ADDIS ABABA UNIVERSITY

COLLEGE OF NATURAL AND COMPUTATIONAL SCIENCE

DEPARTMENT OF ZOOLOGICAL SCIENCE

GENERAL BIOLOGY PROGRAM

Effects of Sucrose Concentration, pH and MS Media Strength on *In vitro* Propagation of *Oreosyce africana (*Hook f.) from Shoot Tip Explant



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

Andualem Manaye

May, 2020

Addis Ababa, Ethiopia

ADDIS ABABA UNIVERSITY

COLLEGE OF NATURAL AND COMPUTATIONAL SCIENCES

DEPARTMENT OF ZOLOGICAL SCIENCE

GENERAL BIOLOGY PROGRAM

Effect of MS strength, pH and Sucrose Concentrations on *In vitro* Propagation of Okra (*Abelmoschus esculentus* L.) from Shoot Tip Explants.

Advisor: Dr. Tileye Feyissa

By:

GIRMA GEBREHIWOT WOLDEYES

A Thesis Submitted to School of Graduate Studies Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biology.

Addis Ababa, Ethiopia

June 2020

ADDIS ABABA UNIVERSITY

COLLEGE OF NATURAL AND COMPUTATIONAL SCIENCES

DEPARTMENT OF ZOLOGICAL SCIENCE

GENERAL BIOLOGY PROGRAM

This is to certify that the thesis prepared by Girma Gebrehiwot W/yes entitled: Effect of MS strength, pH and Sucrose Concentrations on *In vitro* Propagation of Okra (*Abelmoschus esculentus* L.) from Shoot Tip Explants and Submitted to School of Graduate Studies Addis Ababa University in Partial Fulfillment of the Requirements for the Degree of Master of Science in Biology complies with the regulations of the University and meets the standard with respect to originality and quality.

Signed by the Examining Committee:

Examiner_____Signature _____Date _____

Examiner_____Signature _____Date _____

Major Advisors_____Signature _____Date _____

ACKNOWLEDGMENTS

First of all, I bow my head to him, the almighty merciful God for his hand of blessing and bestowing a creative and safe atmosphere during my time of academic study. It is by grace and the Almighty's will that I have this great privilege to express my greetings and appreciation to those who helped me and assisted me in compiling my thesis at various levels.

It is my proud privilege to express my heartfelt indebtedness and deepest sense of gratitude to Dr. Tileye Feyissa for his valuable inspiring guidance, through provoking discussion, constant encouragement, insight, unfailing interest, infinite patience, constructive and enthusiastic nature not only throughout this course of investigation and in the preparation of thesis manuscript but also during my entire PG programme. His fatherly approach, sympathetic understanding and generosity have been much beyond his formal obligations as a mentor of my thesis, for which I am deeply indebted to him.

I am extremely thankful and will be indebted lifelong to my parents for their unremitting encouragement, ceaseless inspiration, infinity times infinity love in this very work as well as back then.

I would like to sincerely express my humble and deep sense of gratitude to Agegnehu Wasse (PhD candidates), Fasika Tegene (MSc), Muluken Birara (PhD candidate), Tesfaye Dilebo (PhD candidates) and many more for their continuous support and encouragement in idea and technical supports.

Even though words are frivolous to express my deep sense of gratitude to Addis Ababa University in general and Institute of Biotechnology Program and Addis Ababa Education Bureau in particular, I do acknowledge my special and deep sense of gratefulness for providing me all academic and monetary supports during the study period.

TABLE OF CONTENTS PAGES

List of Tables

List of Figures

LIST OF ABBREVIATION

2, 4-D	2, 4-dichlorophenoxyacetic acid
ANOVA	Analysis of Variance
BAP	6-Benzyl Amino Purine
GA3	Gibberellic Acid
IAA	Indole 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 herbaceous slender and climbing herbaceous plant that belongs to family Cucurbitaceae, a probably monotypic genus found in tropical Africa. The extract of this plant is found to be very efficient in killing mosquitoes which could be important to control mosquitoes. However, as this plant is endangered, it can be propagated by developing efficient micropropagation protocol. For optimization of the protocol, investigation of different factors that affects the explant in culture is important. Therefore, the objective of this study was to investigate the effect of sucrose concentration, pH and MS salt strength on micropropagation of *O. africana*. Shoots were initiated on MS medium containing 0.5 mg/l BAP and then transferred to different salt strength MS medium (1/4, 1/3, 1/2, full and double full MS) containing different concentrations of sucrose (3%, 2%, 1.5%, and 1%), 0.5 mg/l BAP at different pH (5.0, 5.4, 5.8, 6.2 and 6.6) for shoot multiplication. The multiplied shoots were transferred to half salt strength MS medium containing different concentrations of IAA and IBA for rooting followed by acclimatization. The maximum mean shoot numbers per explant (31.33) was obtained on full salt strength MS medium containing 3% sucrose at 5.8 pH. The highest mean number of leaves (81.03) and the longest mean length of shoot (12.99 cm) per explant were obtained on full strength MS medium after three weeks of culture. The maximum mean root number (23.80) was obtained on ½ MS medium containing 1mg/l IAA and 0.5mg/l NAA after thirty days of acclimatization, 83.33% of plants survived. *O. africana* is too much delicate herb plant because of this it is sensitive to hormone, pH and MS strength. Therefore, appropriate amount of MS strength, pH conditions, and optimum growth regulator should be used and also more emphasis is needed for it to multiply successfully. The protocol optimized can be used for mass propagation and improvement and conservation of *O. africana.*

Key words: salt, strength, sucrose, pH and *O. africana*, micropropagation, *in vitro* propagation

1. INTRODUCTION

Oreosyce africana Hook. f. is herbaceous slender and climbing herbaceous plants growing up to 3 m and also its habitat is in wet or moist Aningeria- Syzygium forest margins, grass land and in plantations at altitude between 1650 and 2000 m (Edwards, 1991). It belongs to family Cucurbitaceae and monotypic genus *Oreosyce*. It is distributed in tropical Africa (Renner *et al.*, 2007).

O. africana is native to Ethiopia and grows in Tigray, Gonder, Gojam, Kefa and Bale. It is distributed in many African countries including Angola, Burundi, Cameroon, Kenya, Madagascar, Malawi, Rwanda, Tanzania, Uganda, Democratic Republic of Congo, Zambia, Zimbabwe and South Africa (Bekele, 2017).

The Cucurbitaceae contributes higher number of medicinal species in Ethiopia (Teklehaymanot and Giday, 2007). Traditional herbal medicine are locally available, accessible, and relatively cheap. In many countries in the tropics including Ethiopia, people have little access to modern medicine. People traditionally use different parts of plants for medicinal values. *O. africana* is also used by many people and traditional medical practitioners as insecticide. such as for mosquito management and control of cattle ticks and other arthropod pests, for intestinal worms (used as an anthelminthic) and for healing a burned body (its leaf also used as medicine) (Bekele, 2018).

Purified fractions of *O. africana* possess a very high adulticidal effect against the principal malaria vector, *Anopheles arabiensis*, and have a potential for development into an affordable botanical mosquitocide as an alternative to the existing inorganic insecticides with environmental toxicity (Bekele *et al.*, 2016a). However, as this plant is endangered, wild and insecticide, it can be propagated by developing efficient micropropagation protocol. For optimization of the protocol, investigation of different factors that affects the explant in culture is important. 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 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. 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 genotype to the parent and thus, keep their intrinsic characteristics (Dann and Wilson, 2011). 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 (Das *et al.*, 2011).

Therefore, *in vitro* propagation technique is the best alternative for the production of large number of clean and healthy planting materials of *O. africana*. Despite its medicinal purposes, mass scale cultivation of *O. africana* is not reported except the effects of hormone on the propagation of *O.africana* by Feyissa (2018) and Tegene (2019). This study differs from them by the applied treatments of plant growth in different PH conditions, different concentrations of sucrose and on different MS (Murashige and Skoog, 1962) media strength.

The extract of this plant is found to be very efficient in killing mosquitoes so that could be used to control mosquitoes. However, as this plant is endangered and wild plant, it can be propagated by developing efficient micropropagation protocol. For optimization of the protocol, investigation of different factors that affects the explant in culture is important because before this study no more studies were done with regard to the conservation process of this plant and in vitro propagation protocol through different factors. Due these factor to produce efficient amount of this plant need to use plant tissue culture technique

2. LITERATURE REVIEW

2.1. Taxonomy and description of *O. africana* Hook. f.

O. africana belongs to Cucurbitaceae family and monotypic genus *Oreosyce*. It contains climbing herbs, with simple leaves and tendrils found in tropical Africa. They have small and 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; sever 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 (Nickrent *et al.*, 1988).

2.2. Ecology and distribution

O. africana has an ecological preference most probably in wet or moist Aningeria- Syzygium forest margins, grass land and in plantations at altitude between 1650 and 2000 m a.s.l.; it is distributed in almost all directions of Ethiopia like Tigray, Gonder, Gojam, Bale and Kefa regions of Ethiopia (Bekele *et al.*, 2012).

O. africana is not only native to Ethiopia but also found other African countries like in Angola, Burundi, Cameroon, Kenya, Madagascar, Malawi, Rwanda, Tanzania, Uganda, Democratic Republic of Congo, Zambia, Zimbabwe and South Africa (Renner *et al.*, 2007).

2.3. **Importance of *O. africana* Hook. f.**

According to World Health Organization report more than 80% of the population in developing countries relies on traditional medicine, and it is now widely accepted that traditional medicines are more affordable, less toxic, and have a wide acceptance around the world as well as in tropical countries like here in our country Ethiopia (Patwardhan *et al.*, 2004).

Although the first malaria eradication campaigns against the mosquito vectors were very successful in the developed parts of the world, particularly with the combination use of DDT and pyrethroid insecticides or coupled application of dichlorodiphenyltrichloroethane (DDT) and pyrethroid insecticides, many countries in Africa, Asia and Latin America lacked the basic health infrastructure, and hence these campaigns could not yield the expected results. In Ethiopia, like other countries, vector

control through application of chemical insecticides was frustrated with the development of strong resistance of mosquitoes to the synthetic insecticides. The discovery of DDT insecticidal properties in 1939 and the subsequent development of organochlorine and organophosphate insecticides limited plant product research since the answers to insect control were thought to have been found (Mabaso *et al.*, 2004).

Increasing of insect vector populations because of the emergence and spread of physiological and behavioral resistance (Chareonviriyaphap *et al.*, 2013) to a wide range of insecticides and environmental changes had been a serious problem in the control of malaria (Ranson and Lissenden, 2016). Besides, this is the higher costs of spraying operations and high degree of refusal by the public to permit indoor spraying have remained challenges to the malaria control programs in developing countries (West *et al.*, 2014). As a result, the drawbacks of the synthetic insecticides, has necessitated the search for an environment friendly and less costly plant products as the best alternative vector control tools for use in the integrated vector management programs. Besides, mosquito control using plant materials can be obtained from local sources and are likely to generate local employment, reduce dependence on expensive imported products, and stimulate efforts to enhance public health. The selection of insecticidal plants could be optimized through initial ethnobotanical screening of plants that indigenous local communities use in their traditional medical systems. It is known that some plant derivatives have been in use as insecticides for quite some time (Bekele *et al.*, 2016a). Among these plant products, pyrethrum produced from *C. cinerariifolium* (Asteraceae), rotenone from *D. elliptica* (Fabaceae), azadirachtin from *A. indica* (Meliaceae), and nicotine from *N. tabacum* (Solanaceae) and *O. africana* (Cucurbitaceae) were widely used (Bekele *et al.*, 2016a). While these botanical bioactive products have the advantage of a relatively low mammalian toxicity and a fairly broad spectrum of activity compared with many synthetic insecticides, their short half-life of activity is the disadvantage. The low persistency of the existing botanical insecticides has been shown to be due to their susceptibility to the effects of light and air (contamination of those plants with fungi, virus and bacteria) as demonstrated for pyrethrum so to free from contaminant those plants should be cultured in plant tissue culture laboratory (El-Wakeil, 2013).

In Ethiopia, people have little access to get modern medicine. So, those people use the family Cucurbitaceae as medicinal value like other African countries. The leaf of *O. africana* has many medicinal value such as to treat an anthelminthic for intestinal worms and its leaf also used as medicine

for a burned part (Tolossa *et al.*, 2013). Similarly in southeast Tanzania, traditional practitioners (healers) make the boil of *O. africana* with the vegetable gruel for pregnant women to drink which helps the birth easy and they also rub themselves with its leaves against trichophytosis. To treat gonorrhea the filtrate solution obtained from *O. africana* was given through hypodermal injection using a syringe (Bekele, 2018).

