred to greenhouse was 100% established as healthy.

Table 6: Percentage of rooting, mean number of roots and mean root length obtained on half strength MS rooting media containing different concentrations of IBA and NAA.

PGR(mg/l)	Rooting (%)	No. of roots/ explants Mean±SE	Root length (cm) Mean±SE
00	00	0.0±00	0.0±0.
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	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 ± standard error (S.E).

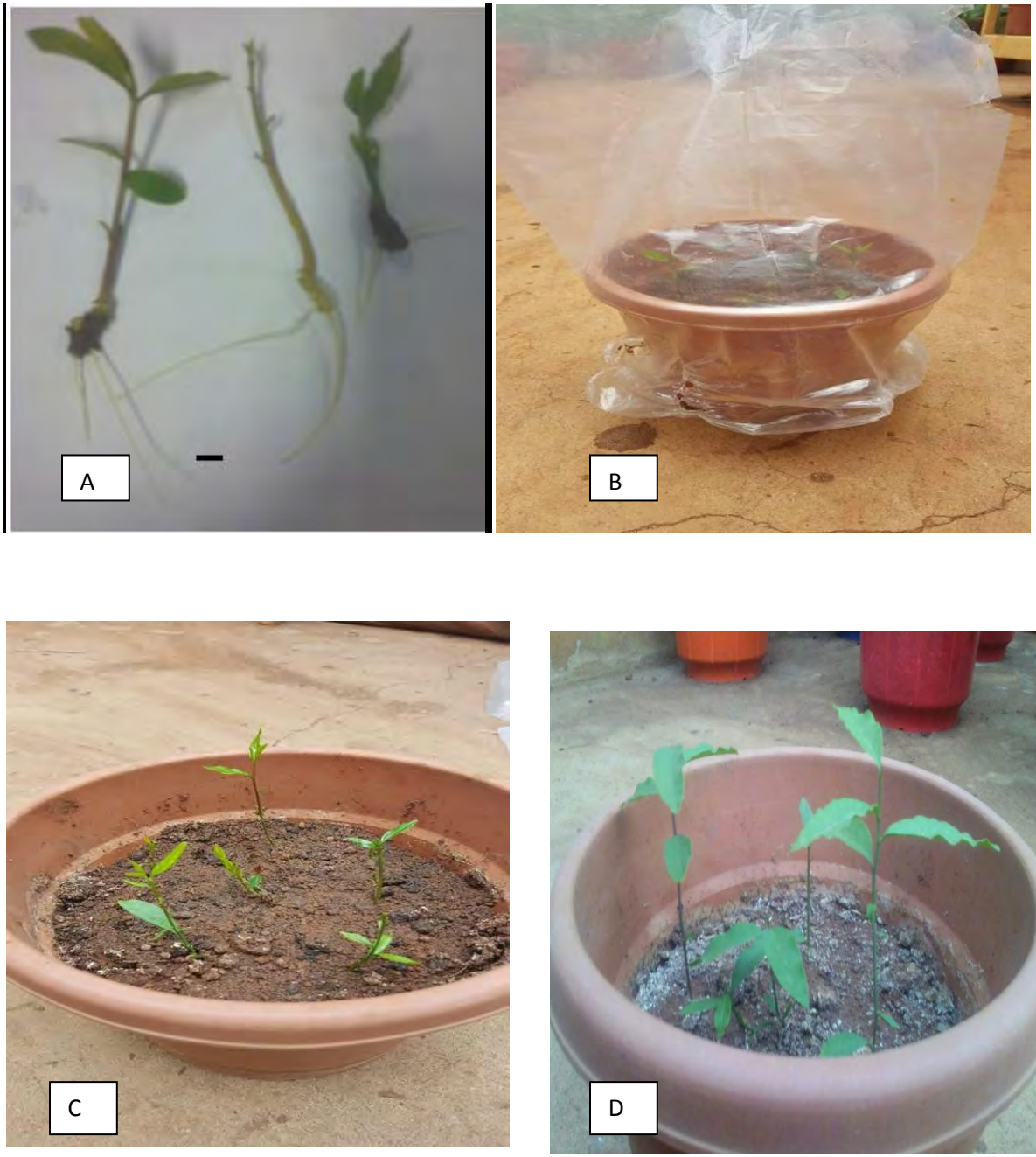

Figure 3: *In vitro* rooting of *X. americana* shoots on 1/2MS medium and acclimatization: A) 0.5 mg/l IBA, B) Plants covered in polyethylene bag after two weeks, (C and D) after 40 and 90 days of acclimatization respectively. Bar represents 1.5cm

