ADDIS ABABA UNIVERSITY

INSTITUTE OF BIOTECHNOLOGY

SCHOOL OF GRADUATE STUDIES

Micropropagation of *Moringa oleifera* from shoot tip explants

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

BY

ZULALIYA JEMAL

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Addis Ababa, Ethiopia

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LIST OF ABBREVIATIONS

BAP----------6-Benzyle Amino Purine

NAA----------Naphthalene Acetic Acid

IBA----------Indol-3-Butyric Acid

KIN ----------Kinetin

MS----------Murashige and Skoog basal medium

PGR----------Plant Growth Regulator

ANOVA----------Analysis of Variance

MICROPROPAGATION OF *MORINGA OLEIFERA* FROM SHOOT TIP EXPLANTS

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ABSTRACT: Moringa oleifera *belonging to the family Moringaceae is regarded as one of the most important and beneficial because it is both a medicinal and nutritional plant.* M. oleifera *is restricted to SNNPs region of Ethiopia. The present study was conducted to develop efficient sterilization,* in vitro *germination and plant propagation protocol for* M. oleifera *from shoot tip. After sterilization of the seeds using different concentrations of calcium hypochlorite at different exposure time, the seeds were cultured on growth regulators free Murashige and Skoog (MS) medium. Shoot tips of* M. oleifera *were excised from* in vitro *grown seedlings and cultured on MS medium supplemented with different concentrations of KIN and BAP for culture initiation. The* in vitro *initiated shoot tips were transferred onto solid MS medium supplemented with different concentrations of KIN in combination with BAP, BAP in combination with IBA and BAP alone were used for shoot multiplication. For rooting half strength MS medium supplemented with different concentrations of NAA and IBA were used. MS medium without PGRs was used as control. 100%* in vitro *germinated clean seedling explants were obtained by using 10 % of calcium hypochlorite and 30 min. All the cultured shoots were initiated on MS medium supplemented with 0.5 mg/l BAP and 0.5 mg/l KN and when these shoots were transferred to shoot multiplication medium, the highest shoot number per explant (5.66±0.44) was obtained on MS medium supplemented with combination of 1.0 mg/l BAP and 0.5 mg/l IBA and Maximum shoot height of 4.51±0.21 was recorded in MS medium supplemented with 0.25 mg/l BAP + 0.1mg/l IBA. Maximum number of healthy roots (7.96±0.69) was obtained on 1/2 strength MS medium containing 0.25 mg/l NAA. Maximum root height of (2.44±0. 04 cm) was obtained on growth regulators free half strength MS medium. Up on acclimatization, 90% plants survived. This* in vitro *propagation protocol of* Moringa oleifera*can be used for mass propagation as well as for its conservation and genetic improvement.*

Key words: *In vitro* propagation, *Moringa oleifera,* Plant Growth Regulators, Shoot multiplication

1. INTRODUCTION

Moringa oleifera Lam. commonly known as the drumstick or ben oil tree is a widely cultivated species of family Moringaceae (Mylene *et al*., 2011) and native to the sub-Himalayan tracts of Northwestern India. It is a fast-growing tropical perennial soft-wooded tree with a long history of traditional medicine and culinary uses (Saini *et al*., 2012). *Moringa* is useful for several purposes including as vegetable, medicine, ornamental, as a source of oil, firewood, as dirt coagulant to clean water, construction of traditional huts, making rope and as fodder for livestock (Hegde and Hegde, 2015).

Most important medicinal and nutritional plant species of Ethiopia are reported to have been threatened and endangered by the overuse and over harvesting for marketing as medicine and food. *Moringa oleifera* also known as Aleko (Konso), Shiferaw (Amharic), Kalan'gi (Hamer-Bena), Ben-oil tree, cabbage tree, horse-radish tree (English). It is deciduous tree that reaches a height of up to 10m, usually smaller, pale feathery foliage. *M. oleifera* originates from India and was introduced to Ethiopia long ago. The tree is now naturalized in many parts of southern Ethiopia. Konso people plant *M. oleifera* around their homesteads and also in the terraced fields (Padayachee and Bajnath, 2012).

M.oleifera is a valuable food that has drawn attention as the 'natural nutrition of the tropics. The fruit, leaves, flowers and immature pods of this tree are highly nutritious and are used as vegetable in many parts of the world, especially in under developed countries in Africa, India,

Pakistan and Philippine. All of its parts are highly nutritious not only for human beings but also for the animals to increase their milk production (Shahzed *et al*., 2014).

Other species of genus *Moringa* is ***M. stenopetala,*** an important crop in Kenya and Ethiopia. ***M.oleifera*** is a promising food source especially its leaves which are rich in nutrients and minerals and the tree has maximum leaves at the end of the dry season when other foods are typically scarce (Fahey, 2005). There are tremendous potential opportunities with ***M. oleifera*** for sustainable agriculture, and the development of cash crops in semiarid regions. It is one of the richest plant sources of Vitamins A, B, C, D, E and K. The vital minerals present in *Moringa* include Calcium, Copper, Iron, Potassium, Magnesium, Manganese and Zinc. *Moringa* is considered the "miracle tree" in some parts of the world because all its parts can be used, from the flowers to the roots. A wide variety of nutritional and medicinal virtues have been attributed to its roots, bark, leaves, flowers, fruits and seeds (Anwar *et al*., 2007). Leaves are eaten as vegetables, and pressed or dried to serve in traditional pharmacology to treat many ailments or to be used as condiment (Kesharwani *et al*., 2014).