The insecticidal properties of plants have been used in Ethiopia, where those plant materials are easily available and their use in health practices is a tradition. The people in Akaki district (east-central Addis Ababa) traditionally used *O. africana's* powder of crushed leaves by sprinkling for mosquito management and control of cattle ticks and other arthropod pests and *O. africana* was used for antimalarial treatment (Bekele, 2018).

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 for 2 days to treat patients suffering from malaria (Chinsembu, 2015).

Bioefficacy of Solvent Fractions of *O. africana* and *Piper capense* against the Malaria Vector, *Anopheles arabiensis* was assessed with high performance liquid chromatography and ultraviolet-visible spectroscopic (Bekele *et al.*, 2016b). The bioassays with dichloromethane fraction of *O. africana* showed higher adulticidal effect against *An. arabiensis* and bioactive chemical constituents from the leaf of *O. africana* 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 (Bekele *et al.*, 2016b). Even though the challenges of malaria control considers the complexity of disease control process, the complexity of the vectors and expensive cost of the control program and variations in disease patterns and in the transmission dynamics from place to place. Besides, there is resistance of the parasite to drugs and increase the spread of insecticide resistance of those specific insects.

2.4. Plant tissue culture

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). The controlled conditions provide the culture an environment conducive for their growth and multiplication

(Hussain *et al.*, 2012a). These conditions include proper supply of nutrients, optimum pH of medium, adequate temperature and proper environment (Saeed and Sun, 2012).

This technology is being widely used for large scale plant multiplication. Apart from their use as a tool of research, plant tissue culture techniques have industrial importance in the area of plant propagation, disease elimination, plant improvement and production of secondary metabolites (Hussain *et al.*, 2012a). A single explant 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 (Oseni *et al.*, 2018). Endangered, threatened and rare species (bamboo) have successfully been grown and conserved by micropropagation because of high efficiency of multiplication and small demands on number of initial plants and space (Hussain *et al.*, 2012a).

Plant tissue culture is an essential for mass multiplication of plant, it also provides the means to multiply and regenerate novel plants from genetically engineered cells (Loyola-Vargas and Vázquez-Flota, 2006). The promising plant thus produced may be readily cloned in cultures under aseptic conditions. Tissue culture is widely used in obtaining disease free plants, rapid propagation of plants those are difficult to propagate even under conventional method, somatic hybridization, and genetic improvement of plants, obtaining androgenic and gynogenic haploid plants for breeding programs (Karp, 1995).

Tissue of plant is an alternative means to vegetative propagation of plants (Rowe, 1986). *In vitro* growing plants are usually important to produce plants free from bacterial and fungal diseases (Debergh and Maene, 1981). Virus eradication and maintenance of plants in virus free stage can also be rapidly achieved in cultures (Roca *et al.*, 1978; Smith and Drew, 1990; Wang and Valkonen, 2008). Three main methods generally used in tissue culture are micropropagation through the enhanced multiplication of axillary bud, organogenesis, and somatic embryogenesis (Martin, 2002).

At present the most successful and commonly used method is enhanced shoot multiplication from the axillary bud. Axillary buds are present in the axis of leaves (Jiménez *et al.*, 2006). In tissue culture, by using optimum concentration of cytokinin or combination of cytokinin and auxin the dormancy of the axillary buds can be broken. Once the dormancy is broken, they develop into shoots (Hewelt *et al.*, 1994). By using media containing optimum concentrations of plant growth regulators, they can be made to multiply very rapidly. Modern plant tissue culture is performed under aseptic conditions under filtered air (Hewelt *et al.*, 1994). Living plant materials from the environment are naturally contaminated on

their surfaces (and sometimes interiors) with microorganisms (Hewelt *et al.*, 1994), so surface sterilization of starting materials (explants) in chemical solutions (usually alcohol) is required. Mercuric chloride is used as a plant sterilizing agent today, as it is dangerous to use, and is difficult to dispose of (Singh *et al.*, 2011).

Explants are then usually placed on the surface of a solid culture medium, but are sometimes placed directly into a liquid medium, particularly when cell suspension cultures are desired. Solid and liquid media are generally composed of inorganic salts plus a few organic nutrients, vitamins and plant hormones. Solid media are prepared from liquid media with the addition of a gelling agent, usually purified agar (Gupta and Pullman, 1991).

The composition of the medium, particularly the plant hormones and the nitrogen source (nitrate versus ammonium salts or amino acids) have profound effects on the morphology of the tissues that grow from the initial explant (Gleddie *et al.*, 1983). An excess of auxin will often result in a proliferation of roots, while an excess of cytokinin may yield shoots (Idowu *et al.*, 2009). A balance of both auxin and cytokinin will often produce an unorganized growth of cells, or callus, but the morphology of the outgrowth will depend on the plant species as well as the medium composition (Idowu *et al.*, 2009). As culture grows, pieces are typically sliced off and transferred to new media (subcultures) to allow for growth or to alter the morphology of the culture (George *et al.*, 2007). The skill and experience of the tissue culturist are important in judging which pieces to culture and which to discard (Idowu *et al.*, 2009). As shoots emerge from a culture, they may be sliced off and rooted with auxin to produce plantlets which, when mature, can be transferred to potting soil for further growth in the greenhouse as normal plants (Idowu *et al.*, 2009).

Moreover, plant tissue culture is considered to be the most powerful technology for crop improvement by the production of somaclonal and gametoclonal variants (james, 1987; Rahman and Rajora, 2001). 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 which leads to the development of commercially important improved varieties (Bairu *et al.*, 2011).

The micropropagation technology has a large power of production of plants with superior quality and separation of useful variants in well-adapted and high yielding genotypes with better disease resistance and stress tolerance capacities (Chindi *et al.*, 2014).

Commercial production of plants through micropropagation techniques has several merits than the traditional or conventional methods of propagation through seed, cutting or pieces of stem, grafting and air-layering, etc. because it is rapid propagation processes that can produce virus free plants (Leakey and Akinnifesi, 2008).

Callus induction and plant regeneration are key tools in plant biotechnology that exploits the totipotent nature of plant cells. Totipotent nature of plant cell means production of the whole plant body from single cell or the ability of single plant cell to produce the whole plant body (Verdeil *et al.*, 2007; Birnbaum and Alvarado, 2008; Mukherjee *et al.*, 2011). Systems of plant regeneration can be categorized as direct (Fan *et al.*, 2012) and indirect (Li *et al.*, 2012). Almost all types of explant tissues are now used as regeneration systems through direct (direct generation from explants) and indirect methods (callus-mediated shoot regeneration). Shoot can be derived either through differentiation of non-meristematic tissues known as adventitious shoot formation or pre-existing meristematic tissues known as axillary shoot formation (Mukherjee *et al.*, 2011). Successful plant regeneration protocol requires appropriate choice of explant, age of the explant, definite media formulations, specific growth regulators, genotype, source of carbohydrate, gelling agent and other physical factors including light hour, temperature, humidity, etc (Mukherjee *et al.*, 2011).

2.4.1. Micropropagation

Micropropagation is one of the most successful techniques of tissue culture. It is the practice of rapidly multiplying stock plant material to produce a large number of progeny plants, using modern plant tissue culture methods (Sahu and Sahu, 2013). It is important to multiply novel plants, such as those that have been genetically modified or breed through conventional plant breeding methods (Dale, 1994). It is also used to provide large number of plantlets for planting from a stock plant which does not produce seeds, or does not respond well to vegetative reproduction (Hoffmann, 1998). During around last three decades progress in this field has been such that multiplication of many ornamental and fruit cultivars is being practiced on commercial feasible method of clonal propagation (Wilkins and Dodds, 1983).

Plant regeneration can be achieved by culturing tissue sections either lacking a preformed meristem (adventitious origin) like axillary bud proliferation approach or from callus and cell cultures. It is the stimulation of axillary buds, which are usually present in the axil of each leaf to develop into a shoot (Hong and Bhatnagar, 2007).

This technique exploits the normal ontogenic route for branch development by lateral (axillary) meristem (Phillips and Hubstenberger, 1995). In nature these buds remain dormant for various periods depending upon the growth pattern of plant (Shimizu-Sato and Mori, 2001). In some species, removal of terminal bud is essential to inhibit apical dominance and stimulate the axillary bud to grow into shoot (Brown *et al.*, 1967). Due to continuous application of cytokine in cultured medium the shoot formed by the bud, which is present on explants develops axillary buds (Romocea *et al.*, 2010). The shoot is then separated and rooted to produce plants or shoots are used as propagules for propagation (Tanaka *et al.*, 2006). The importance of using axillary bud proliferation meristem, shoot tip or bud culture as a means of regeneration is that the incipient shoot has already differentiated *in vivo* (Bornman, 1993*)*. Thus, only elongation and root differentiation are required to establish a complete plant (Pagès *et al.*, 2010). Another major advantage of this technique is that it preserves the precise arrangement of all layers necessary if a chimeral plant genetic section is to be maintained. In a typical chimera, the surface layers of developed meristem are of differing genetic background and it is their contribution in particular arrangement to the plant organ that produces the desired characteristics. As long as the integrity of the meristem remains intact and development is normal *in vitro* then the chimeral pattern will be preserved (Grout, 1999). If however, callus tissue were allowed to form the body of plant and shoot proliferation subsequently was from adventitious origin, then there would be a risk that the chimeral layers of original explants may not all be consistent in the adventitious shoots (de Klerk and Smulders, 2006).

2.4.2. General technique of micropropagation

The process of plant micro-propagation aims to produce clones of plant that has true copies of a plant in large numbers. The process is usually divided into the different stages: Stage 0: this pre-propagation stage requires proper maintenance of the mother plants in the greenhouse under disease and insect free conditions with minimal dust (Cassells and Doyle, 2006). Stage 1: (Initiation) sterilization of explants and establishment of explants (Husain and Anis, 2009). Stage 2: This is the most important stage and the rate of multiplication determines the largely success of micropropagation system this can be achieved by enhanced axillary branching, adventitious bud formation, through callusing enhanced axillary branching (Saini and Jaiwal, 2002). Stage 3: for induction of roots they were transferred to rooting medium and rooting half strength MS medium was used (Pati *et al.*, 2006; Hussain *et al.*, 2012b). Stage 4 (acclimatization): is a gradual process of transporting the plantlets from *in vitro* state to the field conditions (Bhojwani and Dantu, 2013).

2.4.3. Advantages and disadvantages of micropropagation

It is based on the cell doctrine that states a cell is capable of autonomy and is potentially totipotent (Grafi *et al.*, 2011). The most important kinds of organ cultures used for micropropagation are meristem cultures (Zucchi *et al.*, 2002), shoot cultures (Fadzilla *et al.*, 1997), embryo cultures (Gonzalez-Arnao *et al.*, 2008) and isolated root cultures (Medina-Bolivar *et al.*, 2007).

Micropropagation is one form of tissue culture which allows the production of large number of plants from small pieces of the mother plant or explant in relatively short period of time and limited space (Hussain *et al.*, 2012a). It is an aseptic process which requires sophisticated laboratory procedure with unique facilities and special skills (Loberant and Altman, 2009).

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 micro shoots. *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 (Murashige and Skoog, 1962).

Although living cells are considered potentially totipotent, only some cells that are competent divide and give rise to the whole plant body in tissue culture. Furthermore, not all plant species are equally amenable to tissue culture (Vasil and Thorpe, 2013). Production 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 (Feyissa, 2006). 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 (McCown, 2000).

Establishing shoot culture generalized into three stages; aseptic culture establishment (Bohra *et al.*, 2014), multiplication of propagule and preparation for reestablishment of plants in soil (Takayama and Akita, 2005). 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 (McCown, 2000).

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 (Feyissa *et al.*, 2005).

On the other hand, micropropagtion has its drawbacks such as it is very expensive, and can have a labor cost of more than 70%, a monoculture is produced after micropropagation, leading to a lack of overall disease resilience, as all progeny plants may be vulnerable to the same infections and an infected plant sample can produce infected progeny and some plants are very difficult to disinfect of fungal organisms (Rani and Kumar, 2017).

2.5. Source of explants and aseptic techniques

The age, types and position of explants determine the success of tissue culture because all living plant cells have different ability to express totipotency (Harding, 2004). The most commonly used explants are shoot tips, nodal buds and root tips with optimum lengths of that explant types. Large explants can increase chances of contamination and small explants like meristem sometimes might show less growth (Kane, 1996).