6. DISCUSSION

6.1. Sterilization and *in vitro seed* germination of X. *americana*

Various sterilization agents were used to decontaminate the tissues. These sterilants were also toxic to the plant tissues, hence proper concentration of sterilants, duration of exposing the explants to the various sterilants, the sequences of using these sterilants should be standardized to minimize explants injury and achieve better survival (Tola Bayisa *et al.,* 2011). Analysis of variance (ANOVA) revealed that concentration of clorox, exposure time, and their interaction result in significant difference ($P<0.05$) on overcoming contamination of growth media and improving survival and germination level of *X. americana in vitro* seed culture. At shorter exposure time explants death was due to microbial contamination while at prolonged exposure time death was due to scorching by the sterilants. The present study also conform that seed explants responded for different concentration of clorox and exposure time. The highest mean percentage of clean surviving explants (72.33) was obtained on 35% of clorox concentration and exposure time of explants to the sterilant for 20 minute. Further increase in exposure time led to a significant decline in percentage of surviving explants. This is might shows that different explants require different concentration of sterilants and exposure time (Funguomali *et al.*, 2013). The exposure time of sterilization is dependent on the type of tissue; i.e. seed tissue requires longer sterilization time than leaf tissue and (Sharma and Nautiya, 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* using 20% clorox for 20 minutes exposure time and Dahab *et al.* (2010) who reported that the best results in sterilization of nodal explants for *Taxodium distichum* and *Taxodium distichum* var. distichum using 20% clorox for 5 min, followed by 0.2% Mercuric Chloride for 5 min.

Germination started after day 20 and continued till 60 days. It is sluggish and poor may be due to viability problem caused by insufficient pollination. This was the same with Maara *et al*., (2006) who reported that the germination in *X. americana* started on day 16 and continued till day 38, which suggests the existence of some form of physiological dormancy. The difference may be type of germination method. This author suggests rapid germination may be an adaptation to produce seedlings to escape the adverse effects of unfavourable moisture conditions found in the area.

6.2. Shoot Induction

From this study, cultured shoot tips resulted in varying responses to all of the culture media compositions including the control (without plant growth regulators): After germination, a medium containing cytokinins was the most effective for shoot induction. Especially addition of lower concentration of BAP to induction medium was inducing a number of shoots than kinetin. Maximum shoot number per explants (2.06±0.15) was recorded on MS medium supplemented with 0.5 mg/l BAP. These results were similar to those of Hung and Stephen (2011) who obtained highest induction (2.6 ± 0.2 shoots per explants) on full-strength MS medium containing 2.0 mg/l BAP alone. This author also cultured node explants of African mahogany (*Khaya senegalensis)* on medium containing 1.0 mg/l BAP and obtain longer shoots with more nodes than media containing 4.0 mg/l and 8.0 mg/l BAP. The marked effects of BAP on shoot formation compared to kinetin as observed in this study may be due to; high stability of BAP in *in vitro* cultures. BAP is not easily broken down and therefore persists in the medium. Then larger amount of BAP existing in free or ionized forms in the medium are readily available to plant tissues. Also, Danthu *et al***.** **(**2003**)** obtained highest induction (2.4 ± 0.5 shoots per explants) on full-strength MS medium containing 2.0 mg/l BAP and 0.056 mg/l IBA but also on

medium containing 0.5 mg/l BAP recorded (2.3 ± 0.4) shoots per explants of African mahogany (*Khaya senegalensis*) of node explants. Even though, the result reported by these authors is greater than the present study, but the trend was similar. These variable responses could be due to different factor like genotype difference.

