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In vitro mass propagation of *Oreosyce africana* Hook. f. from shoot tip explants

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Biotechnology

BY

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Table of Contents

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBRIVATIONS

2, 4-D	2, 4-dichlorophenoxyacetic acid
ANOVA	Analysis of Variance
BAP	6-Benzyl Amino Purine
GA3	Gibberellic Acid
IAA	Indol Acetic Acid
IBA	Indol-3-Butyric Acid
MS	Murashige and Skoog basal medium
NAA	Naphthalene Acetic Acid
PGR	Plant Growth Regulator
TDZ	Thidiazuron

ABSTRACT

Oreosyce africana *Hook. f. is one of medicinally important plants in Cucurbitaceae family. It is native to Ethiopia and known as "MANABASI" in native languages in Oromifa. Mass scale cultivation of* O. africana *is not found, the natural means of propagation is through seeds. However, the seeds are not available for commercial cultivation and its habitat is mostly in forests. These made its collection difficult and require plant taxonomic knowledge. Therefore,* in vitro *propagation technique is the best alternative for the production of large number of clean and healthy planting materials, for conservation and building up populations of this medicinally important plant species. The objective of this study was to investigate the effect of different growth regulators on* in vitro *propagation of* O. africana*. Leaf segments from four-week-old* in vitro *cultivated shoots were excised and cultured on MS medium supplemented with different concentrations of BAP and NAA combinations for callus induction. The induced calli were transferred on to solid MS medium supplemented with different concentrations of BAP and NAA combinations and different concentration of TDZ. For shoot multiplication, full strength MS medium containing different concentrations of BAP in combination with IBA, for rooting half strength MS medium containing different concentrations of IAA in combination with NAA were used. MS medium without PGRs was used as control. Except for treatment with 0.5 mg/l of BAP alone (70% callus induction), all treatments gave 100% callus induction, however, none of the applied regeneration treatments resulted in shoot regeneration. The highest multiplication of shoots (35.97 per explant) was obtained, at 0.5 mg/l BAP, and the highest mean shoot length (6.17 cm) was obtained on medium containing 0.01 mg/l BAP. Shoot length was decreased with an increase in shoot number and both were affected by the concentration and auxin and cytokinin combination applied. Highest mean number of roots per explant (23.67) was obtained on medium containing 1.0 mg/l IAA combined with 0.5 mg/l NAA and highest mean length of roots (5.08±0.15 cm) was obtained at 0.1 mg/l IAA, which suggested, combining IAA with NAA is better option for good root induction. Up on acclimatization, 95% plants survived. This* in vitro *propagation protocol of* O. africana *can be used for mass propagation as well as for its conservation and genetic improvement.*

Key words: Callus Induction, *Oreosyce africana*, Plant Growth Regulators

1. INTRODUCTION

Oreosyce africana Hook. f. is a slender climbing herb or trailer growing to 3 m and its habitat is in wet or moist *Aningeria- Syzygium* forest margins, grass land and in plantations at altitude between 1650-2000 m (Jeffrey, 1995). It belongs to family Cucurbitaceae, under genus *Oreosyce,* a probably monotypic genus found in tropical Africa and Madagascar.

Oreosyce africana is native to Ethiopia and known as "MANABASI" by local native language in Oromifa (Damtew Bekele, 2017). It is distributed in different regions of Ethiopia i.e. Tigray, Gonder, Gojam, Kefa and Bale regions (Jeffrey, 1995), and also in many countries of Africa i.e. Angola, Burundi, Cameroon, Kenya, Madagascar, Malawi, Rwanda, Tanzania, Uganda, Democratic Republic of Congo, Zambia, Zimbabwe (Neuwinger and Porter, 1996) and South Africa (Geldenhuys, 1992).

The family Cucurbitaceae contributes higher number of medicinal species in Ethiopia (Mirutse Giday and Gobena Ameni, 2003; Balemie Kebu *et al.*, 2004; Haile Yineger and Delenasaw Yewhalaw, 2007; and Hunde Debela *et al*., 2004). Traditional herbal medicine, which uses locally available plants, is accessible, relatively cheap and very personal. In many countries in the tropics including Ethiopia, people have little access to modern medicine (Damtew Bekele, 2017). In our country, people traditionally use different parts of herbs and plants for their medicinal values. *O. africana* also is used by many peoples and traditional medical practitioners believing for its medicinal values for insecticidal properties (for mosquito management and control of cattle ticks and other arthropod pests), for intestinal worms (used as an anthelminthic) and for a burned body (its leaf also used as medicine) (Yamada, 1999).

Purified fractions of *O. africana* possess a very high adulticidal effect against the principal malaria vector, *Anopheles arabiensis*, in Ethiopia and have a potential for development into an affordable botanical mosquitocide as an alternative to the existing inorganic insecticides with environmental toxicity (Damtew Bekele *et al*., 2016).

Despite its medicinal purposes, mass scale cultivation of *O. africana* is not reported except the preliminary work of Abdisa Feyisa (2018), which differs from this research by the applied treatments of plant growth regulators type, concentrations and combinations. The natural means of propagation is through seeds, but it is not available for commercial cultivation and its habitat is mostly in forests. Therefore it requires other propagation methods like *in vitro* mass propagation which could be used for mass scale cultivation and conservation of this medicinally important plant.

Plant tissue culture, also known as *in vitro* culture, is a technique of growing plant cells, tissues or organ in an artificial gel or liquid media supplemented with nutrients, vitamins and plant growth regulators under controlled and sterile conditions (Singh and Kumar, 2009). Plant cells possess sufficient genetic potential to be able to regenerate and give rise to a whole plant (totipotency) – making plant tissue culture an important method in plant biotechnology studies as well as having commercial applications (Thorpe, 2007).

Plants grown via tissue culture are expected to have identical genetic material to the parent and thus, keep their intrinsic characteristics (Azman *et al.*, 2014). This method of establishing genetically identical clones from an organism's tissue, capable of generating into a complete plant is known as clonal propagation or micropropagation (Vizel *et al.*, 2010).

Therefore, *in vitro* propagation technique is the best alternative for the production of large number of clean and healthy planting materials of *O. africana*. And, with the aim of investigating the effect of different growth regulators, the present work was done.

2. LITERATURE REVIEW

2.1 Taxonomy and botanical description of *Oreosyce africana* Hook. f.

O. africana is found under genus Oreosyce Hook.f. in Cucurbitaceae family. The genus Oreosyce Hook.f. is a climbing herb with simple leaves and tendrils. It is probably monotypic genus found in tropical Africa and Madagascar. It has small, monoecious flowers. The male flowers are found in small clusters, rarely solitary; narrowly campanulate hypanthium with stamens inserted on the middle; five small, filiform sepals; five yellow petals, united above the base; short filaments; straight thecae; prominent, elevated disc, free from hypanthium. Female flowers are solitary, pedicellate; tuberculate, setose ovary; several, horizontal ovules; hypanthium and perianth as in male flowers; annular disc, surrounding base of style; three stigmas. It has ovoid, fleshy, tuberculate, setose, fruit dehiscent by expulsion of seeds from the stalk-scar. Seeds are ovate in outline, compressed, with broad margins and depressed faces (Jeffrey, 1995).

Oreosyce africana is a slender climber or trailer which can grow up to 3 m. It has ovate or broadly ovate leaf-blade with cordate base, more or less sinuate-denticulate margin, 3-5-palmately lobed. It has triangular lobes where the central is hugest; 2.5-11.5 cm long petioles that are retrorsely setulose. The male flowers (3 together) are rarely solitary; pedicels are 3-15 mm long; hypanthium is hispid, 2-6.5 mm long; sepals are linear-filiform, 1-6 mm long; petals are bright yellow, 2.5-11 mm long; stamens are as for genus. Female flowers are solitary; pedicels are 3-10 mm long; ovary is ovoid, densely setulose and 4-8 mm long. It has fruit that are ovoid to ellipsoid, usually shortly beaked, tubereulate, green or greenish white. Its seeds are obscurely pitted or foviolate (Jeffrey, 1995).

2.2 Ecology, origin and distribution

Oreosyce africana has an ecological preference of wet or moist *Aningeria- Syzygium* forest margins, grass land and in plantations at altitude between 1650-2000 m asl.; it is distributed in Tigray, Gonder, Gojam, Kefa and Bale regions of Ethiopia (Jeffrey, 1995).

Many herbarium collections, found in the National Herbarium of Ethiopia, Addis Ababa University, indicates the distribution of *O. africana* in different areas of Ethiopia, such as, in coffee plantation in mesophytic to hygrophytic bottom of vegetation, found shaded (in Keffa zone) at 1700-1800m. a.s.l.; in the lower zone of Harenna forest, Bale Mountains National park at 1700m. a.s.l.; in the forest of Bale region on the road to Goba, at 1750m. a.s.l. ; on flat plains and sloppy grounds opposite to Tis Isat Falls (Bahir Dar) at 1650m. a.s.l. (unpublished data).

O. africana is native to Ethiopia but also found in Angola, Burundi, Cameroon, Kenya, Madagascar, Malawi, Rwanda, Tanzania, Uganda, Democratic Republic of Congo, Zambia, Zimbabwe (Neuwinger and Porter, 1996) and South Africa (Geldenhuys, 1992).

2.3 Importance of *Oreosyce africana* Hook. f.

Traditionally, the leaf part of *O. africana* is used as an anthelminthic for intestinal worms and its leaf also used as medicine for a burned part (Yamada, 1999). In south-east Tanzania, traditional medical practitioners make the boil of *O. africana* with the vegetable gruel for pregnant women to drink which makes the labor easy and they also rub themselves with its leaves against trichophytosis (Neuwinger and Porter, 1996).

A study by Haile Yineger and Delenasaw Yewhalaw (2007) showed local healers in Jimma use roots of *O. africana* by crushing and squeezing with water and is applied intravenous as treatment for gonorrhea. Haile Yineger *et al.* (2008) also indicated that the filtrate obtained from *O. africana* was reported to be given through hypodermal injection using a syringe to treat gonorrhea.

The insecticidal properties of plants have been used in Ethiopia, where plant materials are easily available and their use in health practices is a tradition (Damtew Bekele, 2017). According to Damtew Bekele *et al*. (2012), the people in Akaki district (east-central Ethiopia) traditionally used *O. africana*'s powder of crushed leaves by sprinkling for mosquito management and control of cattle ticks and other arthropod pests.

In another study, in Democratic Republic of Congo, peoples traditionally use a crushed handful of the whole *O. africana* plant mixed with a handful of stem barks from *Persea americana* and a decoction is made with 1.5 L. of tap water; a full glass is orally given twice a day during 2 days to treat patients suffering from malaria (Chifundera, 2001).

A study on Bioefficacy of Solvent Fractions of *O. africana* and *Piper capense* against the Malaria Vector, *Anopheles arabiensis* with High Performance Liquid Chromatographic and Ultraviolet-Visible Spectroscopic Analysis by Damtew Bekele *et. al*. (2014) showed that the bioassays with dichloromethane fraction of *O. africana* exhibited higher adulticidal effect against *Anopheles arabiensis.* Another study on Bioactive Chemical Constituents from the leaf of *O. africana* by Damtew Bekele *et al*. (2016) also suggested that the purified fractions of *O. africana* possess a very high adulticidal effect against the principal malaria vector, *An. arabiensis*, in Ethiopia and have a potential for development into an affordable botanical mosquitocide as an alternative to the existing inorganic insecticides with environmental toxicity.