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 (Ochatt *et al.*, 2018; Tegene, 2019).

The fact that source of explant has different capacity of regeneration are well documented (Feyissa *et al.*, 2005). *In vitro* explant in general has better potential to organogenesis than *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 (Feyissa *et al.*, 2005) due to different level of plant hormones present in the plants

Most surface contaminants such as bacteria and fungi can be eliminated by surface sterilizing of the plant material with a suitable sterilizing agent. Surface sterilizing agents are normally applied for 10-15 minutes (Oyebanji *et al.*, 2009) . Under aseptic conditions, the sterilizing solutions are then removed and the plant material is washed 3 or 4 times for 5 minutes each time by agitation in double sterile distilled

water. Washing is important to remove excess sterilizing agent which inhibits the growth of explant. Explants may also be surface sterilized with an aqueous solution of sodium or calcium hypochlorite or mercury chloride. The calcium salt is preferred as it is less phytotoxic whereas mercuric chloride is significantly phytotoxic and it has carcinogenic effects. 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. Sterilization of laboratory instruments is carried out by autoclaving, alcohol washing, baking, radiations, flaming and fumigation (Ahsan *et al.*, 2013).

2.6. 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 commonly composed of the most important chemical substances such as macronutrients, micronutrients, vitamins, other organic components, plant growth regulators, carbon source and some gelling agents in case of solid medium (Murashige, 1977).

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) (Pierik, 1997)

2.6.1. Effects of MS basal media strength on plant growth

Momordica balsamina is herbaceous plant that belongs to the Cucurbitaceae family. The regenerated shoots of *M. balsamina* gave the highest number of shoot (3.75) on full MS strengths medium as compared to ½ or ¼ MS strength. However, maximum elongation of the shoot was observed in ½ MS compared to full and ¼ MS strengths (Thakur *et al.*, 2011).

According to Thakur *et al*. (2011) significant differences in rooting were observed on different concentrations of IBA i.e. 0.5, 1.5, 2.5 mg/l. Maximum root number (2.67) and root length (3.35 cm) were observed on medium containing 1.5 mg/l IBA while at concentration lower than 1.5 mg/l decline in

all parameters. NAA is another vital plant growth regulator which plays an important role in the rooting. Significant differences in rooting were observed on various concentration of NAA i.e. 0.1, 0.3, 0.5 mg/l. Maximum root number (4.75) and root length (5.25 cm) were observed on medium containing 0.3 mg/l NAA. Plants raised *in vitro* are habituated to survive in a high humid environment which often results in poor cuticle development (Thakur *et al.*, 2011). When these plants are transferred to nature, they undergo wilting due to excessive desiccation and death (Thakur *et al.*, 2011) caused by poor stomatal control. For better field survival the plants were acquainted to the external environment gradually by opening the plants for 1 h daily. The fast growth attained by *M. balsamina* in the present work may possibly be attributed to the porosity of soil maintained by providing sand at the upper layer of pots for better root development. This has in turn facilitated high rate of field survival (85%) of the *in vitro* raised plants and similar high field transfer success (90%) has been reported in other *in vitro* raised plant (Thakur *et al.*, 2011)

2.6.2. Effects of sucrose on plant growth and multiplication

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 absence of well-developed leaves in the plantlets, the growth taking place in conditions unsuitable for photosynthesis or without photosynthesis (Jo *et al.*, 2009).

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 (Gauchan, 2012).

The different concentrations of sucrose used enhanced micro-propagation of *Musa* species compared to the control. The maximum number of shoots (7.83) was obtained on medium containing 30 g/l sucrose. Control recorded low number of shoot (1.0) and length (1.8 cm). It was observed that the color of leaves at 60 and 75 g/l sucrose was yellow. Also the highest length of shoots (8.83) and highest length of roots (7.28 cm) and maximum number of roots (12.33) and (9.83) were obtained on medium containing 30

and 45 g/l sucrose, respectively. The maximum mean number of root length of *Musa* species was 7.28 and 7.25 at 30 g/l and 45 g/l respectively (Martin *et al.*, 2007).

2.6.3. Effects of vitamin on plant growth

Four vitamins are ingredients of plant tissue culture medium (Booth and Allan, 2003). These vitamins are myo-inositol, thiamine, nicotinic acid, and pyridoxine which 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 (Saad and Elshahed, 2012).

2.6.4. Effects of pH on plant growth and multiplication

Soil pH affects nutrient availability because the H^+ ions take up space on the negative charges along the soil surface displacing nutrients which means the effect on nutrient availability depends on the size and charge of the nutrient molecules and whether or not they can be lost to leaching (Sharma and Dubey, 2005).

The metal nutrients like copper, iron, manganese, zinc are small molecules when dissolved in water with 2 to 3 positive charges, thus a high charge to size ratio. Due to this they bind strongly to the surface of soil particles (Harmsen and Vlek, 1985; Brümmer, 1986). At high pH, these metal ions stick so tightly they are not readily found in soil solution and thus they are less available for plant uptake (Olaniran *et al.*, 2013).

At low pH, fewer can stick to the soil surface, making them more available for plant uptake (Clarholm and Skyllberg, 2013). Sulfur (S) and the base-forming cations (Ca^{2+}, Mg^{2+}, K^+, and Na^+) are relatively large molecules. Like a large electrostatically charged balloon does not stick well to a wall, these large molecules do not stick tightly to soil particles. Therefore, even at high pH, they easily come off of the soil particle and enter soil solution. At low pH they are displaced by H^+ and may not be available because they have been lost from the soil through leaching or uptake (Jones and Jacobsen, 2005). Nitrate (NO^{3-}) is equally available across soil pH levels because it doesn't bond much to soil (Magdoff and Van Es, 2000).

The cultures on different pH conditions started responding 5-7 days post culture initiation. All explants on the different pH ranges responded well and gave rise to a matured *in vitro* material by the end of the month. Maximum mean number of shoots, number of leaves, and number of nodes and length of shoots were attained at a pH level of 5.6 in case of Kell which are 4.10, 12.10, 4.30 and 5.00, respectively.

While in Qulle Maximum mean number of shoots, number of leaves, number of nodes and length of shoots were attained at a pH level of 6.6 which are 2.40, 9.80, 4.12 and 4.90, respectively (Birhanu, 2011)

2.6.5. Effects of solid media strength on shoot multiplication and root induction

According to Tegene (2019) on *O. africana*, full strength MS medium supplemented with 0.5 mg/l BAP resulted in maximum number of shoots per explant (35.97±0.41) and maximum shoot length of 6.17±0.12 cm was recorded in MS medium supplemented with 0.01 mg/l BAP. 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. 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. Shoots cultured on half strength MS medium supplemented with different concentrations of IAA (0, 0.25 and 0.5 mg/l) produced roots. The maximum mean number of root (7.77±.44) and highest mean length of root (5.42±.45 cm) was obtained on MS medium supplemented with 0.5 mg/l IAA. As concentration of IAA increased, the root number and root length increased.

2.7. Plant growth regulators

The highest rate of micropropagation often depends on the selection of the most suitable explants, on the discovery of the correct combination of growth regulators, and the best nutritional composition of the medium for particular explants (Krikorian, 1995). The growth regulators are required in very small 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 (Bairu *et al.*, 2007) .

Auxins (IAA, IBA, NAA and 2, 4-D) are involved in the regulation of several physiological processes (Saniewski *et al.*, 2002) . These growth regulators generally cause cell elongation, swelling of tissues, cell division, callus formation and the formation of adventitious roots as well as the inhibition of adventitious and axillary shoot formation (Kumar and Reddy, 2011). Also auxins are often added to the culture medium to promote the growth of callus, cell suspensions or organs and to regulate morphogenesis or body formation process, especially in combination with cytokinins (Krikorian, 1995). IBA and IAA are widely used for rooting (Štefančič *et al.*, 2005) and in interaction with cytokinins for root 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 diluted with NaOH (Torres, 1989).

Cytokinins are composed of adenine (aminopurine) and play an important role in the *in vitro* manipulation of plant cells and tissues (Jones and Schreiber, 1997). The most common cytokinins used are kinetin, 6 Benzyl Amino Purine (BAP), thidiazuron (TDZ), zeatin and 2iP. Those hormones are concerned with cell division, modification of apical dominance, shoot differentiation, etc (Piotrowska *et al.*, 2005). 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 (Aremu *et al.*, 2012).

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* (Lang, 1970).

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 (Rangel *et al.*, 2015). Abscisic acid is most common requirement for normal growth and development of somatic embryos and only in its presence do they closely resemble zygotic embryos (Zimmerman, 1993). It is also known to promote morphogenesis in begonia cultures (Rai *et al.*, 2011). 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 (Ziv, 2005).

3. OBJECTIVES

3.1. General objective

- To investigate the effect of different sucrose concentration, pH and MS media strength on *in vitro p*ropagation of *Oreosyce africana (*Hook f.)

3.2. Specific objectives

- To determine optimum concentration of MS salt strength, pH, and sucrose for shoot induction and multiplication of *O. africana* in liquid medium.
- To determine the optimum pH and MS medium strength in liquid medium for rooting of *O. africana*.
- To evaluate the survival of *O. africana* plantlets in greenhouse.

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 *O. africana* Hook. f. were obtained from Plant Tissue Culture and Molecular Biology Laboratory, Institute of Biotechnology, Addis Ababa University. The plants were initially collected from areas around Akaki and Ilu Gelan district, located in 8033'-8057' N latitude and 38043'-38050' E longitude, maintained in Biomedical Sciences Laboratory, Department of Microbial, Cellular and Molecular Biology Department. These plants were obtained from Prof. Beyene Petros and maintained in greenhouse and then transferred to Plant Tissue Culture and Molecular Biology Laboratory of Institute of Biotechnology, Addis Ababa University for multiplication purpose. The microshoots of *in vitro* cultivated stock plants were maintained by subculturing in MS basal medium supplemented with 0.5 mg/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, mixture of Fe-Na-EDTA with FeSO4 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. The nutrients were added step by step, *i.e.* the next nutrient was added after the first one was completely dissolved by using magnetic stirrer. After all the components were fully dissolved, the solution was dispensed in to plastic bottles or test tube and stored at -20°C until used.

4.3. Plant growth regulators stock preparation

In this study, plant growth regulators such as BAP for shoot induction, Indol-Acetic Acid (IAA) and Naphthalene Acetic Acid (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. By using magnetic stirrer, the powder was first dissolved by 2-3 drops of NaOH or HCl based on the requirement of the plant growth regulators (adding HCl on cytokinine powder and adding NaOH on auxin powder for dissolving). Then, the volume or distilled water was adjusted or measured by graduate

cylinder to adding on that measured powder of cytokinin or auxin and stirred until fully dissolved. The growth regulator stock solutions were stored in a refrigerator at +4°C for short term use.

4.4. Culture medium preparation

In order to prepare the medium, 30 g/l of sucrose was dissolved in distilled water and 50 ml of macronutrient, 5 ml of micronutrient, 5 ml of vitamin and 5 ml of Fe-Na-EDTA with FeSO4 mixture per liter were added consecutively. Eventually, different concentrations of sucrose, different strength of MS media were prepared. After adding the growth hormones (0.5 mg/l BAP for shoot multiplication and 1ml/L IAA combined with 0.5ml/L NAA for root induction), pH was adjusted to 5.8. For studying the effect of different level of pH, the media pH value was adjusted to 5.0, 5.4, 5.8, 6.2 and 6.6 using NaOH and/or HCl (by keeping constant the hormone at 0.5mg/l, full MS and 30g/l sucrose). Then the prepared medium in such a way was dispensed in to 30 test tube culture vessels. Each test tubes contain 10 ml MS liquid media and autoclaved at a temperature of 121°C and pressure of 105 KPa for 15 min.

After autoclaving, the dispensed medium by each test tube culture vessel was transferred to a laminar air flow cabinet. For shoot regeneration, full strength MS basal medium were used and the same procedure of preparation were followed. The medium were fortified or prepared with different concentrations of sucrose, different MS strength or basal medium and different pH conditions and also adding of an optimum concentrations of BAP hormone for this plant by referring an optimum hormones from previous and the more recent research work of Tegene (2019) and adjusted pH conditions at 5.8 for each prepared media. After adding the growth hormones, pH was adjusted to different values on media containing full MS, 30 g/l sucrose and 0.5 mg/l BAP hormone to examine the growth effects of pH on this plant using NaOH and/or HCl. Then 10 ml of the prepared medium was dispensed in to a test tube culture vessels and autoclaved at a temperature of 121°C and pressure of 105 KPa for 15 min. 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, half, one third and one fourth strength of MS basal medium containing 30 g/l sucrose and 0.5 mg/l BAP were used. Full strength MS medium containing different concentrations of sucrose (3%, 2%, 1.5% and 1%) and 0.5 mg/l BAP at pH of 5.8 was also used. Full strength MS containing 3% sucrose and 0.5 mg/l BAP at pH of 5.0, 5.4, 5.8, 6.2 and 6.6 were prepared and also the preparation procedure were used for shoot regeneration medium. For rooting, different strength MS basal medium (full MS, half MS, one third MS and one fourth MS) containing 30 g/l sucrose at 5.8 pH

were used. In addition, full strength MS medium containing different concentration of sucrose (3%, 2%, 1.5% and 1%) at different pH value (5.0, 5.4, 5.8, 6.2, and 6.6) was used.