6.3. Shoot multiplication

In the present study, full strength MS medium containing different types and concentrations of PGRs have been used to multiply the already initiated shoots of the *X. americana.* Among all the treatments, the maximum numbers of shoots per explants were recorded on medium containing low concentration of cytokinins. The most rapid and earliest proliferation was observed on media with the lowest concentrations of BAP alone. Proliferation of multiple shoots in different tree plants had been previously characterized with different growth regulators. The finding of Aloufa *et al*. (2003) on *Ximenia americana* showed that the MS medium supplemented with 2.5 mg/l BAP resulted in 6.6 mean numbers of shoots per explants from node explants. The finding of these authors is similar to the present one. In the present study, the maximum mean number of shoots per explants (4.16±0.17) was recorded on MS medium supplemented with 0.5 mg/l BAP. The result reported by Aloufa *et al*. (2003) was higher than the present study. This difference may occur due to the genotype difference and type and source of explants. These author cultured nodal explants of *Ximenia americana* on MS medium containing 0.17 to 3. 38 mg/l BAP and found that the number of shoots per explants was increased with the increase in the level of cytokinines and then declined. The present study also shows this phenomenon. In fact for *Ximenia americana*, highest BAP concentration 2.0 mg/l was used, completely inhibited shoot proliferation, with the final numbers of shoots per explants produced in this medium (1.83±0.16) from the numbers available after shoot induction. As Arab *et al,* (2014) explained by the reason

that the high proliferation rate in MS medium containing 1.0 mg/l BAP compared to the low proliferation rate in MS medium containing 1.25 mg/l BAP caused a reduction in the height of shoots length. Higher concentrations of BAP had an inhibitory effect on proliferation. The current results are in agreement with this author. Borthakur *et al*. (2011) investigated different concentration of BAP alone or in combination with NAA on *Albizia odoratissima* (Bansa) and found that the MS medium supplemented with low concentration of 0.74 mg/l BAP resulted in highest numbers of shoots per explants from apical buds of 7 day old *in vitro* seedlings. In both studies best result was obtained relatively at low concentration of BAP. On the other hand highest shoots per explants (2.83±0. 36) was recorded on MS media supplemented with 0.5 mg/l KIN. But, further increase in the concentration of KIN from 0.5 to 2.0 mg/l significantly decreases from 2.83±0. 36 to 1.80± 0.10 mean shoot number per explants. This also shows the same trends with BAP in inhibiting shoot proliferation as concentration increases.

Shoot explants cultured on MS medium supplemented with 0.75 mg/l BAP in combination with 0.1 mg/l NAA exhibited both the highest mean number of shoot (3.23±09) and mean length of shoots (3.98±0. 28) per explants. 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). When the concentration of BAP was greater than that of NAA, relatively better result was recorded. This is due to the effect of cytokinines as it promotes the axillary branching or axillary bud proliferation (Vieitez and Vieitez, 1980). Explants responded positively with multiple shoots initiated when BAP levels were higher than NAA, beyond that the multiplication rate declined. Propagation of *Azadirachta indica* via axillary buds and nodal segments was investigated by Rodriguez and Ortiz (2001) and found the best medium for shoot regeneration on 3.0 mg/l BAP. The finding of these authors is also contrasting to the

present one. Combining of BAP with NAA to MS medium was comparatively more effective than KIN with NAA for efficient shoot proliferation in shoot tip explants of *Ximenia americana*. These results contrast with the report of (Hung and Stephen, 2011). The positive effects of combined BAP and NAA treatments in *Khaya senegalensis.* But, (Husain and Anis, 2009) agreed with the greater effectiveness of BAP compared with kinetin than kinetin with NAA for shoot proliferation of *Melia azedarach*.

Among explants cultured on MS medium supplemented with different concentration of KIN along with NAA show significant differences. Highest shoots per explants (2.64±0.05) recorded on 0.75 mg/l KIN combining with 0.1 mg/l NAA. This also conform the effect of cytokinines as it promotes the axillary branching (Vieitez and Vieitez, 1980). Further, increase in the concentration of NAA from 0.1 to 0.4mg/l showed a reduction from (2.64±0.05 to 1.31±0.00) in the mean shoot number per explants.