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 (Thorpe, 2007) often to produce the clones of plants. The resultant clones are true-to type of the selected genotype. The controlled conditions provide the culture an environment conducive for their growth and multiplication. These conditions include proper supply of nutrients, pH medium, adequate temperature and proper liquid environment (Hussain *et al*., 2012).

Plant tissue culture techniques are being used for induction of calli and multiple shoots in case of different medicinal herbs and trees and micropropagation may help us to produce clones of a particular plant species that is true to its parents for its genotype, and is faster than the conventional methods (Supriya *et al*., 2017).

Plant tissue culture technology is being widely used for large scale plant multiplication. Apart from their use as a tool of research, plant tissue culture techniques have in recent years, become of major industrial importance in the area of- plant propagation (Hussain *et al*., 2012), disease elimination; production of virus free explants (Habtamu Tegen and Wassu Mohammed, 2016), plant improvement and transformation; transfer of genes with desirable trait into host plants and recovery of transgenic plants, (Hinchee *et al*., 1994) and production of secondary metabolites (Oseni *et al*., 2018).

Small pieces of tissue (named explants) can be used to produce hundreds and thousands of plants in a continuous process. A single explant can be multiplied into several thousand plants in relatively short time period and space under controlled conditions, irrespective of the season and weather on a year round basis (Akin-Idowu *et al*., 2009). Many literatures showed endangered, threatened and rare species have successfully been grown and conserved by micropropagation because of high coefficient of multiplication and small demands on number of initial plants and space (Muluken Enyew and Tileye Feyissa, 2018).

In recent decades, plant cell, tissue, and organ cultures have emerged as an alternative over whole plant cultivation for the production of secondary metabolites which are used as pharmaceuticals, flavors, fragrances, coloring agents, food additives, and agrochemicals (Paek *et al.*, 2014). The term secondary metabolite refers to a compound produced by plants, microorganisms or animals that is not required for their growth (Pickens *et al*., 2011). Plant tissue cultures, in addition to its use in production of active ingredients, also use as experimental materials for studies on synthetic biology. In order to improve the contents of active compounds in medicinal plants, following aspects could be carried out gene interference or gene silencing, gene overexpression, combination with chemical synthesis, application of elicitors, and site-directed mutagenesis of the key enzymes (Juan *et al*., 2017).

In addition, plant tissue culture is considered to be the most efficient technology for crop improvement by the production of somaclonal and gametoclonal variants (Hussain *et al*., 2012). The micropropagation technology has a vast potential to produce plants of superior quality, isolation of useful variants in well-adapted high yielding genotypes with better disease resistance and stress tolerance capacities (Brown and Thorpe, 1995). Certain type of callus cultures give rise to clones that have inheritable characteristics different from those of parent plants due to the possibility of occurrence of somaclonal variability (George, 1993), which leads to the development of commercially important improved varieties.

Plant tissue culture is an integral part of molecular approaches to plant improvement and act as an intermediary whereby advances made by the molecular biologists in gene isolation and modification are transferred to plant cells (Singh and Shetty, 2011).

In plant cell culture, plant tissues and organs are grown *in vitro* on artificial media, under aseptic and controlled environment. The technique depends mainly on the concept of totipotentiality of plant cells (Haberlandt, 1902 as cited in Hussain *et al*., 2012) which refers to the ability of a single cell to give rise to the whole plant. 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). The whole micropropagation process can be divided in to five different stages.

Table 1: Five stages of micropropagation process

Stages	Activities
Stage 0: stock plant preparation	- Growing mother plants under hygienic conditions mother plant should be *ex vitro* cultivated (in greenhouse) under optimal conditions to minimize contamination in the *in vitro* culture (Cassells and Doyle, 2006).
Stage I: Initiation	- Explant selection - Surface sterilization: - Removing contaminants with minimal damage to plant cells (Husain and Anis, 2009). depending on the type of the explant, commonly used disinfectants are sodium hypochlorite (Tilkat, 2009), calcium hypochlorite and ethanol (Muluken Enyew and Tileye Feyissa, 2018) and mercuric chloride ($HgCl_2$) (Cassells and Doyle, 2006). - Cultivation in to nutrient medium under aseptic conditions. - Keep in growth chamber either under light or dark conditions according to the method of propagation.
Stage II: multiplication	- Masses of tissues are repeatedly sub-cultured under aseptic conditions onto new culturing media until the desired (or planned) number of plants is attained. The aim of this phase is to increase the number of propagules (Saini and Jaiwal, 2002)
Stage III: Rooting	- Changing media, including nutritional modification and growth regulator composition to induce rooting and the development of strong root growth (Hussain, *et al*., 2012). It is designed to induce the establishment of fully developed plantlets (Nitish and. Reddy, 2011).
Stage IV: Acclimatiz-ation	- Transferring to *ex vitro* condition (to an appropriate substrate of sand, peat, compost etc.) and gradually hardened under greenhouse. Climatic or environmental adaptation of a plant that has been moved to a new environment.

Plant *in vitro* propagation could be direct or indirect (through callus induction). Exogenous application of auxin induces callus in various plant species. Generally speaking, an intermediate ratio of auxin and cytokinin promotes callus induction, while a high ratio of auxin-to-cytokinin or cytokinin-to-auxin induces root and shoot regeneration, respectively (Skoog and Miller, 1957). Since the discovery of this regeneration system, it has been widely used, for example, in the propagation of economically important traits and the introduction of transgenes and other hormones, such as brassinosteroids or abscisic acid, also induce callus and in some species may substitute auxin or cytokinin in callus formation (Ikeuchi *et al*., 2013).

Generally, biotechnology provides an *in vitro* propagation technique which is the best alternative for the production of large number of clean and healthy planting materials. Therefore, by using tissue culture techniques, we can propagate large number of this valuable plant in short period of time. On the other hand, Espinosa-Leal *et al*. (2018) reported, even if there are extensive researches on plants into secondary metabolites for over such a long period of time, current estimates indicate that only about 6% of higher plants have been systematically studied for their pharmacological potential, and only 15% have been evaluated for phytochemicals in general (Cragg and Newman, 2013 as cited in Espinosa-Leal *et al*., 2018). Therefore, enormous opportunities exist for continued studies in this field. A study on phytochemical analysis of tested plant crude extract of *O. africana* revealed the presence of some secondary metabolites which may be active ingredients for mosquito larvicidal and adulticidal activity (Damtew Bekele *et al*., 2014). This suggests that further biotechnological studies must be conducted. Plant cell, tissue, and organ cultures have emerged as an alternative over whole plant cultivation for the production of secondary metabolites which are used as pharmaceuticals, flavors, fragrances, coloring agents, food additives, and agrochemicals (Paek *et al*., 2014).

Plant tissue cultures, in addition to its use in production of active ingredients, also use as experimental materials for studies on genetic improvement of plants with desirable trait. In order to improve the contents of active compounds in medicinal plants like *O. africana*, aspects like gene interference or gene silencing, gene overexpression, combination with chemical synthesis, application of elicitors, and site-directed mutagenesis of the key enzymes could be carried out (Juan *et al*., 2017).

2.5 Factors affecting *in vitro* propagation

Murashige and Skoog medium (MS medium) is most extensively used for the vegetative propagation of many plant species *in vitro,* because most plants react to it favorably. The pH of the medium is also important that affects both the growth of plants and activity of plant growth regulators. Most tissue cultures are grown at pH 5.2 - 5.8 with pH adjustments being made prior to autoclaving (Skirvin *et al*., 1986). From the different factors affecting *in vitro* propagation of plants, i.e. media, type of explant, genotype, source and orientation of explant, mineral nutrition, growth regulators, carbon source, gelling agents and the main ones are presented below.

2.5.1 Source and type of explant

Source and type of explant is one of the important factors in *in vitro* propagation of plants. Type of explants like leaf, petiole, hypocotyl, epicotyl, embryo, internode and root explant and source of explant i.e. *in vitro* and *in vivo* significantly affect the regeneration of plants (Ali and Mirza, 2006; Kumar *et al*., 2011; Nitish and Reddy, 2011).

This may be due to the different level of endogenous plant hormones present in the plants parts. Tyagi *et al*. (2001) used root, shoot, and leaf explant and maximum regeneration efficiency was observed from leaf explants in *Cajanus cajan*. Alagumanian *et al*. (2004) used leaf and stem explant and maximum regeneration efficiency observed from stem explant in *Solanum trilobotam.* Ali and Mirza (2006) used root, stem, leaf and petiole but maximum responses were observed from stem explant in *Citrus jambhiri* Lush.

The fact that source of explant has different capacity of regeneration are well documented (Tileye Feyissa *et al*., 2005). *In vitro* explant in general has better potential to organogenesis as compared to *in vivo* explant. The difference may be due to the level of endogenous hormones present in the plant explant. Seedling explant is more responsive or meristematic than mature plants (Tileye Feyissa *et al*., 2005) due to different level of plant hormones present in the plants.

2.5.2 Type and composition of culture medium

Various basal media like White medium, Nitsch and Nitsch medium, B5 medium and Gamborg medium have been used for micropropagation (Diallo *et al*., 2008). 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 (Murashige and Skoog, 1962). Selection, strength and combination of media are also one of important parameter for optimizing the regeneration protocol of *in vitro* propagation (Diallo *et al*., 2008).

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 macroelements, 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 Fossard, 1976).

Sucrose is by far the most used carbon source, for several reasons. It is cheap, readily available, relatively stable to autoclaving, and readily assimilated by plants; 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 (Pierik, 1997). Other carbohydrates can be also used, such as glucose, maltose and galactose as well as the sugar-alcohols glycerol and sorbitol (Fowler, 2000).

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.

2.5.3 Plant growth regulators

Growth regulators are organic compounds naturally synthesized in higher plants, which influence growth and development. Apart from the natural compounds, synthetic chemicals with similar physiological activities have been developed which correspond to the natural ones (Pierik, 1997).

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).

There are several classes of plant growth regulators, as e.g. cytokinins, auxins, gibberellins, ethylene and abscisic acid. The auxins, cytokinins and gibberellins are most commonly used plant growth regulators (Oseni *et al*., 2018). Growth and morphogenesis *in vitro* are regulated by the interaction and balance between the growth regulators supplied in the medium, and the growth substances produced endogenously (George, 1993).

Auxins (IAA, IBA, NAA or 2, 4-D) are involved in the regulation of several physiological processes, for example, apical dominance and formation of lateral and adventitious roots. 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 cytokinin (George, 1993). IBA and IAA are widely used for rooting and, in interaction with a cytokinin, for shoot proliferation. 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).

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).