4.5. Culture initiation

The sterilized shoot explants were trimmed to 2-2.5 cm long and used for shoot and node culture. The explants had three or four nodes. The explants were cultured on 15 ml full strength MS medium containing 0.5 mg/l BAP, 3% sucrose and 0.7% agar in culture vessels. The pH was adjusted to 5.8 before the addition of agar and autoclaved at 121°C and 0.15 KPa pressure for 15 min. The cultures were maintained at a temperature of 25±2°C and light intensity of 23 µmol $m^{-2}s^{-1}$ under 16 h photoperiod. Unless indicated, all cultures were maintained under these growth conditions. Five explants per culture vessel were used. In another experiment, shoot explants were cultured in 15 ml liquid MS medium in 25 mm × 100 mm test tubes and kept on an orbital shaker at 110 rpm for a week.

4.6. Shoot multiplication

The shoots that were initiated on solid MS medium were transferred to liquid multiplication medium. While the shoots from solid medium were transferred to the liquid medium to multiplication treatment was considered different treatment protocols such as MS medium strength (full MS, half MS, one third MS and one fourth MS), different pH conditions (5.0, 5.4, 5.8, 6.2, 6.6) and different concentration of sucrose (3%, 2%, 1.5% and 1%) were evaluated for their effect on shoot multiplication after four weeks growth period. All of these shoot multiplication media were supplemented with 0.5mg/L BAP for shoot multiplication and 1mg/L IAA combined with 0.5mg/L NAA for rooting. A treatment consisting of one in a tube were used to count number of shoots, number of leaf and shoot lengths per explant were recorded after four weeks.

4.7. Rooting

For root induction, microshoots of about 2.5 to 3.5 cm long were excised and transferred to the rooting media. The rooting media was full, half, one third and one fourth strength MS basal medium supplemented with 1mg/l IAA combined with 0.5 mg/l NAA and another treatment at different pH (5.0, 5.4, 5.8, 6.2, and 6.6) by keeping the other components of media normal or standard. The cultures were maintained at 25 ± 2°C with 16 h photoperiod at light intensity of 22 µmol m-2s-1 for 30 days. All experiments were performed by using test tube. After thirty days of culture, the number and length of roots per explant were recorded.

4.8. Acclimatization

Plantlets with developed roots were carefully and systematically transferred from culture vessels to potted soil. 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 plants survived and established as healthy. After 4 weeks of culturing in rooting media, plantlets were removed from culture vessel and the roots were thoroughly washed under 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, clay 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. Thirty plantlets were planted for acclimatization and the number of survived plants was recorded after a month.

4.9. Experimental design and data analysis

A completely randomized design (CRD) was used for all experiments. Data of all treatments were subjected to one-way 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. Shoot initiation

The explant shoot tips were took from rooting growth culture medium of previous researcher Tegene (2019) and transferred to new multiplication medium containing 50 ml full strength MS medium with 0.5 ml/l BAP alone, 30g/l sucrose and 7g/l agar and 33±0.532 shoot number explants were produced after one month of culturing.

Figure 1. Shoot initiation of *O. africana* on solid MS medium containing 0.5 mg/l BAP. Bar represents 1.5 cm.

5.2. Effect of MS salt strength on shoot multiplication

There was a significant difference among the four MS media strength in the number of shoots, number of leaves and length of shoot. The highest mean number of shoots per explant (31.33±0.99) was obtained on full salt strength MS medium. The highest mean number of leaves (81.03±4.96) and the longest mean length of shoot (12.99±0.95 cm) per explant were obtained on full strength MS medium whereas the lowest mean number of shoot (2.35±0.25), mean number of leaves (9.40±0.93) and shortest mean length of shoot (3.32±0.14 cm) per explant were recorded. When we compare each MS treatment based on necrosis there were significance difference between one fourth MS and full MS treatment because one fourth treatment becomes necrotized after three week eventually which means for *O. africana in vitro* propagation was good at full MS medium. But on double full MS (2x) treatment all explants were dried completely.

Table 1: Effect of MS salt strength on mean number of new shoots, leaves and length of shoots per explant of *O. africana* on media containing 0.5 mg/l BAP

MS strength	No. of shoots	No. of leaves	Length of shoots
Full	31.33 ±0.990[a]	81.03 ± 4.96[a]	12.95± 0.399[a]
1/2	7.53 ±0.922[b]	26.57 ±3.45[b]	3.74±0.143[b]
1/3	4.47±0.527[c]	16.23 ±1.04[b]	9.35±0.203[b]
¼	2.35 ±0.257[c]	9.40 ± 0.93[c]	3.32±0.145[b]
Double full (2x)	0.00±0.00[d]	0.00±0.00[c]	0.00±0.00[c]

Numbers connected by the same subscript letters in the same column are not significantly different at $p<0.05$.

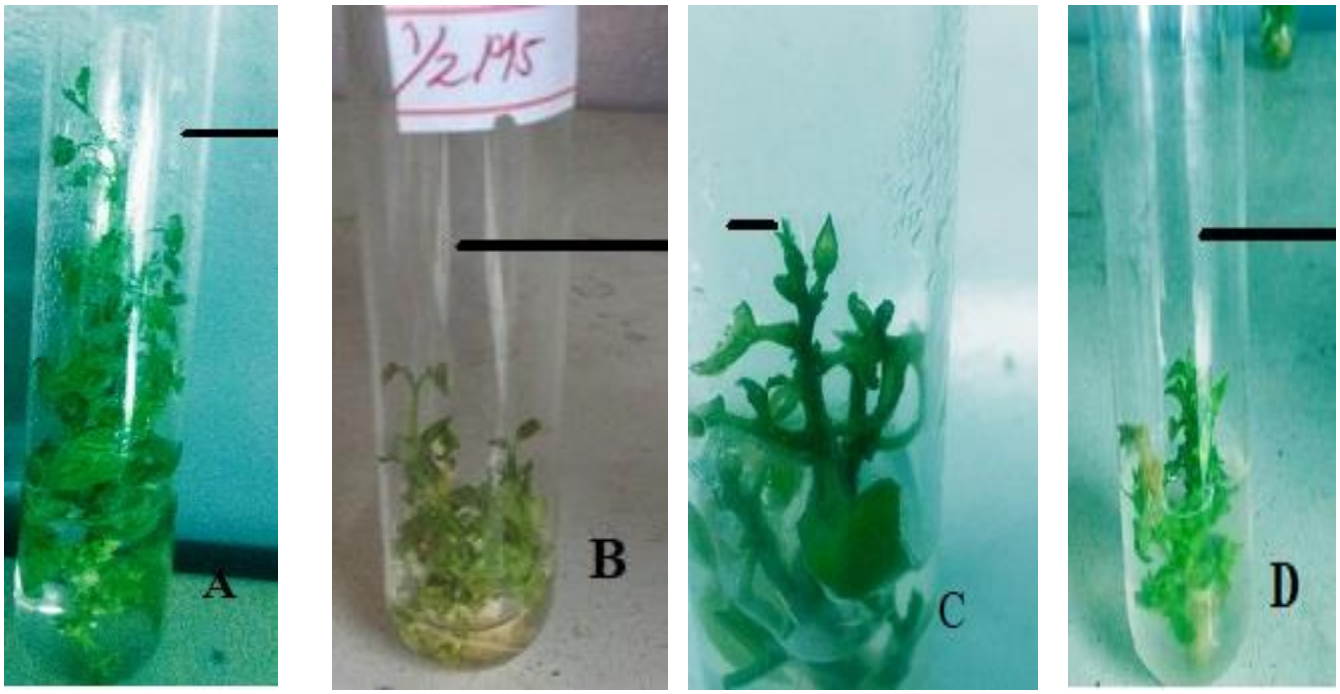

Figure 2. Effect of different MS strength on shoot multiplication of *O. africana*: A) full MS, B) ½ MS, C) 1/3MS and D) ¼ MS strength. Bar represents 1.5 cm.

5.3. Effect of pH on shoot multiplication

There was significant difference among number of shoots, number of leaves and length of shoot per explant at pH 5.0, 5.4, 5.8, 6.2 and 6.6 in *O. africana.* The highest mean number of shoots (31.33±0.99), leaves (81.03±4.96) and length of shoots (12.99±0.39 cm) per explant were obtained at a pH of 5.8 from *O. africana*. The lowest mean number of shoots per explant were obtained at pH of 6.6 at (Table 2) because all explants dried.

Table 2: Effect of different pH levels on mean number of shoots, leaves and length of shoots per explant of *O. africana* on MS medium containing 0.5 mg/l BAP

pH	No. of shoots	No. of leaves	Length of shoots (cm)
5.0	3.07 ± 0.439^{b}	6.83 ± 0.656^{c}	3.61 ± 0.245^{c}
5.4	5.07 ± 0.540^{b}	32.57 ± 3.416^{b}	4.87 ± 0.329^{b}
5.8	31.33 ± 0.99^{a}	81.03 ± 4.963^{a}	12.99 ± 0.390^{a}
6.2	3.57 ± 0.495^{b}	27.80 ± 2.072^{b}	4.27 ± 0.263^{bc}
6.6	0.00 ± 0.000^{c}	0.00 ± 0.000^{d}	0.00 ± 0.000^{d}

Numbers connected by the same subscript letters in the same column are not significantly different at $p<0.05$.

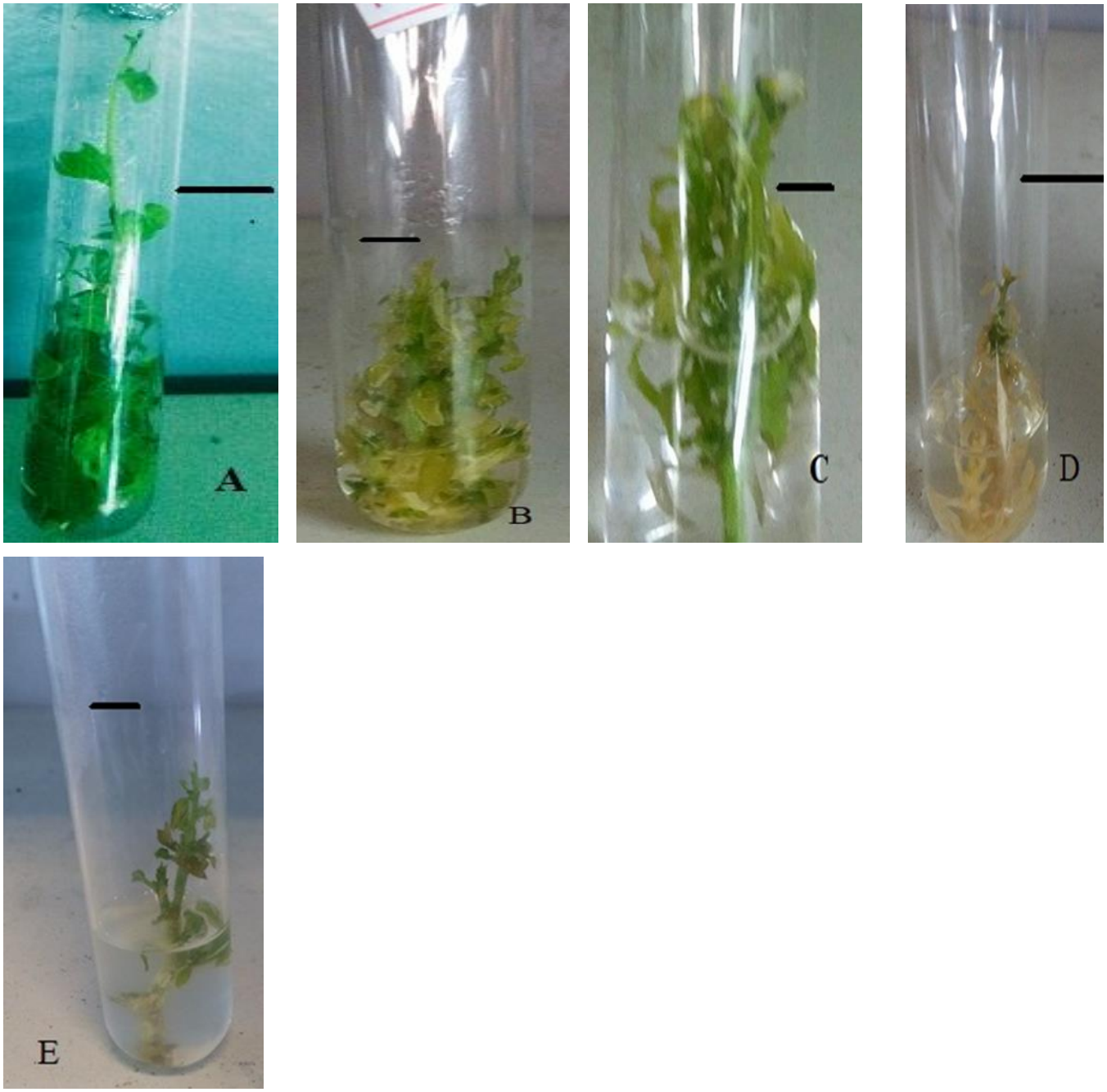

Figure 3. Effect of different pH on shoot multiplication of shoot tip explnts of *O. africana*: A) 5.8 pH, B) 5.4 pH, C) 6.2 pH, D) 6.6 pH and E) 5.0 pH. Bar represents 1.5 cm.