Generally, the present study were showed that the explants responded positively with multiple shoots initiated when BAP alone or KIN alone were used and combining of these growth regulators with lower concentrations of NAA. Using BAP in combination with NAA was comparatively more effective than using KIN in combination with NAA for efficient shoot multiplication.

6.4. Rooting

Auxins are mainly used in root induction and their effect varies with type and concentrations used in different plant species (Swamy and Singh, 2002). In the present study, the analysis of variance 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 and roots produced were thin. The highest number of mean root per shoot (3.36±0. 69) was recorded on 0.5 mg/l IBA and mean root length (2.21±0.40) was recorded on 2.0 mg/l IBA. This was 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 was applied. However, further increase in the concentration of IBA to 2.0 mg/l showed a reduction in the mean root number per shoot in agreement with Dahab *et al.* (2010) and Tileye Feyissa *et al*. (2005). As the present data shows that using lower levels of auxin (IBA) is significantly better than higher levels for root induction and elongation. Weiler (1984) also reported the inhibition of root elongation by higher concentration of growth regulators and stated ethylene deposition as the reason. Auxins of all types stimulate plant cell to produce ethylene, especially when high amount of synthetic auxins are used. Ethylene retard 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 is may be between 0.1 and 0.5 mg/l as the present result indicated. In the present study comparison was made between the rooting hormones and IBA was found to be more effective in increasing root number and length than NAA.

6.5. Acclimatization

Rooted plantlets were transferred to polyethylene bags containing autoclaved garden soil and placed inside the growth chamber for two weeks. After 2 weeks, the covers were removed. Then, they were taken into the greenhouse where 100% plants survived and established as healthy. This result was much better than previous report of Aloufa *et al*. (2003) where only 80% of the plants taken from regulator supplemented media were acclimated versus 15% of those taken from auxins free media.

7. CONCLUSIONS

Ximenia americana propagates by seed which is incompetent due to seed viability loss after short period, and time consuming for large scale production and conservation of this species. 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. Thirty five percent concentration of commercial bleach clorox and 20 minutes exposure time was the best sterilization protocol for seed explants of *Ximenia americana* as this treatment resulted in highest percentage of survived clean explants (72.33%). BAP is the most important cytokinins for initiation and multiplication of *Ximenia americana.* The highest mean number of shoots per explants was (4.16±0. 17) on full strength MS medium supplemented with 0.5 mg/l BAP alone for shoot multiplication and half MS strength supplemented with 0.5 mg/l IBA for root induction.

8. RECOMMENDATIONS

Future perspectives, based on the present study, should focus on the following area:

A. The effect of different nutrient and physiological dormancy of the seed on *in vitro* germination of *Ximenia americana* seed with respect to low germination rate and longevity.

B. Different rooting hormone and rooting response of multiplied shoots at each sub-culturing stage should be investigated.

C. The effect of different concentrations of auxins and cytokinins on callus induction and regeneration of shoots and roots should be further studied

D. The effect of different concentrations of cytokinins and auxins on direct regeneration of shoots and roots should be further studied

E. Determining the genetic stability whether repeated sub-culturing stage affects stability of cultures should be assessed

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10. APPENDIX

APPENDIX 1: Stock solution for MS (Murashige and Skoog1962)

Components	Concentration (g/L)	m/L during media preparation
Micronutrients		
ZnSO4.7H2O	1.72	
H3BO3	1.124	
* MnSO2.4H2O	3.38	
* MnSO4.H2O	0.05	5 ml/l
KI	0.166	
NaMoO4.2H2O	0.05	
CoCl2.6h2O	0.05	
Na2EDTA	7.472	
FeSO4.7H2O	5.56	
Macronutrients		
NH4NO3	33	
KNO3	38	
Cacl2.2H2O	8.8	50ml/l
MgSO4.7H2O	7.4	
KH2PO4	3.4	
Vitamins		
Myo-inositol	20	
Glycin (glycocoll)	0.4	
Nicotinic acid (NaOH)	0.1	5ml/l
Pyridoxin (B6)	0.1	
Thiamin (B1)	0.02	

***** Alternatives

 Na2EDTA and FeSO4.7H2O prepared alone