The high concentration of auxins generally favors root formation, whereas the high concentration of cytokinins promotes shoot regeneration. Cytokinins generally promote cell division and induce shoot formation and axillary shoot proliferation. A balance of both auxin and cytokinin leads to the development of mass of undifferentiated cells known as callus. High cytokinin to auxin ratio promotes shoot proliferation while high auxin to cytokinins ratio results in root formation (Rout, 2004).

Gibberellic acid (GA3) is the most common gibberellin used. It induces the elongation of internodes and the growth of meristems or buds *in vitro* (Pierik, 1997).

3. OBJECTIVES

3.1 General objective

To investigate the effect of different growth regulators on *in vitro* propagation of *Oreosyce africana* Hook. f.

3.2 Specific objectives

- ✓ To optimize growth regulators concentration for callus induction and regeneration
- ✓ To optimize growth regulators concentration for shoot multiplication
- ✓ To optimize concentration of auxins for root initiation and development
- ✓ To evaluate the survival rate of *in vitro* grown plantlets in *ex vitro* environment.

4. MATERIALS AND METHODS

All the activities of this experiment were conducted at Plant Tissue Culture and Molecular Biology Laboratory of the Institute of Biotechnology, Addis Ababa University.

4.1 Plant material

In vitro cultivated plants of *Oreosyce africana* Hook. f. were obtained from Plant Tissue Culture and Molecular Biology Laboratory, Institute of Biotechnology, Addis Ababa University. The used plants were initially collected from areas around Akaki and Ilu Gelan district, located in $8^0$33'-$8^0$57' N latitude and $38^0$43'-$38^0$50' E longitude, for other research purposes, and maintained in Plant Tissue Culture and Molecular Biology Laboratory of Addis Ababa University for future research use. The microshoots of *in vitro* cultivated stock plants were maintained by subculturing in MS basal medium supplemented with 0.5mg/L 6-Benzyl Amino-purine (BAP).

4.2 MS medium stock solution preparation

Throughout the research activity, Murashige and Skoog (1962) (MS) medium was used. Full strength stock solution of macronutrients, micronutrients, Fe-Na-EDTA and FeSO4 mixture and vitamins were prepared separately. In order to prepare these solutions, appropriate amount of each nutrient was weighed in grams per liter (appendix 1) and dissolved in distilled water consecutively. Using magnetic stirrer, the nutrients were added step by step, i.e. the next nutrient was added after the first one was completely dissolved. After all the components were fully dissolved, the solution was dispensed in to plastic bottles and stored at -20°C until used.

4.3 Plant growth regulators stock solution preparation

In this study, plant growth regulators such as 6-Benzyl Amino-purine (BAP), Naphthalene Acetic Acid (NAA) and Thidiazurone (TDZ) for callus induction and regeneration; BAP, NAA and Indol-Butyric Acid (IBA) for shoot induction and Indol-Acetic Acid (IAA) and NAA for rooting were used. All of the plant growth regulators stock solutions were prepared by weighing and dissolving the powder in distilled water at 1:1 (1mg/ml) ratio. Using magnetic stirrer, the powders were first dissolved by 2-3 drops of NaOH and HCl based on the requirement of the plant growth regulators. Then, the volume was adjusted by adding distilled water and stirred until fully dissolved. The growth regulator stock solutions were stored in a refrigerator at +4°C for short term use.

For all activities, in this research, growth regulator free MS medium were used as control.

4.4 Culture medium preparation

The culture medium for callus induction contained full strength MS basal medium. In order to prepare the medium, using a magnetic stirrer, 30 g/l of sucrose was dissolved in distilled water and 50 ml of macronutrient, 5 ml of micronutrient, 5 ml of vitamin and 5ml of Fe-Na-EDTA and FeSO4 mixture per liter were added consecutively. Then, different concentrations of BAP and NAA combinations were added. After adding the growth hormones, pH was adjusted to 5.8 using NaOH and/or HCl. Then, 7.0 g/L agar was added and boiled until the agar was melted. Then the prepared medium was dispensed in to Magenta GA-7 culture vessels and autoclaved at a temperature of 121°C and pressure of 105 KPa for 15 minutes. Immediately after autoclaving, the medium was transferred to a laminar air flow cabinet and dispensed in to sterile Petri dishes. Each Petri dish contained 25ml of the autoclaved culture medium kept under laminar air flow cabinet for immediate use.

For shoot regeneration, full strength MS basal medium were used and the same procedure of preparation were followed. The medium were fortified with different concentrations of BAP and NAA combinations and different concentration of TDZ. After adding the growth hormones, pH was adjusted to 5.8 using NaOH and/or HCl. Then, 7.0 g/L agar was added and boiled until the agar was melted (still using a magnetic stirrer). Then 50 ml of the prepared medium was dispensed in to Magenta GA-7 culture vessels and autoclaved at a temperature of 121°C and pressure of 105 KPa for 15 minutes. Immediately after autoclaving, the medium was taken in to a laminar air flow cabinet or kept in refrigerator for one or two days until used.

For shoot multiplication, full strength MS basal medium were used and the preparation procedure were as of shoot regeneration medium. The growth regulators used for shoot multiplication were different concentrations of BAP and IBA combinations.

For rooting, half strength MS basal medium was used. The same preparation procedure but half of the medium nutrients were used. Different concentrations of NAA and IAA combinations were used for rooting.

4.5 Callus induction

For callus induction, leaf segments from shoots after four weeks of culture were used. Using scalpel and forceps, the leaves were excised, wounded and cultured in 9 cm diameter Petri dishes containing 25 ml MS medium supplemented with different concentrations of BAP (0.0, 0.5, 1.0, 1.5 and 2.0 mg/L) in combination with NAA (0.0, 0.01, 0.05, 0.1, and 0.5mg/L), and sucrose (30 g/L). Growth regulator free MS medium were used as control. Explants were placed with their abaxial surface in contact with the medium. Five explants per Petri dish with six replicates per treatment (for total of 21 treatments) were used.

The cultures were maintained in full darkness for four weeks at room temperature. Data on the percentage of explants that produced callus were scored after four weeks.

4.6 Shoot regeneration

After four weeks, the calli produced were transferred to shoot regeneration medium containing various concentrations of BAP (0.0, 0.5, 1.0, 2.0, 3.0, and 5.0) in combination with NAA (0.0, 0.01 and 0.1) and TDZ (0.0, 0.1, 0.5, 1.0, and 1.5). Then the cultures were maintained in both dark and light conditions for four months. Sub-culturing to fresh medium was done at four week interval. After the four weeks of the third sub-culture, data were recorded if there are any shoots regenerated. All experiments (total of 19 treatments) were performed by using five explants per culture vessel with 6 replications.

4.7 Shoot multiplication

Since the induced callus did not regenerate and gave shoots, shoots from *in vitro* propagated plantlets were used as a source of explant. The shoots were trimmed to about 1.5 cm length and cultured on 50 ml of full strength MS medium in Magenta GA-7 culture vessels (10 x 6 cm) supplemented with different concentrations of BAP (0.0, 0.01, 0.1, 0.5, 1.0, mg/L) in combination with IBA (0.0, 0.01, 0.1, 0.5, mg/L). All experiments (total of 13 treatments) were performed by using five explants per culture vessel with 6 replications. The cultures were maintained at 25 ± 2°C with 16 h photoperiod and light intensity of 22 μ mol $m^{-2}s^{-1}$ for 30 days.

The number and length of shoots per explant were recorded after four weeks of culture.

4.8 Rooting

For root induction, micro shoots about 2.5 to 3.5 cm long were used as explant. The explants were excised and transferred to the rooting media. The rooting media was half strength MS basal medium supplemented with different concentrations of IAA (0.0, 0.1, 0.5, 1.0 mg/L) in combination with NAA (0.0, 0.25, 0.5, mg/L). The cultures were maintained at 25 ± 2°C with 16 h photoperiod at light intensity of 22 μmol $m^{-2}s^{-1}$ for 30 days. All experiments were performed by using five explants per culture vessel with six replications.

After forty days of culture, the number and length of roots per explant were recorded.

4.9 Acclimatization

After 4 weeks of culturing in rooting media, well rooted plantlets were thoroughly washed with running tap water, without damaging the roots, in order to remove all the residues of culture medium. Then, plantlets were planted in plastic pots containing a sterile mixture of sand, red soil and compost at ratio of 1:2:1 (w/w) respectively. To ensure high humidity, the pots were covered with transparent polyethylene bags. Adequate watering was done at an interval of two-to-three days. After two weeks, the plants were shifted to a natural growth environment and the plastic covers were gradually removed. Finally, the plants were fully exposed to the environment and watering was done when required.

From the total of fifty plantlets planted for acclimatization, the number of survived plants was recorded after forty days of acclimatization.

4.10 Experimental design and data analysis

The study was conducted at Plant Tissue Culture and Molecular Biology Laboratory, Institute of Biotechnology, Addis Ababa University. A completely randomized design (CRD) was used for all experiments. Data of all treatments were subjected to one-way analysis of variance (ANOVA) to detect the presence of significant differences among treatments. To detect homogeneity of variance, the means of different treatments were analyzed by using Tukey test using statistical data analysis software SPSS 20.0 version at 0.05 probability levels.

5. RESULTS

5.1 Callus induction and Shoot regeneration

Young leaf explants excised from well-developed shoots was used for shoot regeneration. Leaf explants responded differently to different concentrations of BAP combined with NAA. With an increase in concentrations of BAP from 0.5 up to 1.0 mg/l, an increase from 1.5 up to 2.5 mg/l, decrease in callus proliferation was observed (Table 2 and Figure 1).