5.4. Effect of different sucrose concentrations on shoot multiplication

There was significant difference in mean number of shoots, leaves and shoot length among shoots cultured on MS medium containing different concentrations of sucrose. The highest mean number of shoots (31.33±0.989), mean number of leaves (81.03±4.96) and length of shoots (12.99±0.39 cm) per explant were obtained on medium containing 3% sucrose (Table 2). On the other hand, the lowest mean number of shoots (2.44±0.21), leaves (11.03±0.60) and length of shoots (3.64± 0.56 cm) per explant were recorded on medium containing 1% sucrose. The shoots had better appearance at 3% sucrose as compared to those cultured on medium containing 2% sucrose, 1.5% and 1% and also there was no sign of necrosis at all sucrose concentrations.

Table 3: Effect of different concentrations of sucrose on mean number of shoots, leaves, and length of shoots per explant of *O. africana* on MS medium containing 0.5 mg/l BAP

Sucrose conc. (%)	No. of shoots	No. of leaves	length of shoots
3%	**31.33 ± 0.989^{a}**	**81.03 ±4.963^{a}**	**12.95±0.390^{a}**
2%	12.90± 0.737^{b}	36.03 ± 2.465^{b}	4.40±0.165^{b}
1.5%	6.730 ±0.565^{c}	18.83 ± 1.094^{c}	3.69 ±0.199^{b}
1%	2.44 ±0.218^{d}	11.03 ± 0.605^{c}	3.64 ±0.560^{b}

Numbers connected by the same subscript letters in the same column are not significantly different at $p<0.05$.

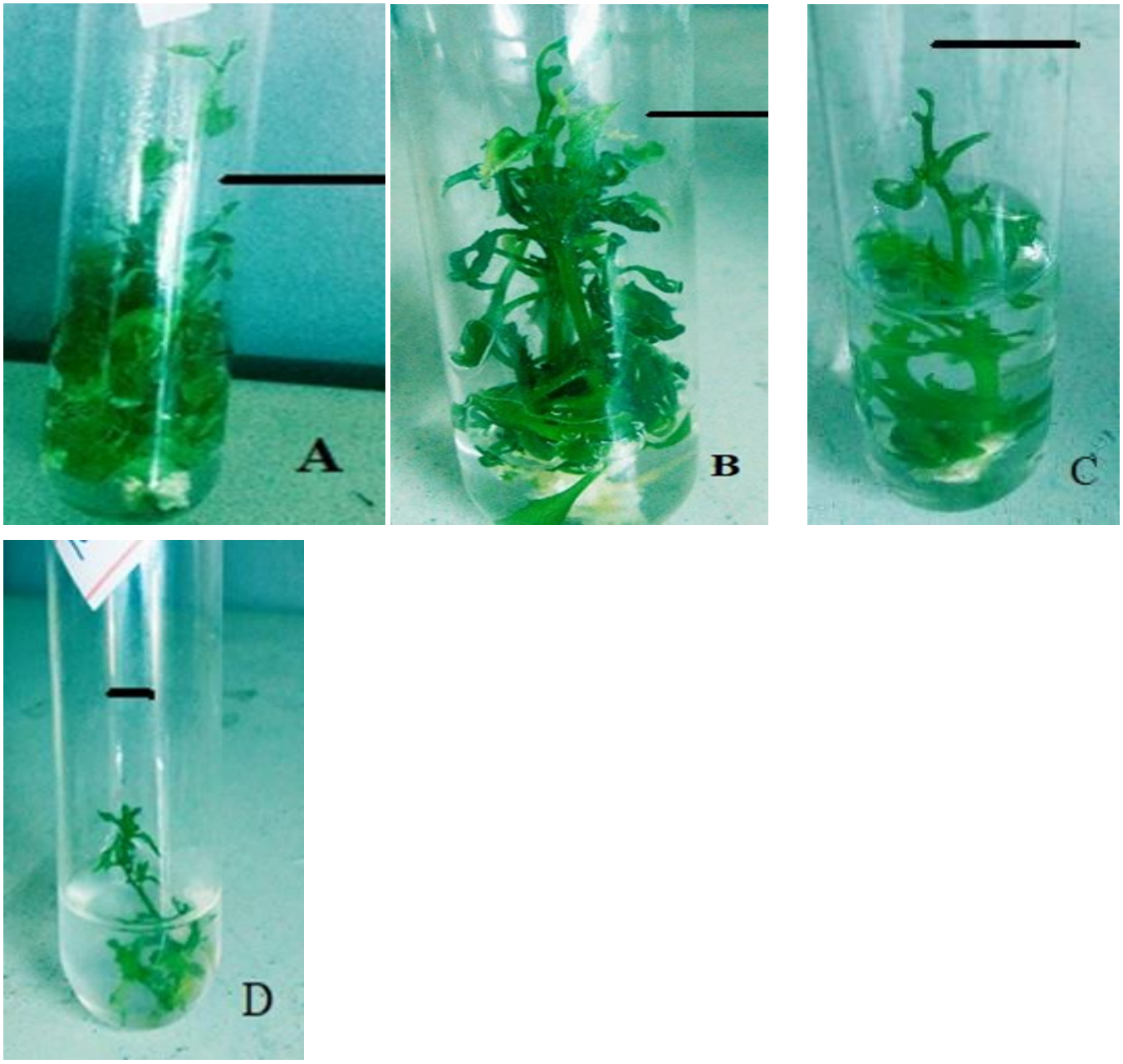

Figure 4. Effect of different sucrose concentration on shoot multiplication of *O. africana*: A) 3%, B) 2%, C) 1.5% and D) 1%. Bar represents 1.5 cm.

5.5 Rooting

5.5.1. Effects of different MS salt strength on root induction

There was significant difference in mean number and length of shoots on different MS salt strength. The highest mean root number (22.38±1.54) and length (9.38±1.54) were recorded on ½ MS strength containing 1mg/l IAA and 0.5mg/l NAA. In this treatment, 23 (76.66%) survived and the remaining 7 (24.44%) explants died. The lowest mean number (2.40±0.245) and length (2.26±0.01 cm) of roots were recorded on full strength MS medium supplemented with 1mg/l IAA and 0.5mg/l NAA. However, all shoots cultured on ¼ MS strength supplemented with 1mg/l IAA and 0.5mg/l NAA died.

Table 4: Effect of different MS salt strengths on mean root number and length of roots on medium containing 1mg/l IAA in combination with 0.5mg/l NAA

MS media	No. of roots	length of root	present of survived explant
Full	2.40 ± 0.245^{b}	2.26 ± 0.01^{b}	100%
½	23.80 ± 0.583^{a}	9.38 ± 1.54^{a}	76.66%
1/3	2.80 ± 0.340^{b}	2.72 ± 0.72^{b}	30%
¼	0.00 ± 000^{c}	0.00 ± 000^{c}	0.00%

Numbers connected by the same subscript letters in the same column are not significantly different at $p<0.5$.

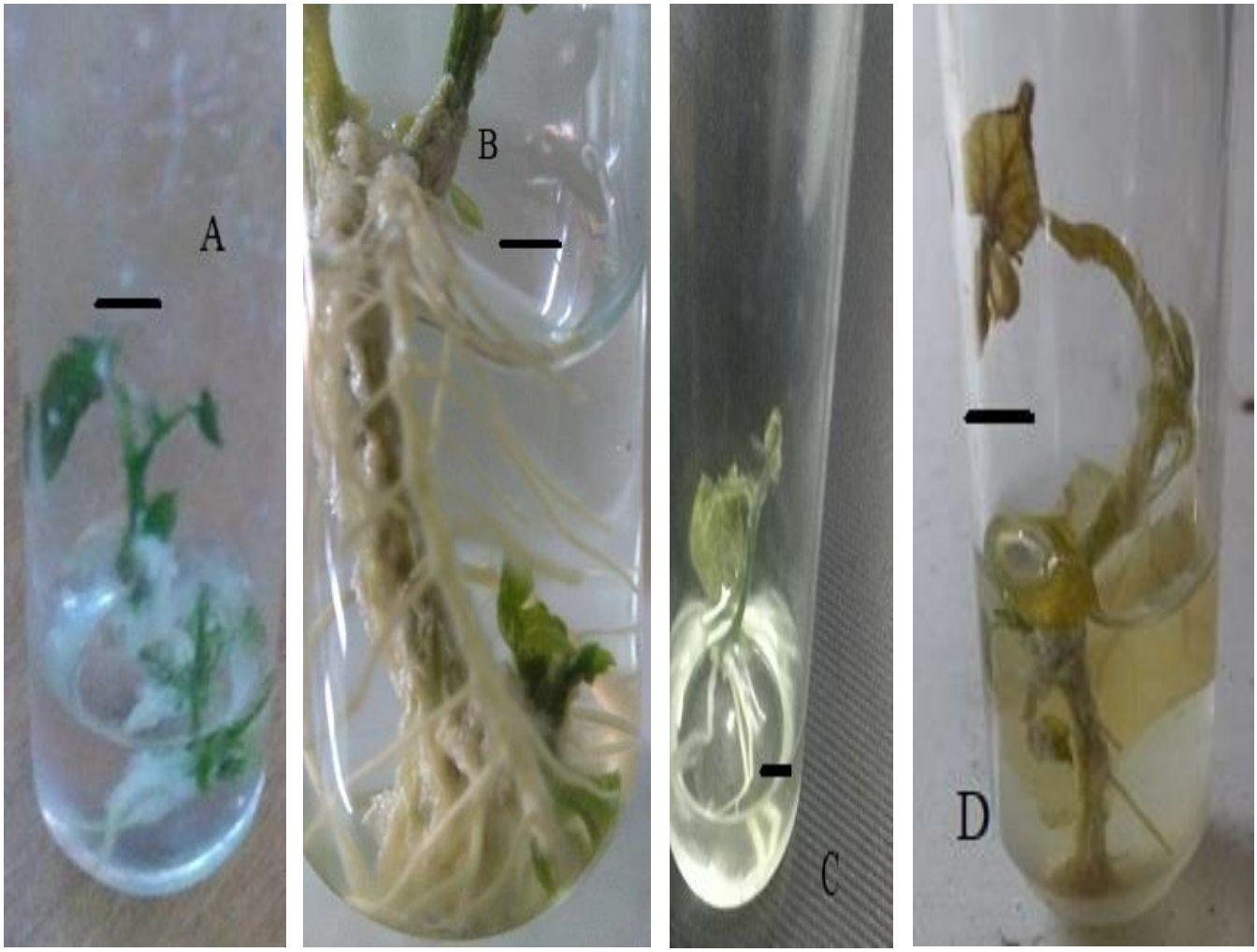

Figure 5: *In vitro* rooting of *O. africana* shoots on different salt strength MS medium containing 1.0 mg/l IAA in combination with 0.5 mg/L NAA: A) full MS, B) ½ MS, C) 1/3MS and D) ¼ MS strength. Bar represents 1.5cm

5.5.2. Effects of pH on *in vitro* root induction of *O. africana*

Among shoots cultured on medium of different pH values, only shoots cultured on half strength MS medium adjusted at 5.8 pH showed root induction resulting in 23.80±0.583 mean root number. All shoots cultured on medium adjusted at other pH values died after three weeks of culture without producing any root.

5.6. Acclimatization

After thirty days of acclimatization, the survival rate of total plants transferred to the greenhouse was 83.33%.

Figure 6. Acclimatization of *in vitro* rooted shoots of *O. africana*. Bar represents 2 cm.

6. DISCUSSION

6.1. Shoot initiation

The result of the present study on shoot number (33 shoots per explant) is strongly supported by recent studies on micropropagation of *O. africana* by Feyissa (2018) who obtained 36.65±2.03 mean number of shoots per explants and Tegene (2019) who obtained 35.97±0.41 mean number of shoots per explant.

6.2. Effects of MS strength on shoot multiplication and root induction of *O. africana*

Full salt strength MS medium was found to be the most suitable growth and multiplication medium for mass propagation of *O. africana*. However, with a decrease in the concentration of MS salt strength the leaves became yellowish after two weeks and shoots cultured on ¼ salt strength MS medium showed slight shoot tip necrosis. However, shoots cultured on 2x salt strength MS medium dried. The number of shoots, leaves and length of shoots per explant significantly decreased indicating that using liquid media to for mass *in vitro* propagation has a number of merits such as to saving time, reduce cost, getting large number of explants and thrive growth and multiplication of explant shoots. The lowest mean number of shoots, leaves and lengths of shoots were recorded on ¼ strength MS medium. This study is in agreement with the study of Tegene (2019) who obtained 35.97±0.41 shoots per explant on full strength MS medium and Feyissa (2018) who obtained 36.66±2.03 mean number of shoots per explant 4.26±0.10 cm mean length of shoots.

Thakur *et al*. (2011) reported a continuous decrease in concentration of MS salt strength (from full to ½MS, 1/3MS and ¼ MS mg/l) resulted in continuous decrease in the mean shoot number of *M. balsamina*. This situation might have happened because of genotype similarity of these two plant species, the treatment that utilized in these explant culture is the same and might be cultured in the same season or temperature (cold season or warm season). If comparison based on leaf number, all the previous studies did not record leaf number per explant. In terms of length this study strongly disagree with Feyissa (2018) and Tegene (2019) studies, the shoot length difference of the present study and that of two previous studies was the cause of media difference which means the present study used liquid media and those previous studies used solid media. According to the Rezali (2016) reports of *T. flagelliforme* the highest mean number of shoots and length of shoots were (4.40±0.986) and (24.467±5.273) respectively on full strength MS liquid medium. The lowest mean number of shoots (3.600±1.75) and height (17.75±10.39) of this plant were recorded on ¼ strength MS liquid medium. The present study agrees with the culture of *T. flagelliforme* because both experiments on full strength

MS liquid medium showed the highest mean number of shoots and length of shoots. On the other hand, these two studies show that the lowest mean number of shoots and length of shoots were recorded on ¼ strength MS medium. This indicates that MS media strength has effects on explant multiplication and growth even if the plants are from different genotypes. The highest mean number of leaves (14.911±9.17) and number of roots (36.55±24.80) of *T. flagelliforme* were recorded on full strength MS liquid medium. Even though the results of the experiment on *T. flagelliforme* and *O. africana* in liquid medium disagree with the present study because in the present study the highest mean number of root was recorded on ½ strength MS medium whereas in *T. flagelliforme* the highest mean number of root was recorded on full strength MS medium.

So, full MS salt strength may provide sufficient nutrients (macronutrients, micronutrients &amino acids), respiratory substrates and regulate biochemical metabolism that enhance shoot formation. In vitro root induction was enhanced when the concentration of salts in the medium was reduced by half. Perhaps its reason can be explained that due to decrease in the concentration of mineral salts, reduction in hyperhydricity and low osmotic pressure was resulted in increasing rooting rate, initiating roots, the number and length of roots.

6.3. Effects of different concentrations of sucrose on shoot multiplication and root induction of *O. africana*

The highest mean number of shoots per explant (31.33±0.989) on medium containing 30 g/l supplemented with 0.5 mg/l BAP in liquid medium after three weeks of culture. This result agrees with the results of Tegene (2019) where the highest mean shoot number of *O. africana* (35.97±0.41) was obtained on solid MS medium containing 30 g/l sucrose and Feyissa (2018) who obtained the highest mean number of *O. africana* shoots per explant (36.66±2.03) on solid MS medium containing 30 g/l sucrose.

The second maximum mean number was (12.95±0.737) shoots per explant on 2% sucrose concentration supplemented with 0.5 mg/l BAP after four weeks for culture. Further decrease in concentration of sucrose from 3% to 2%, 1.5%, and 1% resulted in decrease in rate of shoot proliferation. In ”Kello“ and ”Qulle“ varieties of cassava sucrose concentration of 1.5% showed the highest mean number of shoots (3.7 ± 1.55), number of leaves (12.15 ± 6.57) and length of shoot (5.10 ± 1.22 cm) per explant and minimum shoot number (1.95 ± 1.05) as reported by Berhanu (2011). In this case the present study strongly disagrees with the Roza study because these plant species are different. Thus 3% sucrose

concentration in the basal medium may satisfy the energy requirements, assist in water conservation, and maintain a stable osmotic potential of cells, speed up cell division.

6.4. Effects of pH on shoot multiplication and root induction

The pH increase as well as decrease from the standard pH value can cause the deficiency of nutrients in the media because if it is acidic surface the media is filled in H^+ due this nutrient available decrease and also the media near to basic it filled with OH^- and the nutrient availability to plant decrease extremely (Thomas, 1996). The results of the present study show these effects on plants because when the media is acidic the explant leaf became yellowish and showed stunted growth and the same effect was occurred when the media was basic. This means both acidic and basic media cause to the deficiency of nitrates and other metals (Lucena, 2000). The highest mean number of shoots (31.33±0.989), number of leaves (81.03±4.95) and length of shoots (12.99±0.39 cm) per explant were obtained at a pH of 5.8 from *O. africana* species. But, at pH of 6.6 treatment the explants were not survive.

According to Birhanu (2011) the maximum mean number of shoots, number of leaves, and number of nodes and length of shoots were attained at a pH level of 5.6 in case of "Kello" which are 4.10, 12.10, 4.30 and 5.00, respectively. While in "Qulle" maximum mean number of shoots, number of leaves, and number of nodes and length of shoots were attained at a pH level of 6.6 which are 2.40, 9.80, 4.12 and 4.90, respectively on the solid media. Even if *O. africana* and"Kello" variety of cassava were cultured in different media type and they are different species their result strongly agreed at pH of 5.8 and 5.6 respectively. However, *O. africana* and"Qulle" showed strongly different results which might be genotypic difference and media type difference. The pH 5.8 on shoot multiplication probably regulates the activities of enzymes, growth regulation, up take of nutrients and the function of the cell membrane as well as the buffered pH of the cytoplasmic activity that enhances cell division and the growth of shoots.

Root induction of *O. africana* on liquid medium in different MS salt strength, the highest mean number and length of root were recorded in ½ MS strength supplemented with 1.0mg/l IAA and 0.5 mg/l NAA which were 22.38±1.54 and 5.08±0.15 respectively. In this treatment 76.66% shoots survived and the remaining 24.44% died. According to Tegene (2019) 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 mg/l NAA. The present study agrees with Tegene study. Culturing of shoots on roorting medium of

different pH values resulted in death of the shoots except those at pH of 5.8. This indicates that both the acidic and basic effects cause nutrient shortage to the explant that leads to death of shoots.

6.5. Acclimatization

Rooted plantlets were transferred to polyethylene bags containing autoclaved garden soil and placed inside the growth chamber for two weeks for hardening. After 2 weeks, the cover was removed. Then, they were taken into the greenhouse where 83.33% plants survived and no aberrant plants were observed. This result agrees with the result of Tegene (2019) who reported 95% survival of cassava plants after forty days of acclimatization in greenhouse. Hence, it can be concluded that the composition of forest soil, compost and sand in the mixture might have well drained with high water holding capacity, good aeration and nutrients present that contributed to the survival rates.

7. CONCLUSIONS

Based on this study, it can be concluded that, full salt strength MS medium containing 0.5 mg/l BAP is found to be the best salt strength for shoot multiplication of *O.africana* in liquid medium. The best multiplication of shoots (31.33±0.99 per explant) was obtained, and the highest shoot length (12.95±0.39 cm) was obtained on full salt strength MS medium. The highest mean number of roots (23.80±0.53) per explant and the highest mean length of roots (22.38±1.5 cm) was obtained on half salt strength MS medium containing 1.0 mg/l IAA in combination with 0.5 mg/l NAA. Appropriate PH for this plant multiplication is very specific range because the best result was recorded only at 5.8 pH with 31.33± 0.989 shoots per explant. The *in vitro* grown plantlets survived in the *ex vitro* environment, when they are exposed to direct sunlight for some hours. By doing so 83.33 % plantlets were managed to survive. The optimized culture medium is useful for the rapid and large-scale propagation of *O. africana* *O. africana* is too much delicate herb plant because of this it is sensitive to hormone, pH and MS strength. Therefore, appropriate amount of MS strength, pH conditions, and optimum growth regulator should be used and also more emphasis is needed for it to multiply successfully.

8. RECOMMENDATIONS

The following are recommendations given based on this result of this study.

- The use of liquid media help to get abundant shoot multiplication of *O. africana* within short time at minimized cost.
- In terms of yield and other qualities, there is a need to evaluate the performance of *in vitro* propagated plants in field in comparison with the sexually propagated plants.
- Explants other than shoot tips culture for *in vitro* propagation of *O.africana* should be considered to investigate if they can offer better and more efficient responses.
- Optimum number of subculture cycles need to be worked out using BAP and also other PGRs for scaling up of tissue culture production of *O. africana*
- Suitable DNA markers like ISSR may be used for analysis of genetic fidelity of tissue culture propagated plantlets
- Future researcher should assess the effects of multiple subculturing on genetic instability of *O. africana* Even if in 3% sucrose concentration there was too minimum root induction to assess the effect sucrose concentration on *O. africana* should need further studied by varying its concentration.

9. REFERENCES

Ahsan, T., Amjad, N., Iqbal, A., and Javed, A. (2013). A review: tissue culturing of important medicinal plants. *International Journal of Water Resources and Environmental Sciences.* **2**(4)**:** 76-79.

Aremu, A. O., Bairu, M. W., Doležal, K., Finnie, J. F., and Van Staden, J. (2012). Topolins: a panacea to plant tissue culture challenges? *Plant Cell, Tissue and Organ Culture (PCTOC).* **108**(1)**:** 1-16.

Bairu, M. W., Aremu, A. O., and Van Staden, J. (2011). Somaclonal variation in plants: causes and detection methods. *Plant Growth Regulation.* **63**(2)**:** 147-173.

Bairu, M. W., Stirk, W. A., Dolezal, K., and Van Staden, J. (2007). Optimizing the micropropagation protocol for the endangered Aloe polyphylla: can meta-topolin and its derivatives serve as replacement for benzyladenine and zeatin? *Plant Cell, Tissue and Organ Culture.* **90**(1)**:** 15-23.

Bekele, D. (2018). The Biomosquitocides Oreosyce africana (Cucurbitaceae) in the Control of Malaria Vector, Anopheles arabiensis.

Bekele, D., Asfaw, Z., Petros, B., and Tekie, H. (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**(2)**:** 12-24.

Bekele, D., Tekie, H., Asfaw, Z., and Petros, B. (2016a). 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. *J Fertil Pestic.* **7**(2).

Bekele, D., Tekie, H., Asfaw, Z., and Petros, B. (2016b). Bioefficacy of solvent fractions of Oreosyce africana and Piper capense against the Malaria vector, Anopheles arabiensis with High performance liquid chromatographic and ultraviolet-visible spectroscopic analysis. *Biochem Anal Biochem.* **5**(294)**:** 2161-1009.1000294.

Bhojwani, S. S., and Dantu, P. K. (2013). Micropropagation. **In**: *Plant tissue culture: An introductory text,* pp. 245-274. Springer.

Birnbaum, K. D., and Alvarado, A. S. (2008). Slicing across kingdoms: regeneration in plants and animals. *Cell.* **132**(4)**:** 697-710.

Birhanu (2011). *Factors influencing micropropagation and somatic embryogenesis of two, Kello and Qulle, cassava varieties.* Addis Abeba University.