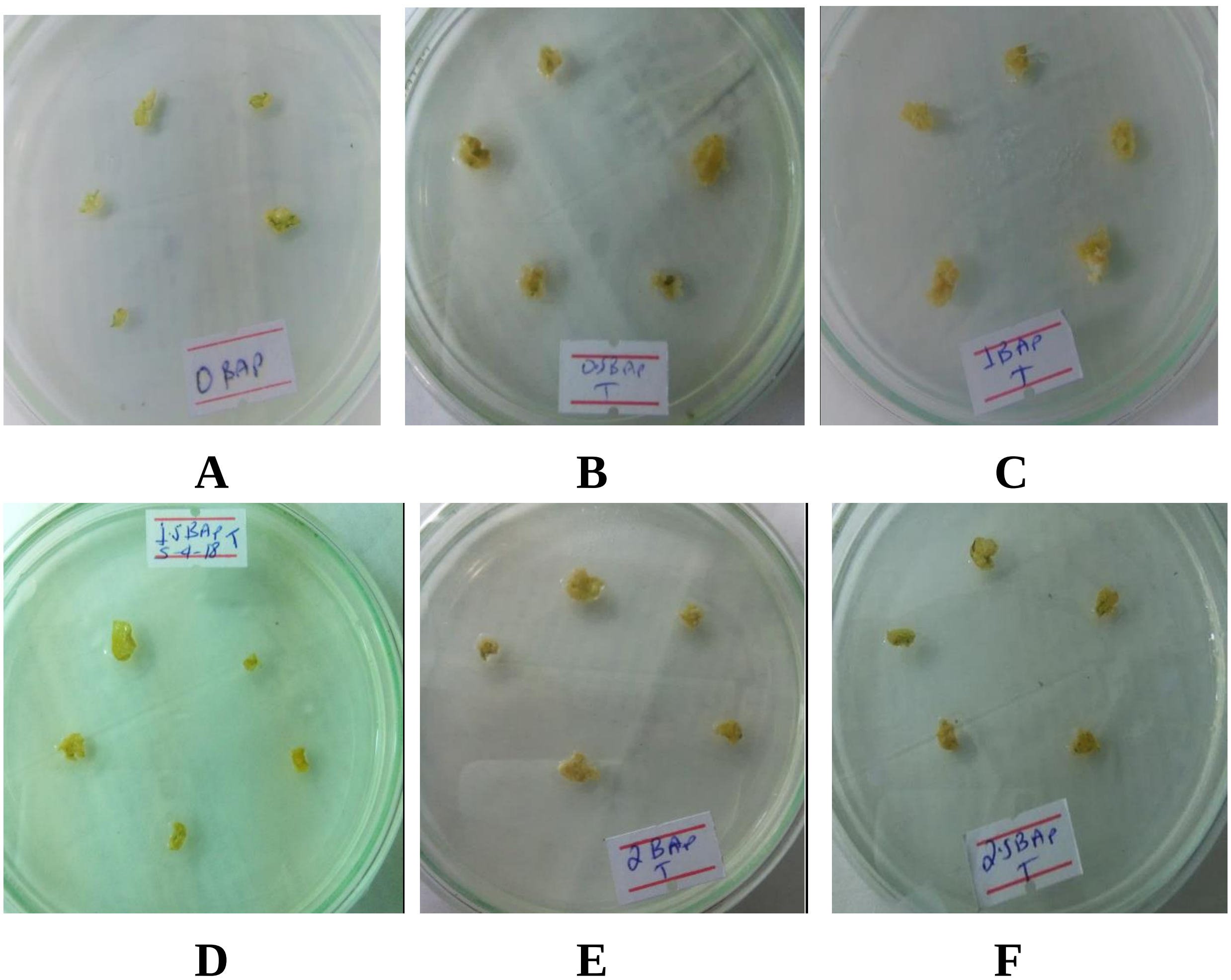


Figure 1: Induced callus from leaf explants of *O. africana* on different concentrations of BAP after 30 days of growth in the dark

A= control	**B**= 0.5 mg/l BAP	**C**= 1.0 mg/l BAP
D= 1.5 mg/l BAP	**E**= 2.0 mg/l BAP	**F**= 2.5 mg/l BAP

Table 2: Effect of different concentrations of BAP in combination with NAA on callus induction and proliferation

Treatment			
BAP	NAA	Callus induction (%)	Callus condition (proliferation)
0.0	0.0	46.7	Very Poor
0.5	0.0	70	Very Poor
1.0	0.0	100	Very good
1.5	0.0	100	Very good
2.0	0.0	100	Good
2.5	0.0	100	Poor
0.5	0.01	100	Poor
0.5	0.05	100	Good
0.5	0.1	100	Good
0.5	0.5	100	Very good
1.0	0.01	100	Poor
1.0	0.05	100	Good
1.0	0.1	100	Good
1.0	0.5	100	Excellent
1.5	0.01	100	Poor
1.5	0.05	100	Good
1.5	0.1	100	Good
1.5	0.5	100	Very good
2.0	0.01	100	Poor
2.0	0.05	100	Good
2.0	0.1	100	Good
2.0	0.5	100	Very good

N.B. - **very poor**- represents **many** of the explants did not induce callus, and the callus did not show any increase until the 30th day. In contrasting with others, this is the least of all.

- **Poor**- represents **all** of the explants induce callus, and the produced callus did not show any increase until the 30th day. In contrasting with others, this is greater than ‘’very poor’’ but others.
- **Good**- represents **all** of the explants induced callus, but visible increment in the callus proliferation was not observed until 30th day. In contrasting with others, this is greater than ‘very poor’ and ‘poor’ callus conditions but others.
- **Very good**- represents **all** of the explants induced callus, and some of it showed visible callus proliferation. In contrast with others, this is greater than ‘’very poor’’, ‘poor’ and ‘good’ but ‘excellent’.

- **Excellent-** represents the entire explants induced callus and showed high callus proliferation when compared with others.

In the combination of (0.0, 0.5, 1.0, 1.5, and 2.0 mg/L) BAP and (0.0, 0.01, 0.05, 0.1, and 0.5mg/L) NAA, with an increase in the concentration of NAA from 0.01 to 0.5 mg/L, the callus induction and proliferation was continuously increased (Figure 2).

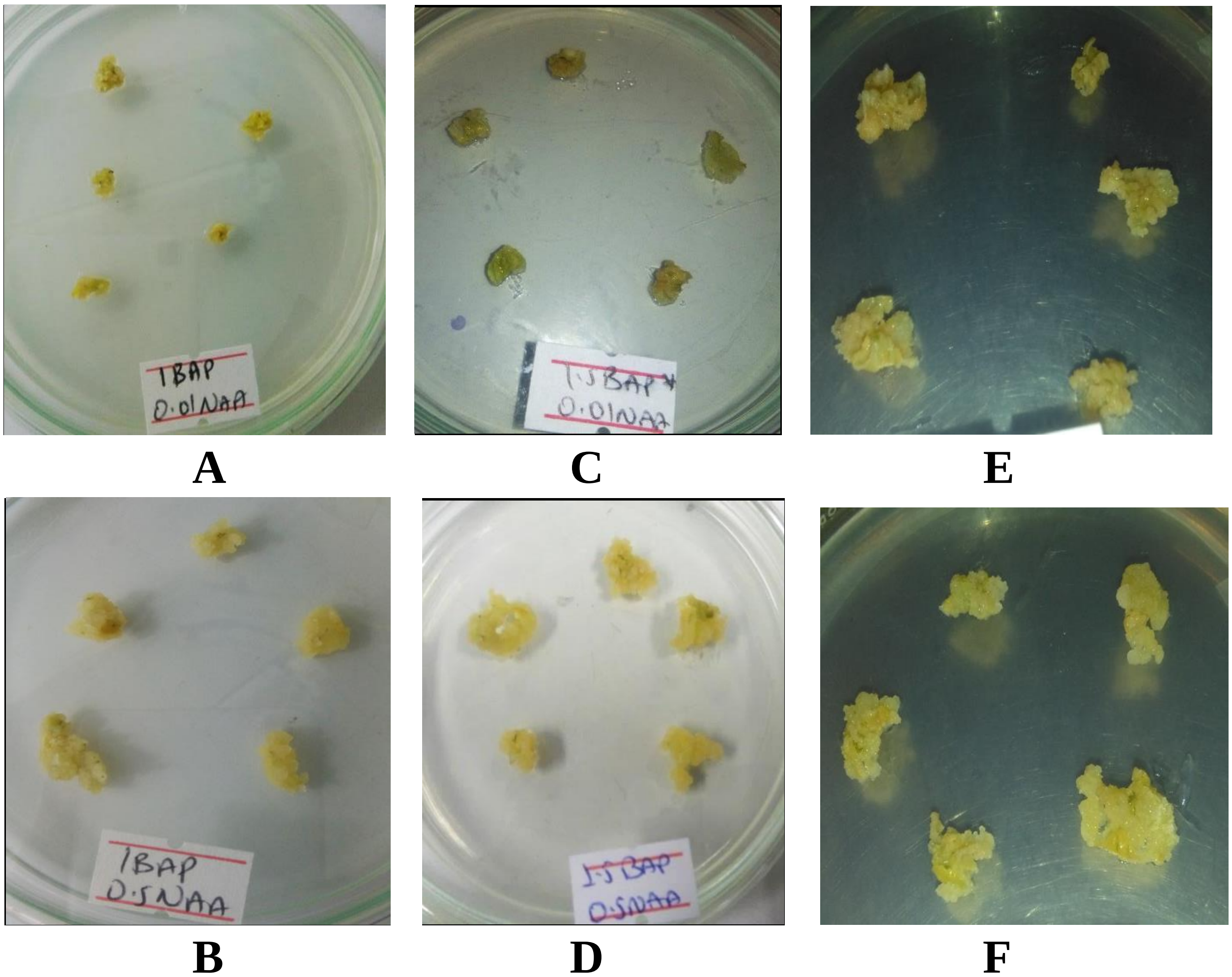


Figure 2: Callus induction from leaf explants of *O. africana* on full strength MS medium containing different concentrations of BAP in combination with NAA after 30 days of growth in the dark

A= 1.0 mg/l BAP and 0.01 mg/l NAA; **B**= 1.0 mg/l BAP and 0.05 mg/l NAA

C= 1.5 mg/l BAP and 0.01 mg/l NAA; **D**= 1.5 mg/l BAP and 0.05 mg/l NAA

E= 2.0 mg/l BAP and 0.01 mg/l NAA; **F**= 2.0 mg/l BAP and 0.05 mg/l NAA

All leaf explants in all conducted treatments, except 0.5 mg/L BAP, induced callus. But the size of the callus was different for all treatments. Depending on an observation with an eye, grading the callus proliferation condition was tried and from all treatments, 1.0 mg/L BAP combined with 0.5 mg/L NAA shows the best callus induction and proliferation (Table 2). Even if they induce callus, all explants of all treatments have not been regenerated.

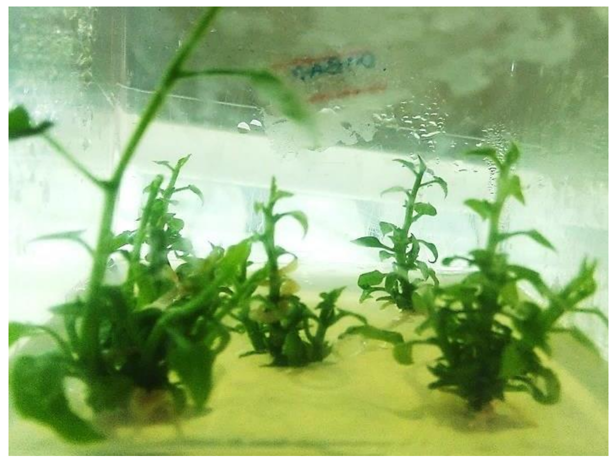
5.2 Shoot multiplication

There was variation in shoot multiplication in response to varying concentration and combination of growth regulators in medium.(Figures 3 and 4).