Bohra, P., Waman, A. A., Sathyanarayana, B., Umesha, K., Anu, S., Swetha, H., and Gourish, R. (2014). Aseptic culture establishment using antibiotics with reference to their efficiency and

phytotoxicity in difficult-to-establish native Ney Poovan banana (Musa, AB). *Proceedings of the National Academy of Sciences, India Section B: Biological Sciences.* **84**(2)**:** 257-263.

Booth, M., and Allan, G. (2003). Utilization of digestible nitrogen and energy from four agricultural ingredients by juvenile silver perch Bidyanus bidyanus. *Aquaculture Nutrition.* **9**(5)**:** 317-326.

Bornman, C. H. (1993). Micropropagation and somatic embryogenesis. **In**: *Plant Breeding*, pp. 246-260. Springer.

Brown, C. L., McAlpine, R. G., and Kormanik, P. P. (1967). Apical dominance and form in woody plants: a reappraisal. *American Journal of Botany.* **54**(2)**:** 153-162.

Brümmer, G. (1986). Heavy metal species, mobility and availability in soils. **In**: *The importance of chemical "speciation" in environmental processes*, pp. 169-192. Springer.

Cassells, A. C., and Doyle, B. M. (2006). Pathogen and biological contamination management. **In**: *Plant cell culture protocols*, pp. 35-50. Springer.

Chareonviriyaphap, T., Bangs, M. J., Suwonkerd, W., Kongmee, M., Corbel, V., and Ngoen-Klan, R. (2013). Review of insecticide resistance and behavioral avoidance of vectors of human diseases in Thailand. *Parasites & vectors.* **6**(1)**:** 280.

Chindi, A., Giorgis, G. W., Solomon, A., Tessama, L., and Negash, K. (2014). Rapid Multiplication Techniques (RlVITs): A Tool for the Production of Quality Seed Potato (Solanum tuberosum L.) in Ethiopia. *Asian Journal of Crop Science.* **6**(3)**:** 176-185.

Chinsembu, K. C. (2015). Plants as antimalarial agents in Sub-Saharan Africa. *Acta tropica.* **152:** 32-48.

Clarholm, M., and Skyllberg, U. (2013). Translocation of metals by trees and fungi regulates pH, soil organic matter turnover and nitrogen availability in acidic forest soils. *Soil Biology and Biochemistry.* **63:** 142-153.

Dale, P. (1994). The impact of hybrids between genetically modified crop plants and their related species: general considerations. *Molecular Ecology.* **3**(1)**:** 31-36.

Dann, A. L., and Wilson, C. R. (2011). Comparative assessment of genetic and epigenetic variation among regenerants of potato (Solanum tuberosum) derived from long-term nodal tissue-culture and cell selection. *Plant cell reports.* **30**(4)**:** 631-639.

Das, A., Gantait, S., and Mandal, N. (2011). Micropropagation of an elite medicinal plant: Stevia rebaudiana Bert. *International Journal of Agricultural Research.* **6**(1)**:** 40-48.

de Klerk, G., and Smulders, M. (2006). Epigenetic mechanisms cause aberrant plants. *Prophyta Annual.* **2006:** 34-37.

Debergh, P., and Maene, L. (1981). A scheme for commercial propagation of ornamental plants by tissue culture. *Scientia horticulturae.* **14**(4)**:** 335-345.

Edwards, S. B. (1991). Crops with wild relatives found in Ethiopia. *Plant genetic resources of Ethiopia*. 42-47.

El-Wakeil, N. E. (2013). RETRACTED ARTICLE: Botanical Pesticides and Their Mode of Action. *Gesunde Pflanzen.* **65**(4)**:** 125-149.

Fadzilla, N. a. M., Finch, R. P., and Burdon, R. H. (1997). Salinity, oxidative stress and antioxidant responses in shoot cultures of rice. *Journal of Experimental Botany.* **48**(2)**:** 325-331.

Fan, M., Xu, C., Xu, K., and Hu, Y. (2012). LATERAL ORGAN BOUNDARIES DOMAIN transcription factors direct callus formation in Arabidopsis regeneration. *Cell research.* **22**(7)**:** 1169.

Feyissa, T. (2006). *Micropropagation, transformation and genetic diversity of Hagenia abyssinica (Bruce) JF Gmel*. 2006.

Feyissa, T., Welander, M., and Negash, L. (2005). Micropropagation of Hagenia abyssinica: a multipurpose tree. *Plant cell, tissue and organ culture.* **80**(2)**:** 119-127.

Gauchan, D. (2012). Effect of different sugars on shoot regeneration of maize (Zea mays L.). *Kathmandu University Journal of Science, Engineering and Technology.* **8**(1)**:** 119-124.

George, E. F., Hall, M. A., and De Klerk, G.-J. (2007). *Plant propagation by tissue culture: volume 1. the background*. 1 Springer Science & Business Media.

Gleddie, S., Keller, W., and Setterfield, G. (1983). Somatic embryogenesis and plant regeneration from leaf explants and cell suspensions of Solanum melongena (eggplant). *Canadian Journal of Botany.* **61**(3)**:** 656-666.

Gonzalez-Arnao, M. T., Panta, A., Roca, W. M., Escobar, R. H., and Engelmann, F. (2008). Development and large scale application of cryopreservation techniques for shoot and somatic embryo cultures of tropical crops. *Plant Cell, Tissue and Organ Culture.* **92**(1)**:** 1-13.

Grafi, G., Florentin, A., Ransbotyn, V., and Morgenstern, Y. (2011). The stem cell state in plant development and in response to stress. *Frontiers in plant science.* **2:** 53.

Grout, B. W. (1999). Meristem-tip culture for propagation and virus elimination. **In**: *Plant cell culture protocols*, pp. 115-125. Springer.

Gupta, P. K., and Pullman, G. S. (1991). Method for reproducing coniferous plants by somatic embryogenesis using abscisic acid and osmotic potential variation. Google Patents.

Harding, K. (2004). Genetic integrity of cryopreserved plant cells: a review. *CryoLetters.* **25**(1)**:** 3-22.

Harmsen, K., and Vlek, P. (1985). The chemistry of micronutrients in soil. **In**: *Micronutrients in tropical food crop production*, pp. 1-42. Springer.

Hewelt, A., Prinsen, E., Schell, J., Van Onckelen, H., and Schmülling, T. (1994). Promoter tagging with a promoterless ipt gene leads to cytokinin-induced phenotypic variability in transgenic tobacco plants: implications of gene dosage effects. *The Plant Journal.* **6**(6)**:** 879-891.

Hoffmann, W. A. (1998). Post-burn reproduction of woody plants in a neotropical savanna: the relative importance of sexual and vegetative reproduction. *Journal of applied ecology.* **35**(3)**:** 422-433.

Hong, Y., and Bhatnagar, S. (2007). II. 4 Tropical Tree Legumes. *Edited by T Nagata (Managing Editor) H Lörz.* 407.

Husain, M. K., and Anis, M. (2009). Rapid in vitro multiplication of Melia azedarach L.(a multipurpose woody tree). *Acta Physiologiae Plantarum.* **31**(4)**:** 765-772.

Hussain, A., Qarshi, I. A., Nazir, H., and Ullah, I. (2012a). Plant tissue culture: current status and opportunities. **In**: *Recent advances in plant in vitro culture*. IntechOpen.

Hussain, I., Shah, T., Gondal, M. A., and Mahmood, H. (2012b). Analysis of S-box in image encryption using root mean square error method. *Zeitschrift für Naturforschung A.* **67**(6-7)**:** 327-332.

Idowu, P., Ibitoye, D., and Ademoyegun, O. (2009). Tissue culture as a plant production technique for horticultural crops. *African Journal of Biotechnology.* **8**(16).

james, D. J. (1987). Cell and tissue culture technology for the genetic manipulation of temperate fruit trees. *Biotechnology and genetic engineering reviews.* **5**(1)**:** 33-80.

Jiménez, V. M., Castillo, J., Tavares, E., Guevara, E., and Montiel, M. (2006). In vitro propagation of the neotropical giant bamboo, Guadua angustifolia Kunth, through axillary shoot proliferation. *Plant Cell, Tissue and Organ Culture.* **86**(3)**:** 389-395.

Jo, E.-A., Tewari, R. K., Hahn, E.-J., and Paek, K.-Y. (2009). In vitro sucrose concentration affects growth and acclimatization of Alocasia amazonica plantlets. *Plant Cell, Tissue and Organ Culture (PCTOC).* **96**(3)**:** 307.

Jones, C., and Jacobsen, J. (2005). Plant nutrition and soil fertility. *Nutrient management module.* **2**(11).

Jones, R. J., and Schreiber, B. M. (1997). Role and function of cytokinin oxidase in plants. *Plant Growth Regulation.* **23**(1-2)**:** 123-134.

Kane, M. E. (1996). Propagation from preexisting meristems. *Plant tissue culture concepts and laboratory exercises.* 61-71.

Karp, A. (1995). Somaclonal variation as a tool for crop improvement. *Euphytica.* **85**(1-3)**:** 295-302.

Koch, K. (1996). Carbohydrate-modulated gene expression in plants. *Annual review of plant biology.* **47**(1)**:** 509-540.

Krikorian, A. D. (1995). Hormones in tissue culture and micropropagation. **In**: *Plant hormones*, pp. 774-796. Springer.

Kumar, N., and Reddy, M. (2011). In vitro plant propagation: a review. *Journal of forest and environmental science.* **27**(2)**:** 61-72.

Lang, A. (1970). Gibberellins: structure and metabolism. *Annual Review of Plant Physiology.* **21**(1)**:** 537-570.

Leakey, R., and Akinnifesi, F. (2008). Towards a domestication strategy for indigenous fruit trees in the tropics. *Indigenous fruit trees in the tropics: domestication, utilization and commercialization CAB International, Wallingford, UK, in association with the World Agroforestry Centre, Nairobi, Kenya.* 28-49.

Li, Z.-G., Gong, M., Yang, S.-Z., and Long, W.-B. (2012). Efficient callus induction and indirect plant regeneration from various tissues of Jatropha curcas. *African Journal of Biotechnology.* **11**(31)**:** 7843-7849.

Loberant, B., and Altman, A. (2009). Micropropagation of plants. *Encyclopedia of industrial biotechnology: bioprocess, bioseparation, and cell technology.* 1-17.

Loyola-Vargas, V. M., and Vázquez-Flota, F. (2006). An introduction to plant cell culture. **In**: *Plant cell culture protocols*, pp. 3-8. Springer.

Lucena, J. J. (2000). Effects of bicarbonate, nitrate and other environmental factors on iron deficiency chlorosis. A review. *Journal of Plant Nutrition.* **23**(11-12)**:** 1591-1606.

Mabaso, M. L., Sharp, B., and Lengeler, C. (2004). Historical review of malarial control in southern African with emphasis on the use of indoor residual house-spraying. *Tropical Medicine & International Health.* **9**(8)**:** 846-856.

Magdoff, F., and Van Es, H. (2000). *Building soils for better crops*.Sustainable Agriculture Network Beltsville.

Martin, K. (2002). Rapid propagation of Holostemma ada-kodien Schult., a rare medicinal plant, through axillary bud multiplication and indirect organogenesis. *Plant Cell Reports.* **21**(2)**:** 112-117.

Martin, K. P., Zhang, C.-L., Slater, A., and Madassery, J. (2007). Control of shoot necrosis and plant death during micro-propagation of banana and plantains (Musa spp.). *Plant Cell, Tissue and Organ Culture.* **88**(1)**:** 51-59.

Mboera, L. E., Mazigo, H. D., Rumisha, S. F., and Kramer, R. A. (2013). Towards malaria elimination and its implication for vector control, disease management and livelihoods in Tanzania. *MalariaWorld Journal.* **4**(19).

McCown, B. H. (2000). Special symposium: In vitro plant recalcitrance recalcitrance of woody and herbaceous perennial plants: Dealing with genetic predeterminism. *In Vitro Cellular & Developmental Biology-Plant.* **36**(3)**:** 149-154.

Medina-Bolivar, F., Condori, J., Rimando, A. M., Hubstenberger, J., Shelton, K., O'Keefe, S. F., Bennett, S., and Dolan, M. C. (2007). Production and secretion of resveratrol in hairy root cultures of peanut. *Phytochemistry.* **68**(14)**:** 1992-2003.

Mukherjee, P., Varshney, A., Johnson, T. S., and Jha, T. B. (2011). Jatropha curcas: a review on biotechnological status and challenges. *Plant Biotechnology Reports.* **5**(3)**:** 197-215.

Murashige, T. (1977). Clonal crops through tissue culture. **In**: *Plant tissue culture and its bio-technological application*, pp. 392-403. Springer.

Murashige, T., and Skoog, F. (1962). A revised medium for rapid growth and bio assays with tobacco tissue cultures. *Physiologia plantarum.* **15**(3)**:** 473-497.

Nickrent, D. L., Eshbaugh, W. H., and Wilson, T. K. (1988). *The vascular flora of Andros Island, Bahamas*. Kendall/Hunt Dubuque, IA.

Ochatt, S., Conreux, C., Mcolo, R. M., Despierre, G., Magnin-Robert, J.-B., and Raffiot, B. (2018). Phytosulfokine-alpha, an enhancer of in vitro regeneration competence in recalcitrant legumes. *Plant Cell, Tissue and Organ Culture (PCTOC).* **135**(2)**:** 189-201.

Olaniran, A. O., Balgobind, A., and Pillay, B. (2013). Bioavailability of heavy metals in soil: impact on microbial biodegradation of organic compounds and possible improvement strategies. *International journal of molecular sciences.* **14**(5)**:** 10197-10228.

Oseni, O. M., Pande, V., and Nailwal, T. K. (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**(7)**:** 3778-3786.

Osman, K. T. (2013). Plant nutrients and soil fertility management. **In**: *Soils*, pp. 129-159. Springer.

Oyebanji, O., Nweke, O., Odebunmi, O., Galadima, N., Idris, M., Nnodi, U., Afolabi, A., and Ogbadu, G. (2009). Simple, effective and economical explant-surface sterilization protocol for cowpea, rice and sorghum seeds. *African Journal of Biotechnology*. **8**(20).

Pagès, L., Serra, V., Draye, X., Doussan, C., and Pierret, A. (2010). Estimating root elongation rates from morphological measurements of the root tip. *Plant and Soil*. **328**(1-2)**:** 35-44.

Pati, P. K., Rath, S. P., Sharma, M., Sood, A., and Ahuja, P. S. (2006). In vitro propagation of rose—a review. *Biotechnology advances*. **24**(1)**:** 94-114.

Patwardhan, B., Vaidya, A. D., and Chorghade, M. (2004). Ayurveda and natural products drug discovery. *Current science*. 789-799.

Phillips, G. C., and Hubstenberger, J. F. (1995). Micropropagation by proliferation of axillary buds. **In**: *Plant Cell, Tissue and Organ Culture*, pp. 45-54. Springer.

Pierik, R. L. M. (1997). *In vitro culture of higher plants*. Springer Science & Business Media.

Piotrowska, A., Czerpak, R., Adamowicz, J., Biedrzycka, A., and Potocka, M. (2005). Comparison of stimulatory effect of cytokinins adenine and urea derivatives on the level of some components in Wolffia arrhiza [L.] Wimm.[Lemnaceae]. *Acta Societatis Botanicorum Poloniae*. **74**(2).

Rahman, M., and Rajora, O. (2001). Microsatellite DNA somaclonal variation in micropropagated trembling aspen (Populus tremuloides). *Plant Cell Reports*. **20**(6)**:** 531-536.

Rai, M. K., Shekhawat, N., Gupta, A. K., Phulwaria, M., Ram, K., and Jaiswal, U. (2011). The role of abscisic acid in plant tissue culture: a review of recent progress. *Plant Cell, Tissue and Organ Culture (PCTOC)*. **106**(2)**:** 179-190.

Rangel, D. E., Braga, G. U., Fernandes, É. K., Keyser, C. A., Hallsworth, J. E., and Roberts, D. W. (2015). Stress tolerance and virulence of insect-pathogenic fungi are determined by environmental conditions during conidial formation. *Current genetics*. **61**(3)**:** 383-404.

Rani, A., and Kumar, S. (2017). *International Conference on Innovative Research in Science, Technology and Management*.

Ranson, H., and Lissenden, N. (2016). Insecticide resistance in African Anopheles mosquitoes: a worsening situation that needs urgent action to maintain malaria control. *Trends in parasitology*. **32**(3)**:** 187-196.

Renner, S. S., Schaefer, H., and Kocyan, A. (2007). Phylogenetics of Cucumis (Cucurbitaceae): Cucumber (C. sativus) belongs in an Asian/Australian clade far from melon (C. melo). *BMC Evolutionary Biology*. **7**(1)**:** 58.

Roca, W. M., Espinoza, N., Roca, M., and Bryan, J. (1978). A tissue culture method for the rapid propagation of potatoes. *American potato journal*. **55**(12)**:** 691-701.

Romocea, J.-E., Pop, L., and Gergely, I. (2010). Initiation of Begonia erythrophylla L. vitroculture from axillary buds. *Analele UniversitÄĹŁii din Oradea Fascicula Biol.* **17**(2)**:** 324-328.

Rowe, W. J. (1986). New technologies in plant tissue culture. **In**: *Tissue culture as a plant production system for horticultural crops*, pp. 35-51. Springer.

Saad, A. I., and Elshahed, A. M. (2012). Plant tissue culture media. **In**: *Recent advances in plant in vitro culture*. IntechOpen.

Saeed, T., and Sun, G. (2012). A review on nitrogen and organics removal mechanisms in subsurface flow constructed wetlands: dependency on environmental parameters, operating conditions and supporting media. *Journal of environmental management.* **112:** 429-448.

Sahu, J., and Sahu, R. K. (2013). A review on low cost methods for in vitro micropropagation of plant through tissue culture technique. *UK Journal of Pharmaceutical Biosciences.* **1**(1)**:** 38-41.

Saini, R., and Jaiwal, P. K. (2002). Age, position in mother seedling, orientation, and polarity of the epicotyl segments of blackgram (Vigna mungo L. Hepper) determines its morphogenic response. *Plant Science.* **163**(1)**:** 101-109.

Saniewski, M., Ueda, J., and Miyamoto, K. (2002). Relationships between jasmonates and auxin in regulation of some physiological processes in higher plants. *Acta Physiologiae Plantarum.* **24**(2)**:** 211.

ScHAEfEr, H. (2007). Cucumis (Cucurbitaceae) must include Cucumella, Dicoelospermum, Mukia, Myrmecosicyos, and Oreosyce: a recircumscription based on nuclear and plastid DNA data. *Blumea-Biodiversity, Evolution and Biogeography of Plants.* **52**(1)**:** 165-177.

Schmidt, E. L. (1982). Nitrification in soil. *Nitrogen in agricultural soils.*(nitrogeninagrics)**:** 253-288.

Sharma, P., and Dubey, R. S. (2005). Lead toxicity in plants. *Brazilian journal of plant physiology.* **17**(1)**:** 35-52.

Shimizu-Sato, S., and Mori, H. (2001). Control of outgrowth and dormancy in axillary buds. *Plant Physiology.* **127**(4)**:** 1405-1413.

Singh, B., Goswami, A., and Naresh, R. (2011). Role of tissue culture for enhancing productivity of horticultural crops through plant production techniques. *Annals of Horticulture.* **4**(1)**:** 20-27.

Sinha, R. K., Agarwal, S., Chauhan, K., and Valani, D. (2010). The wonders of earthworms and its vermicompost in farm production: Charles Darwin's 'friends of farmers', with potential to replace destructive chemical fertilizers from agriculture. *Agricultural sciences.* **1**(2)**:** 76-94.

Smith, M., and Drew, R. (1990). Current applications of tissue culture in plant propagation and improvement. *Functional Plant Biology.* **17**(3)**:** 267-289.

Štefančič, M., Štampar, F., and Osterc, G. (2005). Influence of IAA and IBA on root development and quality of Prunus' GiSelA 5'leafy cuttings. *HortScience.* **40**(7)**:** 2052-2055.

Takayama, S., and Akita, M. (2005). Practical aspects of bioreactor application in mass propagation of plants. **In**: *Liquid culture systems for in vitro plant propagation*, pp. 61-78. Springer.

Tanaka, M., Takei, K., Kojima, M., Sakakibara, H., and Mori, H. (2006). Auxin controls local cytokinin biosynthesis in the nodal stem in apical dominance. *The Plant Journal.* **45**(6)**:** 1028-1036.

Tegene, F. (2019).*In Vitro Mass Propagation of Oreosyce Africana Hook. F. From Shoot Tip Explants*. Addis Ababa University.

Teklehaymanot, T., and Giday, M. (2007). Ethnobotanical study of medicinal plants used by people in Zegie Peninsula, Northwestern Ethiopia. *Journal of ethnobiology and Ethnomedicine.* **3**(1)**:** 12.

Thakur, G. S., Pandey, M., Sharma, R., Sanodiya, B. S., Prasad, G., and Bisen, P. S. (2011). Factors affecting in vitro propagation of Momordica balsamina: a medicinal and nutritional climber. *Physiology and molecular biology of Plants.* **17**(2)**:** 193-197.

Thomas, G. W. (1996). Soil pH and soil acidity. *Methods of soil analysis: part 3 chemical methods.* **5:** 475-490.

Thorpe, T. A. (2007). History of plant tissue culture. *Molecular biotechnology*. **37**(2)**:** 169-180.

Tolossa, K., Debela, E., Athanasiadou, S., Tolera, A., Ganga, G., and Houdijk, J. G. (2013). Ethno-medicinal study of plants used for treatment of human and livestock ailments by traditional healers in South Omo, Southern Ethiopia. *Journal of Ethnobiology and Ethnomedicine.* **9**(1)**:** 32.

Torres, K. C. (1989). Tissue Culture Media—Composition and Preparation. **In**: *Tissue Culture Techniques for Horticultural Crops*, pp. 26-51. Springer.

Vasil, I. K., and Thorpe, T. A. (2013). *Plant cell and tissue culture*. Springer Science & Business Media.

Verdeil, J.-L., Alemanno, L., Niemenak, N., and Tranbarger, T. J. (2007). Pluripotent versus totipotent plant stem cells: dependence versus autonomy? *Trends in plant science.* **12**(6)**:** 245-252.

Wang, Q., and Valkonen, J. (2008). Elimination of two viruses which interact synergistically from sweetpotato by shoot tip culture and cryotherapy. *Journal of Virological Methods.* **154**(1-2)**:** 135-145.

West, P. A., Protopopoff, N., Wright, A., Kivaju, Z., Tigererwa, R., Mosha, F. W., Kisinza, W., Rowland, M., and Kleinschmidt, I. (2014). Indoor residual spraying in combination with insecticide-treated nets compared to insecticide-treated nets alone for protection against malaria: a cluster randomised trial in Tanzania. *PLoS medicine.* **11**(4)**:** e1001630.

Wilkins, C. P., and Dodds, J. H. (1983). Tissue culture propagation of temperate fruit trees. **In**: *Tissue Culture of Trees*, pp. 56-79. Springer.

Zimmerman, J. L. (1993). Somatic embryogenesis: a model for early development in higher plants. *The Plant Cell.* **5**(10)**:** 1411.

Ziv, M. (2005). Simple bioreactors for mass propagation of plants. **In**: *Liquid culture systems for in vitro plant propagation*, pp. 79-93. Springer.

Zucchi, M. I., Arizono, H., Morais, V. A., Fungaro, M. H. P., and Vieira, M. L. C. (2002). Genetic instability of sugarcane plants derived from meristem cultures. *Genetics and Molecular Biology.* **25**(1)**:** 91-96.

10. APPENDEX

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

Components	Concentration (g/L)	ml/L during media preparation
Macronutrients		
ZnSO4.2H2O	1.72	5
H3BO3	1.124	
MnSO4.H2O	0.05	
KI	0.166	
NaMoO4.2H2O	0.05	
CoCl2.6H2O	0.05	
Na2EDTA	7.472	
FeSO4.7H2O	5.56	
Macronutrients		
NH4NO3	33	
KNO3	38	50
MgSO4.7H2O	7.4	
CaCl2.2H2O	8.8	
KH2PO4	3.4	
vitamins		
Myo-inositol	20	
Glycine	0.4	
Nicotinic acid	0.1	5
Pyridoxine	0.1	
Thymine	0.02	

- FeSO4.7H2O and Na2EDTA are prepared alone.

Declaration

This thesis is my original work and has not been presented for MSc degree in any other University and that all the sources and materials used for the thesis have been properly acknowledged.

Declared By: Andualem manaye

Signature: _____

Date: _____

This is to certify that the above declaration made by the candidate is correct to the best of my knowledge as an advisor.

Dr. Tilye Feyissa _____ _____

(Advisor) Signature Date