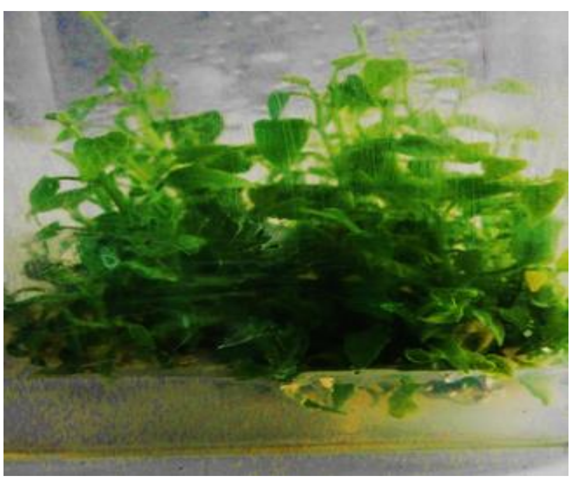
5.2.1 Effect of BAP and IBA on shoot multiplication

The response of explants cultured on MS medium supplemented with different concentrations of BAP (0.0, 0.01, 0.1, 0.5, 1.0, mg/L) in combination with IBA (0.0, 0.01, 0.1, 0.5, mg/L) is presented in Table 3. Full strength MS medium supplemented with 0.5 mg/l BAP resulted in maximum number of shoots per explant (35.97±0.41), followed by medium containing 0.5 mg/l BAP + 0.01 mg/l IBA (10.67±0.22).

A



B



C

D

Figure 3: Shoot multiplication from shoot tip explants of *O. africana* on MS medium containing different concentrations of BAP.

(A) 0.01 mg/l BAP, (B) 0.1 mg/l BAP, (C) 0.5 mg/l BAP and (D) 1.0 mg/l BAP.

In all combinations of BAP with IBA, with the increase in the concentration of IBA from 0.01 to 0.1 and 0.5, the number and length of shoots decreased. And, calli formation at the base of the explants was observed (Figure 4).

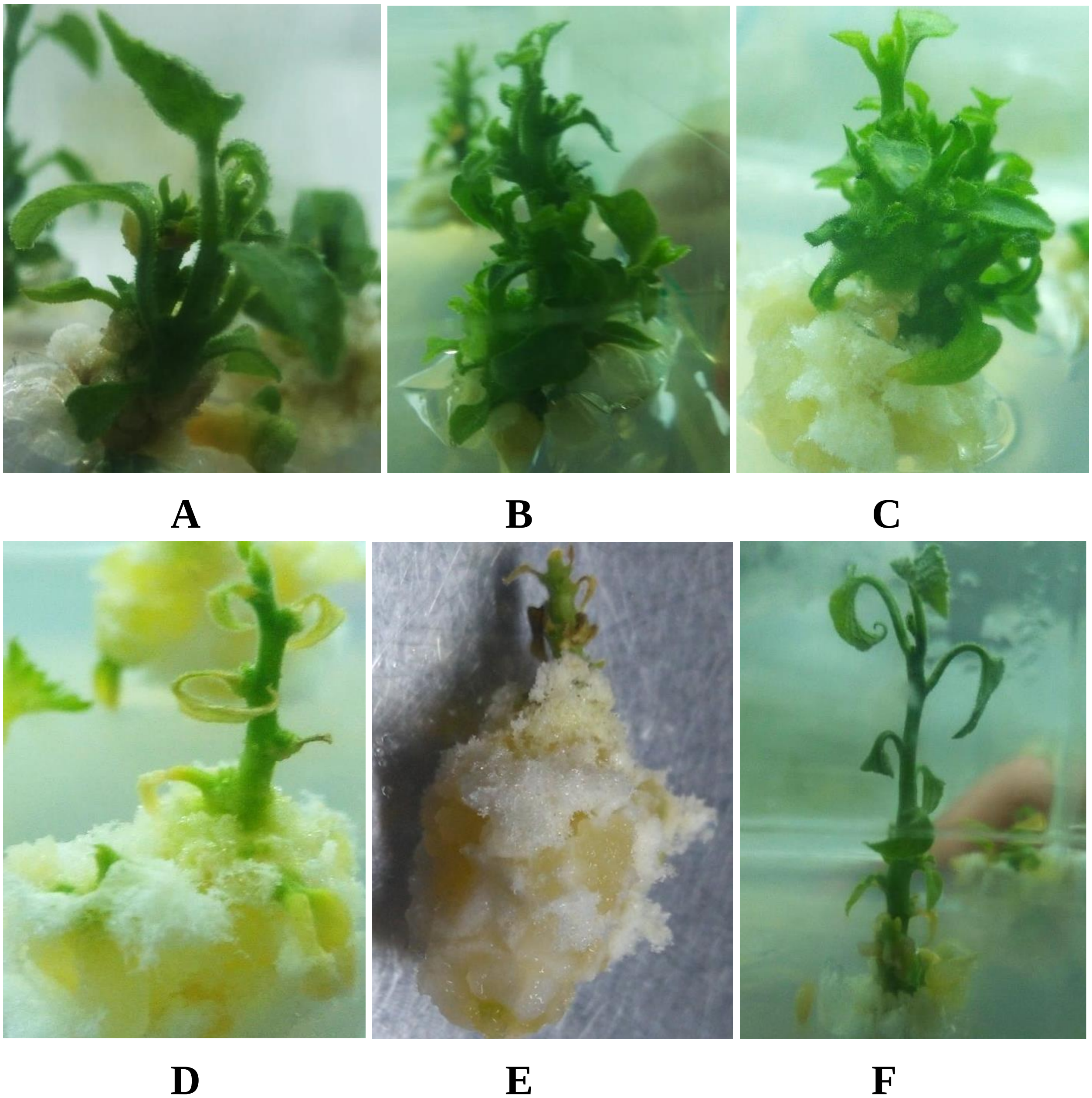


Figure 4: Shoot multiplication from shoot tip explants of *O. africana* on MS medium containing BAP in combination with IBA

(**A**) 0.1mg/l BAP + 0.01 mg/l IBA, (**B**) 0.5 mg/l BAP + 0.01 mg/l IBA, (**C**) 1.0 mg/l BAP + 0.01 mg/l IBA, (**D**) 0.1 mg/l BAP + 0.1 mg/l IBA, (**E**) 1.0 mg/l BAP + 0.5 mg/l IBA and (**F**) control

Maximum shoot length of 6.17±0.12 cm was recorded in MS medium supplemented with 0.01 mg/l BAP followed by 3.25±0.11 cm in MS medium containing 0.1 mg/l BAP. The control, MS medium containing no PGR, resulted in the third longest explants (2.58±0.09) (Table 3).

Table 3: Effect of different concentrations of BAP in combination with IBA on shoot multiplication of *O. africana*

Mean values of shoot number and shoot lengths are indicated as ±SE.

BAP	IBA	No. of shoots/ explants Mean±SE	Shoot length(cm) Mean±SE
0.0	0	1.17±0.69^{ef}	2.58±0.09^{c}
0.01	0	1.8±0.11^{de}	**6.17±0.12^{a}**
0.1	0	4.7±0.16^{c}	3.25±0.11^{b}
0.5	0	**35.97±0.41^{a}**	2.08±0.08^{d}
1.0	0	4.2±0.17^{c}	1.12±0.04^{ef}
0.01	0.01	1.13±0.06^{ef}	0.63±0.03^{g}
0.1	0.01	1.57±0.1^{def}	0.97±0.03^{f}
0.1	0.1	1.0±0.0^{f}	0.55±0.01^{gh}
0.5	0.01	10.67±0.22^{b}	2.08±0.05^{d}
0.5	0.1	1.0±0.0^{f}	0.3±0.01^{h}
0.5	0.5	1.0±0.0^{f}	0.35±0.00^{gh}
1.0	0.01	2.07±0.15^{d}	1.33±0.02^{e}
1.0	0.1	1.0±0.0^{f}	0.32±0.02^{h}
1.0	0.5	1.0±0.0^{f}	0.25±0.00^{h}

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

5.3 Rooting and Acclimatization

5.3.1 Effect of IAA and NAA on rooting

The shoots cultured on half strength MS basal media supplemented with different concentrations of IAA, NAA and combination of IAA and NAA resulted in different rooting responses including the control (Figure 5).

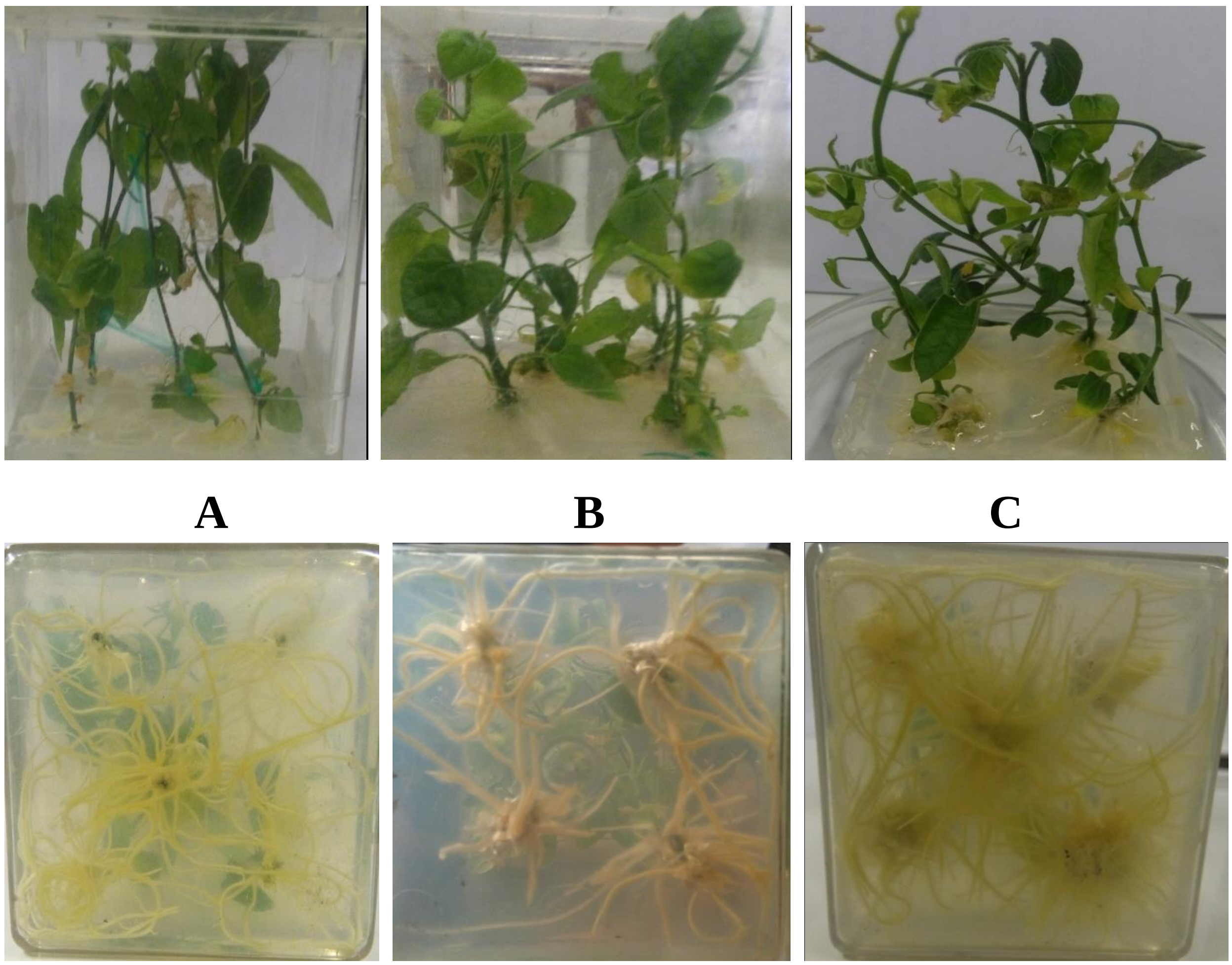


Figure 5: *In vitro* rooting of *O. africana* shoots on half strength MS medium containing different concentrations of IAA, NAA, and no PGR (control).

(**A** and **a**) control, (**B** and **b**) 0.1 mg/l IAA, and (**C** and **c**) 1.0 mg/l NAA + 0.25 mg/l NAA.

The highest mean number of roots per shoot (23.67±0.19) was obtained on 1/2 strength MS medium containing 1.0 mg/l IAA in combination with 0.5 NAA followed by 0.5 mg/l IAA (19.6±0.21) and 1.0 mg/l IAA in combination with 0.25 mg/l NAA (19.67±0.38) (Table 4). The highest mean length of root per explant (5.08±0.15 cm) was obtained on 1/2 strength MS medium containing 0.1 mg/l IAA followed by growth regulators free half strength MS medium (3.42±0.64 cm) which was used as control and 0.5 mg/l IAA (3.58±0.13 cm).

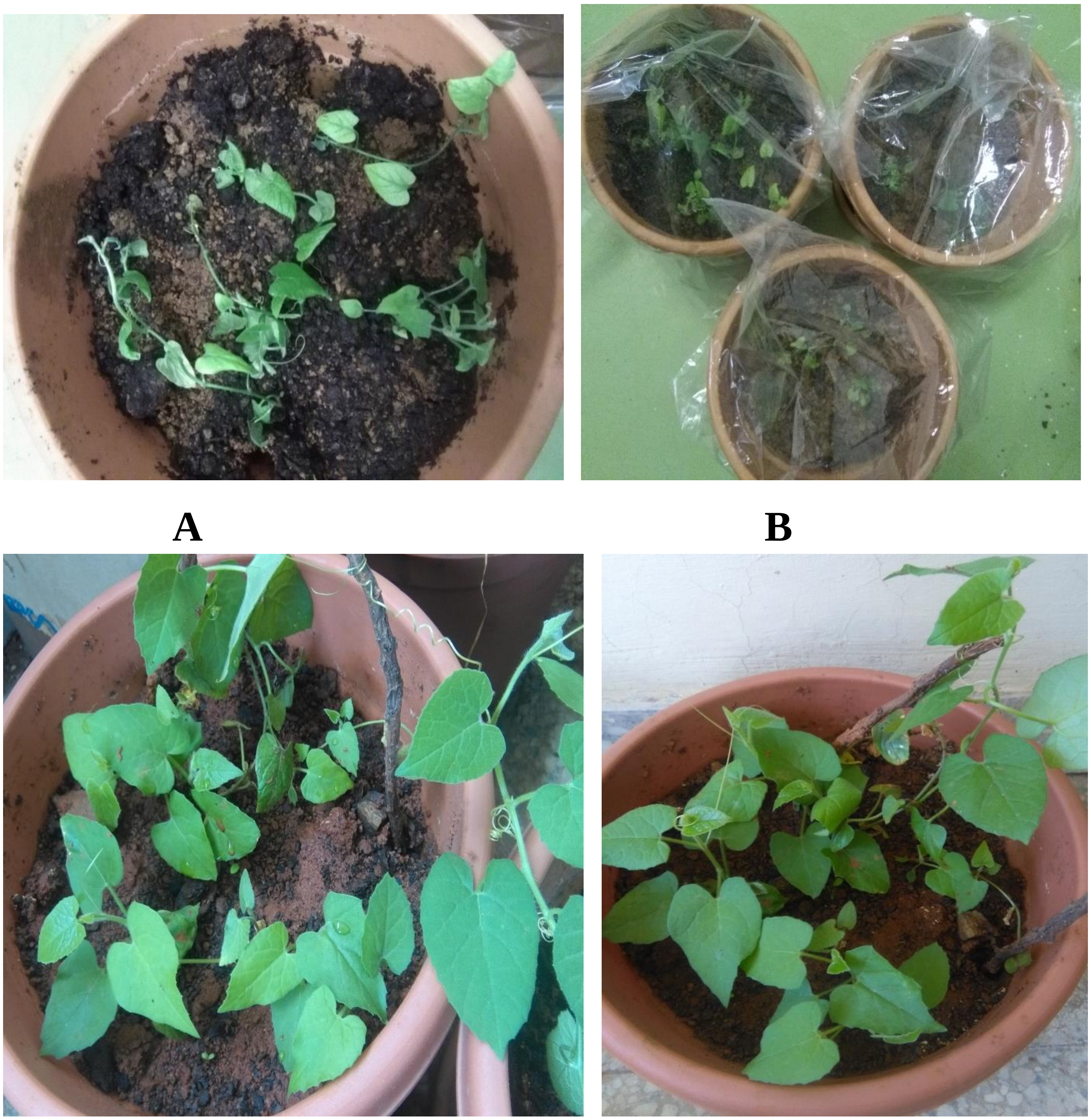
Table 4: Mean number and length of roots obtained on half strength MS rooting medium containing different concentrations of IAA, NAA and combinations of IAA and NAA.

Mean values are indicated as ±SE

IAA	NAA	No. of root/ explant Mean±SE	Root length (cm) Mean±SE
0.0	0.0	5.0±0.17^{g}	3.42±0.64^{b}
0.1	0.0	11.23±0.19^{e}	**5.08±0.15^{a}**
0.5	0.0	19.6±0.21^{b}	3.58±0.13^{b}
1.0	0.0	12.7±0.17^{d}	2.17±0.06^{de}
0.0	0.25	11.67±0.27^{de}	1.45±0.07^{f}
0.0	0.5	15.4±0.23^{c}	2.22±0.08^{d}
0.1	0.25	15.63±0.32^{c}	1.75±0.11^{ef}
0.5	0.25	15.93±0.19^{c}	1.95±0.10^{de}
1.0	0.25	19.67±0.38^{b}	2.92±0.12^{c}
0.1	0.5	6.75±0.15^{f}	1.42±0.05^{f}
0.5	0.5	15.33±0.13^{c}	1.92±0.05^{de}
1.0	0.5	**23.67±0.19^{a}**	1.75±0.04^{ef}

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

Plantlets with developed roots were carefully and systematically removed from culture vessels and washed by running tap water, then immediately planted in potted soils. After forty days of acclimatization, the survival rate of total plants transferred to greenhouse was 95% (Figure 6).



A **B** **C** **D**

Figure 6: Acclimatization of *in vitro* rooted shoots of *O. africana*.

(A) Plants transferred from the medium to the pots; (B) Plants covered with plastic bags and (C and D) after 40 days of acclimatization

6. DISCUSSION

6.1 Callus induction and regeneration

In this study, *in vitro* regeneration of *O. africana* from leaf explants was carried out for establishment of culture conditions under hormonal control. Many factors influence plant *in vitro* regeneration. The factors considered in this study were the effect of cytokinin and auxin concentrations on plant callus induction and regeneration from leaf explants.

In this study, except for treatment with 0.5 mg/l of BAP alone (70% callus induction), all treatments resulted in 100% callus induction. However, A study by Paulsamy *et al.* (2012) on related species *Mukia maderaspatana* shows, the percentage callus induction from leaf explants of this plant increased with an increase in concentration of BAP (from 0.5 to 1, 1.5, 2.0, and 2.5 mg/l) in combination with 0.5 mg/l of NAA, and decreased in concentration of BAP at 3 mg/l in combination with 0.5 mg/l of NAA. From all the treatments, 1.0 mg/l BAP in combination with all concentrations of NAA (0.0, 0.01, 0.05, 0.1, and 0.5mg/L), resulted in better callus with regard to callus proliferation condition. In all treatments of combinations of NAA, the callus proliferation increases with an increase in the concentrations of NAA, and 0.5 mg/l were resulted in better callus induction and proliferation. This suggests that, both cytokinin and auxin are crucial for better induction and proliferation of callus than cytokinin alone.

The induced calli were friable, yellow to brown color. After being transferred to regeneration medium, subculturing to fresh similar medium were carried out with 4 weeks interval, for 4 months. The calli in all treatments were gradually turned to full brown color and died/ dried.

Exogenous application of auxin and cytokinin induces callus in various plant species. Generally speaking, an intermediate ratio of auxin and cytokinin promotes callus induction, while a high ratio of auxin-to-cytokinin or cytokinin-to-auxin induces root and shoot regeneration, respectively (Skoog and Miller, 1957). The same is true for the callus induction of *O. africana* from leaf explants.

An intermediate ratio of auxin (0.5 mg/l NAA) and cytokinin (1.0 mg/l BAP) induced an excellent callus. However, in the case of shoot regeneration, from the all treatments, different concentrations of BAP, in combination with NAA, and different concentrations of TDZ alone, did not result in shoot regeneration. This could be due to other factors that affect plant regeneration capacity like:- genotype, explant type used, type of PGRs and concentration, the growth medium type used and composition, and light intensity.

Different plants and different types of explants (leaf, node, cotyledon, stem etc.) have different capacity of callus induction and ability of regeneration from the induced callus. This could be due to the plants indigenous hormone content and the plant's genetic effect (Tileye Feyissa *et al*., 2005).

The type and concentrations of PGRs also have different effect on the callus induction and regeneration of plants. This could be due to the different ways of its expression (way of action of the growth hormones) in different plants.

Even if MS medium is the mostly used and efficient growth medium, others like GB5 (Gamborg's B-5), LS (Linsmaier and Skoog) and CHE' (Chee and Pool) (Verma *et al.,* 2016) could be used and because of the difference in the composition of the medium, its effect on the plants callus induction and regeneration varies.

6.2 Shoot multiplication

In this study different concentrations of BAP and combination with IBA were used for shoot multiplication. Full strength MS medium containing BAP alone (0.5 mg/l), and then in combination with tiny concentration of IBA (0.01) was found to be the most suitable plant growth regulators for mass micropropagation of *O. africana*. With an increase in the concentration of BAP (from 0.01 to 0.1 and 0.5) the number of shoots per explant continuously increased significantly, and then decreased at 1.0 mg/l BAP. This is in contrast with Vasudevan *et al*. (2001) who reported a continuous increase in concentration of BAP (from 0.1 to 1.0 mg/l) will continuously increase the mean number of *Cucumis sativus* shoots per shoot tip explants.

In other way, our result is in agreement with finding of Alam *et al*. (2015) who reported that increase in the concentration of BAP (from 0.5 to 1.5 mg/l) resulted in continuous increase in shoot proliferation percentage of *Cucumis sativus* nodal explants, and then decreased continuously on the next increased BAP concentrations (2.0 to 3.0 mg/l). Schaller *et al*. (2014) also suggested that, for many processes involving cytokinin, as with other hormones, a bell-shaped dose response curve is observed, with an optimal concentration above or below which a diminished response is evoked. Therefore, in this study, the effect of BAP on shoot multiplication process of *O. africana* was readily observed, where an optimal cytokinin concentration exists for shoot multiplication, the production of new shoots initially increasing with increasing levels of exogenous cytokinin but then decreasing once the optimal level is exceeded.

Maximum shoot proliferation (35.97±0.41) was observed on MS medium supplemented with 0.5 mg/l BAP alone followed by 10.67±0.22 shoots per explant on MS medium supplemented with 0.5 mg/l BAP combined with 0.01 mg/l IBA (which is the smallest concentration from all treatments). Further increase in concentration of IBA to 0.1 and 0.5 mg/l did not improve any of the parameter but reduced the proliferation of shoots and showed a negative effect on shoot multiplication, which is formation of callus at the basal tip of explants. Abdisa Feyisa (2018) also got the maximum mean number of *O. africana* shoots per explant (36.66±2.03) in MS medium supplemented with 0.5 mg/l BAP and all shoots cultured on medium containing different concentrations of BAP and KIN in combination with IBA formed callus. This is in contrast with Paulsamy *et al.* (2012) who reported the mean number of *Mukia maderaspatana* shoots increased with an increase in the concentration of auxin (NAA) in combination with BAP, and no negative effect were reported.

Another study on multiple shoot induction from the shoot tip explants of *Cucumis sativus* by Vasudevan *et al*. (2001**)** reported that, at higher concentrations of auxin (NAA) combined with BAP; calli were produced and shoot bud formation were prevented. Also, in this study, all treatments of BAP combined with 0.1 and 0.5 mg/l IBA resulted in no additional shoot growth on the cultured explants and gradually turned in to full callus cells. This revealed the inhibitory action of auxin on multiple shoot development.

Another parameter used to analyze the effect of PGRs on multiplication of shoots was shoot length. The length of multiplied shoots was different in media containing various concentrations of auxin and cytokinins. The mean length of shoots decreased with an increase in the concentration of BAP (from 0.01 to 1.0 mg/l). This is in contrast with Vasudevan *et al*. (2001) who reported increase in mean length of *Cucumis sativus* shoots (from 4.8 to 6.1 cm) with an increase in concentration of BAP (from 0.2 to 1 mg/l).

The highest mean shoot length (6.17±0.12 cm) was obtained from medium supplemented with 0.01 mg/l BAP followed by 3.25±0.11 cm on medium containing 0.1 mg/l BAP and then 2.58±0.09 cm on medium containing no PGR (the control). This is in contrast with Abdisa Feyisa (2018), who got the maximum mean shoot length of *O. africana* (4.26±0.10 cm) on growth regulators free MS medium. Further increase in concentration of BAP, resulted in decrease in length of the multiplied shoots while combination of BAP with different concentrations of IBA decreased, even, the length of the cultured explants due to production of callus at the basal end tip. Vasudevan *et al*. (2001) and Abdisa Feyisa (2018) also reported callus were produced at the basal end of explants cultured in MS medium containing BAP in combination with higher concentrations of auxin.

Another research by Paulsamy *et al.* (2012) on *Mukia maderaspatana* reported the length of shoots increased from 2.23±0.19 to 4.23±0.19 cm with an increase in concentration of cytokinin (from 0.5 to 2.0 mg/l BAP) and auxin from 0.3 to 0.5mg/l of NAA combinations then decreased forward and minimum shoot length (1.93±0.76) was recorded in MS medium containing 2.0 mg/l BAP combined with 0.5 mg/l NAA.

Shoot number and shoot length of explants in this study had inverse relationship. Shoot length decreased with an increase in shoot number.

Generally speaking, the reason for obtaining different results from this related species for the applied exogenous PGRs could be due to the genetic factor or the difference in genetic makeup of plants, indigenous hormone level variation of the species, and the used explant types.

6.3 Rooting and Acclimatization

Auxins (IAA, IBA, NAA or 2, 4-D) are involved in the regulation of several physiological processes, for example, apical dominance and formation of lateral and adventitious roots; mainly used in root induction. Their effect varies with type and concentrations used in different plant species (Swamy and Singh, 2002). IAA was reported as potential auxin for rooting in several plants including *Arachis stenosperma* and *A. villosa* (Vijayalakshmi and Giri, 2003). Significant difference on supplementation of half strength MS medium containing different concentration of IAA and NAA combinations on rooting was observed. A maximum mean number of roots (23.67±0.19) was obtained in a half strength MS rooting medium containing 1.0 mg/l IAA combined with 0.5 mg/l NAA and mean root length (5.08±0.15 cm) was observed on half strength MS medium supplemented with 0.1 mg/l IAA. This is a better result while comparing with Abdisa Feyisa (2018), who reported the maximum mean number of root (7.77±.44) and highest mean length of root (5.42±.45 cm) in MS medium supplemented with 0.5mg/l IAA on *O. Africana.*

Maximum mean number of roots and maximum length of roots were obtained from explants in different concentration of auxins. This is in contrast with Paulsamy *et al.* (2012), who reported higher number of roots (11 roots/shoot) and greater root length (6 cm) in MS medium containing the same concentration of auxin (1.0 mg/l IBA) on *Mukia maderaspatana*. Alam *et al*. (2015) also reported maximum number of root (2.46) and maximum length of roots (2.2 cm) in rooting medium containing the same concentration of auxin (0.5 mg/l NAA) on *Cucumis sativus*. Another study by Anand and Jeyachandran (2004) reported maximum number of roots (5.6) per explant and maximum length of roots (2.2 cm) per explant in rooting medium supplemented with 2.0 mg/l NAA.

The result of this study reviled that Auxins have a great role in the regulation of physiological processes, i.e. formation of lateral and adventitious roots. Their effect varies with type and concentrations used in different plant species (Vijayalakshmi and Giri, 2003). This could be the reason for getting different result of rooting for the mentioned related specious. Acclimatization of *in vitro* rooted plantlets was successful, where 95% plants survived and established as healthy plants. This result was better than a report by Paulsamy *et al.* (2012) who got 54% of survived plants of *Mukia maderaspatana* in hardening medium of red soil, sand, and vermicompost (at

1:1:1 ratio), and Ahmad and Anis (2005) got 60% survived plants of *Cucumis sativus* in Soilrite medium. However, Abdisa Feyisa (2018) got 98% survived plantlets of *O. africana*. This could be due to the plants disease resistance capacity variation, the applied care during washing the explants for removing the medium residue and sterilization of the hardening medium.

7. CONCLUSION

Based on this study, it can be concluded that,

- ✓ BAP alone and combined with low concentration of IBA (0.01 mg/l) is the most important cytokine for the multiplication of *O. africana*.

- ✓ The best multiplication of shoots (35.97 per explant) was obtained, at 0.5 mg/l BAP, and the highest shoot length (6.17 cm) was obtained at 0.01 mg/l BAP.

- ✓ Highest mean number of roots produced was 23.67 roots per explant at 1.0 mg/l IAA combined with 0.5 mg/l NAA and highest mean length of roots (5.08±0.15 cm) was obtained at 0.1 mg/l IAA, which suggested, combining IAA with NAA is better option for good root induction.

- ✓ Although calli could be induced, the plant regeneration from leaf explants was not successful in all of the applied growth hormones types and concentrations.

- ✓ The *in vitro* grown plantlets survived in the *ex vitro* environment, when they are exposed to direct sunlight for some hours. By doing so 95 % plantlets were managed to survive.

8. RECOMMENDATION

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

- The effect of different concentrations of auxins and cytokinins, and subculturing on callus induction and regeneration of shoots should be further studied, and explants other than leaf should be considered to investigate if they can offer efficient regeneration response.

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

9. REFERENCES

Abdisa Feyisa (2018). *Micropropagation of Oreosyce africana from shoot tips*. M.Sc. Thesis, Addis Ababa University, Addis Ababa, pp. 14–19.

Ahmad, N. and Anis, M. (2005). *In vitro* mass propagation of *Cucumis sativus* L. from nodal segments. *Turk Journal of Botany*, **29**: 237–240.

Akin-Idowu, P., Ibitoye, D., Ademoyegun, O. (2009). Tissue culture as a plant production technique for horticultural crops. *African Journal of Biotechnology*, **8**: 3782–3788.

Alam. M. F., Amin, R., Uddin M. E., Biswas. S. k. and Islam M. (2015). Regeneration of shoot from nodal explants of *Cucumis sativus* considering different hormonal concentration. *International Research Journal of Biological Sciences*, **4**: 48–52.

Ali, S. and Mirza, B. (2006). Micropropagation of rough lemon (Citrus jambhiri Lush.): Effect of explant type and hormone concentration. *Acta Botanica Croatica*, **65**, 137–146.

Anand, S. and Jeyachandran, R. (2004). *In vitro* Multiple Shoot Regeneration from Nodal Explants of *Zehneria scabra* (L.f.) Sonder – An Important Medicinal Climber. *Plant Tissue Culture*, **14**: 101–106.

Azman, A., Mhiri, C., Grandbastien, M. and Tam, S. (2014). Transposable elements and the detection of somaclonal variation in plant tissue culture: a review. *Malaysian Applied Biology*, **43**: 1–12.

Balemie Kebu, Ensermu Kelbessa, and Zemede Asfaw. (2004). Indigenous medicinal plant utilization, management and threats in Fentalle area, Eastern Shewa, Ethiopia. *Ethiopian Journal of Biological Sciences*, **3**: 37–58.

Bhojwani, S. and M. Razdan, (1996). *Plant Tissue Culture: Theory and Practice: Developments in Crop Science.* Elsevier, Amsterdam.

Brown, D. and Thorpe, T. (1995). Crop improvement through tissue culture. *World Journal of Microbiology and Biotechnology*, **11**: 409–415.

Cassells A. and Doyle, B. (2005). Plant Cell Culture Protocols. **In**: *Pathogen and biological contamination management: the road ahead*, pp. 35–50, (Loyola-Vargas, VM, Vázquez-Flota, F, eds). Humana Press, New York.

Chifundera, K. (2001). Contribution to the inventory of medicinal plants from the Bushi area, South Kivu Province, Democratic Republic of Congo. *Fitoterapia*, **72**: 351–368.

Damtew Bekele, (2017). A systematic review of *oreosyce africana* Hook.f. (family cucurbitaceae) plant profile, bioactivities and chemical constituents. *International Journal of Modern Pharmaceutical Research*, **6**: 35–43.

Damtew Bekele, Beyene Petros, Habte Tekie and Zemede Asfaw (2014). Larvicidal and adulticidal effects of extracts from some indigenous plants against the malaria vector, *Anopheles arabiensis* (Diptera: Culicidae) in Ethiopia. *Journal of Biofertilizers and Biopesticides*, **5**:144.

Damtew Bekele, Habte Tekie, Zemede Asfaw and Beyene Petros (2016) Bioactive Chemical Constituents from the Leaf of *Oreosyce africana* Hook.f (Cucurbitaceae) with Mosquitocidal Activities against Adult *Anopheles arabiensis*, the Principal Malaria Vector in Ethiopia. *Journal of Fertilizers & Pesticides*, **7**: 159.

Damtew Bekele, Zemede Asfaw, Beyene Petros, and Habte Tekie (2012). Ethnobotanical study of plants used for protection against insect bite and for the treatment of livestock health problems in rural areas of Akaki district, Eastern Shewa, Ethiopia. *Topclass Journal of Herbal Medicine*, **1**: 40–52.

De Fossard, R. (1976). *Tissue culture for plant propagators*. Department of Botany, university of New England.

Diallo, M., Ndiaye, A., Sagna, M., and Gassama-Dia, Y. (2008). Plants regeneration from African cowpea variety (*Vigna unguiculata* L. Walp.). *African Journal of Biotechnology*, **7**:8

DOI: 10.1080/11263504.2018.1448014.

Espinosa-Leal, C., Puente-Garza, C. and García-Lara, S. (2018). In vitro plant tissue culture: means for production of biological active compounds. *Planta*, **248**: 1–18.

Fowler, M. (2000). Plant cell culture, laboratory techniques. **In**: *Encyclopedia of Cell Technology*, pp. 994–1002, (Spier, R. ed). JohnWiley & Sons, New York.

Geldenhuys, C. (1992). Richness, composition and relationships of the floras of selected forests in southern Africa. *Bothalia*, **22**: 205–233.

George, E. (1993). Plant propagation by Tissue Culture. *Eastern Press*, Eversley.

Habtamu Tegen and Wassu Mohammed (2016). The role of plant tissue culture to supply disease free planting materials of major horticultural crops in Ethiopia. *Journal of Biology, Agriculture and Healthcare*, **6**: 122–129.

Haile Yineger and Delenasaw Yewhalaw. (2007). Traditional medicinal plant knowledge and use by local healers in Sekoru district, Jimma Zone, south-west Ethiopia. *Journal of Ethnobiology and Ethnomedicine*, **3**: 24. https://doi:10.1186/1746-4269-3-24.

Haile Yineger, Delenasaw Yewhalaw and Demel Teketay (2008). Ethnomedicinal plant knowledge and practice of the Oromo ethnic group in southwestern Ethiopia. *Journal of Ethnobiology and Ethnomedicine*, **4**: 11.

Hinchee, M., Corbin, D., Armstrong, C., Fry, J., Sato, S., Deboer, D., Petersen, W., Armstrong, T., Connor-Wand, D., Layton, J., and Horsch, R. (1994). Plant transformation. **In:** *Plant Cell and Tissue Culture*, pp. 231–270, (Vasil, L. and Thorpe, T., eds). Kluwer Academic, Dordrecht.

Hunde Debela, Zemede Asfaw, and Ensermu Kelbessa, (2004). Use and management of ethnoveterinary medicinal plants by indigenous people in 'Boosat', Wolenchiti area. *Ethiopian Journal of Biological Sciences*, **3**: 113–132.

Husain, M., and Anis, M. (2009). Rapid *in vitro* multiplication of *Melia azedarach* L. (a multipurpose woody tree). *Acta physiologiae plantarum*, **31**: 765–772.

Hussain, A., Qarshi, I., Nazir, H., and Ullah, I. (2012). Plant tissue culture: current status and opportunities. In *Recent advances in plant in vitro culture*. InTech. DOI: 10.5772/50568

Ikeuchi, M., Sugimoto, K. and Iwase, A. (2013). Plant Callus: Mechanisms of Induction and Repression (Review). *The Plant Cell*, **25**: 3159–3173.

Jeffrey, C. (1995). Cucurbitaceae. **In**: *Flora of Ethiopia and Eritrea, Canellaceae to Euphorbiaceae*. **2**(2), pp. 17–59, (Edwards, S., Tadesse, Mesfin and Hedberg, I. eds). The National Herbarium, Addis Ababa, Ethiopia and the Department of Systematic Botany, Uppsala, Sweden.

Juan W., Jian-li L., Jing L., Jin-xin L., Shu-jie L., Lu-qi H., and Wen-yuan G. (2017). Production of Active Compounds in Medicinal Plants: From Plant Tissue Culture to Biosynthesis. *Chinese Herbal Medicines*, **9**: 115–125.

Kumar, N., Vijayanand, K. and Reddy, M. (2011). Plant regeneration in non-toxic *Jatropha curcas*- impacts of plant growth regulators, source and type of explants. *Journal of Plant Biochemistry and Biotechnology,* **20**:125–133.

Mirutse Giday, and Gobena Ameni (2003). An ethnobotanical survey of plants of veterinary importance in two woredas of southern Tigray, Northern Ethiopia. *SINET: Ethiopian Journal of Science*, **26**: 123–136.

Muluken Birara (2016). *Protocol optimization of* in vitro *propagation of an endangered endemic medicinal plant,* Echinops kebericho*, through shoot tip culture*. M.Sc. Thesis, Addis Ababa University, Addis Ababa, pp. 13.

Muluken Enyew and Tileye Feyissa (2018). *In vitro* shoot regeneration from leaf explants of *Echinops kebericho*: an endangered endemic medicinal plant, *Plant Biosystems* - An International Journal Dealing with all Aspects of Plant Biology,
DOI: 10.1080/11263504.2018.1448014.

Neuwinger, H. and Porter, A. (1996). *African Ethnobotany: Poisons and Drugs: Chemistry, Pharmacology, Toxicology.* Chapman and Hall, London, pp. 941.

Nitish k. and. Reddy M. (2011). In vitro Plant Propagation: A Review. *Journal of Forest Science*, **27**: 61–72.

Oseni, O., Pande, V. and Nailwal T. (2018) A Review on Plant Tissue Culture, A Technique for Propagation and Conservation of Endangered Plant Species. *International Journal of Current Microbiology and Applied Sciences,* **7**: 3778–3786.

Paek, K., Murthy, H. and Zhong, J. (2014). *Production of biomass and bioactive compounds using bioreactor technology.* Springer publishers, New York.

Paulsamy, S., Mallikadevi, T., Karthika, K. and Jamuna, S. (2012). Protocol for *in vitro* culture from leaf and nodal explants of *Mukia maderaspatana* (L.) M. Roem. - an important traditional medicinal climber. *Journal of Scientific Research in Pharmacy,* **1**: 36–40.

Pickens, L., Tang, Y., and Chooi, Y. (2011). Metabolic engineering for the production of natural products. *Annual review of chemical and biomolecular engineering*, **2**: 211–236.

Pierik, R. (1997). In vitro *Cultures of Higher Plants*. Springer, New York, pp. 89–125

Rout, G. (2004). Effect of cytokinins and auxin on micropropagation of *Clitoria ternatea L. Biology Leters,* **41**: 21–26.

Saini R, and Jaiwal P (2002). Age, position in mother seedling, orientation, and polarity of the epicotyl segments of blackgram (*Vigna mungo* L. Hepper) determines its morphogenic response. *Plant Sci*ence, **163**: 101–109.

Schaller, G., Street, I. and Kieber, J. (2014). Cytokinin and the cell cycle. *Current Opinion in Plant Biology*, **21**:7–15.

Singh, G. and Shetty, S. (2011). Impact of tissue culture on agriculture in India. *Biotechnology, Bioinformatics and Bioengineering,* **3:** 279–288.

Singh, M. and Kumar, S. (2009). *Principal and methodology in plant tissue culture*. APH Publishing, India. pp. 9–56.

Skirvin, R., Chu, M., Mann, M., Young, H., Sullivan, J. and Fermnian, T. (1986). Stability of tissue culture medium pH as a function of autoclaving, time and cultured plant material. *Plant Cell Reports,* **5**: 292-294.

Skoog, F., and Miller, C. (1957). Chemical regulation of growth and organ formation in plant tissues cultured in vitro. *Symposia of the Society for Experimental Biol*ogy, **11**: 118–130.

Supriya, T., Ritika, K, Shukla, L. and Deepak, K. (2017). Tissue culture study of *Ocimum sanctum* for initiation of microshoot on nodal explants. *Indian Journal of Life Science,* **6**: 1–5.

Thorpe, T (2007). History of plant tissue culture. *Journal of Molecular and Microbial Biotechnol*ogy, **37**: 169–180.

Tileye Feyissa, Welander, M., and Legesse Negash, (2005). Micropropagation of *Hagenia abyssinica*: a multipurpose tree. *Plant Cell, Tissue and Organ Culture*, **80**: 119–127.

Tilkat, E., Onay, A., Yıldırım, H., and Ayaz, E. (2009). Direct plant regeneration from mature leaf explants of pistachio, *Pistacia vera* L. *Scientia Horticulturae,* **121**: 361–365.

Tyagi, A., Comai, L., and Byers, B. (2001). Comparison of plant regeneration from root, shoot, and leaf explants in pigeon pea (*Cajanus cajan*) cultivars. *SABRAO Journal of Breeding and Genetics*, **33**: 59–71.

Vasudevan, A., Selvaraj, N., Sureshkumar, P. and Ganapathi A. (2001). Multiple shoot induction from the shoot tip explants of Cucumber *(Cucumis sativus* L*.). Cucurbit Genetics Cooperative Report,* **24**: 8–12.

Verma. S., Kumar, D., Cingoz, G., Uslu, E. and Gurel, E. (2016). Influence of nutrient media on callus induction, somatic embryogenesis and plant regeneration in selected Turkish crocus species. *Biotechnology Reports,* **10**: 66–74.

Vijayalakshmi G, Giri C (2003) Plant regeneration via organogenesis from shoot base derived callus of *Arachis stenosperma* and *A. villosa*. *Current Science,* **85**: 1624–1629.

Vizel, M., Loya, Y., Downs, C. and Kramarsky- Winter, E. (2010). A novel method for coral explant culture and micropropagation. *Marine Biotechnology*, **13**: 423–432.

Wadood, A., Ghufran, M., Jamal, S., Naeem, M., Khan, A., and Ghaffar, R. (2013). Phytochemical analysis of medicinal plants occurring in local area of Mardan. *Biochemical and Analytical Biochemistry*, **2**: 1–4.

Yamada, T., (1999). A report on the ethnobotany of the Nyindu in the eastern part of the former Zaire. *African Study Monographs*, **20**: 1–72.

10 APPENDIX

APPENDIX 1 Stock solution for MS (Murashige and Skoog, 1962**)** medium.

Components	Concentration(g/L)	ml/L during media preparation
Micronutrients		5ml/L
$ZnSO_4.7H_2O$	1.72	
H_3BO_3	1.124	
***** $MnSO_2.4H_2O$	3.38	
$MnSO_4.H_2O$	0.05	
KI	0.166	
$NaMoO_4.2H_2O$	0.05	
$CoCl_2.6H_2O$	0.05	
Na_2EDTA	7.472	
$FeSO_4.7H_2O$	5.56	
Macronutrients		50ml/L
NH_4NO_3	33	
KNO_3	38	
$CaCl_2.2H_2O$	8.8	
$MgSO_4.7H_2O$	7.4	
KH_2PO_4	3.4	
Vitamins		5ml/L
Myo-inositol	20	
Glycin (glycocoll)	0.4	
Nicotinic acid (NaOH)	0.1	
Pyridoxin (B6)	0.1	
Thiamin (B1)	0.02	

***** Alternatives

- Na_2EDTA and $FeSO_4.7H_2O$ prepared alone