Moringa is particularly suitable for dry regions, as it can be grown using rainwater without expensive irrigation techniques. In seasonally cool regions, flowering only occurs once a year between April and June. In more constant seasonal temperatures and with constant rainfall, flowering can happen twice or even all year-round. The fruit is a hanging, three-sided brown capsule of 20–45 cm size which holds dark brown, globular seeds with a diameter around 1 cm. The seeds have three whitish papery wings and are dispersed by wind and water. *Moringa* leaves have a characteristic distinctive, strong, mustard-like taste; they are a good source of provitamin

A, vitamins B and C, minerals (particularly iron) and the sulphur containing amino acids methionine and cystine, and are eaten as a supplement to the major staple foods. In the Northern part of Nigeria, the cooked leaves of *Moringa* are frequently eaten as the principal ingredient of a sauce. These leaves are generally harvested from trees found within household gardens or planted as part of hedges around gardens (Hegde and Hegde, 2015).

M. oleifera is restricted to SNNPs region of Ethiopia and sexual propagation of some of these species is tedious and not even be possible without having enough individual plants for cross-pollination. Traditional propagation of drumstick tree is by seeds. This method results in one plant per seedling. Drumstick trees obtained from seeding vary depending on the genotype and hence in their phenotypes leading to variation in fruit (pod) production and nutritional values. Selected characteristics of plants may be maintained via *in vitro* propagation. As a widely used method for the rapid clonal propagation of many economically important crops, it could help to satisfy the demand for *M.oleifera* planting materials. Amplification of these rare individuals by *in vitro* propagation would make them more widely available and less likely to become lost to cultivation. Micropropagation is used to propagate plants within short period of time and for *in vitro* conservation of genetic resource, understanding of gene structure, function in molecular biology and plant improvement through transgenic technology. The response of different plant genotypes differ under *in vitro* culture conditions. Therefore, each genotype of a plant species require its own micropropagation protocol.

2. LITERATURE REVIEW

2.1. An overview of *Moringa oleifera*

The Moringaceae or Horseradish tree is the family of trees that consist of 13 different species, of which *Moringa oleifera* is the most widely cultivated. *Moringa* is native to the sub- Himalayan parts of Northern India, Pakistan, Bangladesh and Afghanistan (Oyeyinka and Oyeyinka, 2016). *M.oleifera*is a multi-purpose food plant, which originated, produced and used in many African countries, South America and New Zealand. However, it has been cultivated in many parts of the world and can now be found in almost all tropical countries (Hegde and Hegde, 2015).

M. oleifera typically grows in semi-dry, desert or tropical soils. Almost all parts of this plant: root, bark, gum, leaf, fruit (pods), flowers, seed and seed oil have been used for treating various ailments (Kesharwani *et al*., 2014) such as skin infections, anemia, coughs, diarrhea, swelling, headaches, gout, acute rheumatism, hysteria, cholera, heart complaints, fever, respiratory disorders, inflammation, digestive disorders, asthma, intestinal complaints, diabetes and rheumatism in the indigenous system of medicine (Padayachee and Bajnath, 2012). The different parts of *M. oleifera* such as the roots, leaves, flowers, fruits and seeds are also known to be good sources of phytochemicals. *M. oleifera* is regarded as one of the most important and beneficial because it is both a medicinal and nutritional plant (Padayachee and Bajnath, 2012).

M.oleifera leaves contain more vitamin A compared to carrots, more calcium than milk, more iron than spinach, more vitamin C than oranges, and more potassium than bananas, and that the protein quality of *Moringa* leaves rivals that of milk and eggs (Fahey, 2005). It is very nutritious and has a variety of potential uses. A fast-growing vegetable crop, it is drought-resistant and

grows in almost all types of well-drained soil. Presently, both the business and scientific communities have shown interest in this crop because of its economic potential (Marfori, 2010). Extracts from all parts of drumstick tree also show pharmacological properties including anti-inflammatory, anti-bacterial and anti-spasmodic activity. Other applications of drumstick tree are water purification, biogas production, biopesticide, and domestic cleaning agent (Riyathong *et al*., 2008). Fruits, seeds and roots are used for human consumption and their stems are used for animal feed. The species has become very important because it contains 18 of the 20 amino acids that the human body requires and is one of the plant species with high seed oil content, between 30-40% (Catres and Delaveau, 2014).

M. oleifera is used as a cleaning agent, fertilizer, gum, honey clarifier, natural pesticide and pulp for paper (HDRA, 2002; Fuglie, 1999). Given its close relationship to *M. oleifera*, *M. stenopetala* could possibly be used for all these purposes, and these aspects very well deserve detailed scientific investigations (Eyassu Seifu, 2014).

Moringa leaves and pods can do much to preserve the mother's health and pass on strength to the fetus or nursing child. One hundred gram portion of leaves could provide a woman with over a third of her daily need of calcium and give her important quantities of iron, protein, copper, sulfur and B-vitamins and during pregnancy and breast-feeding, women are most at risk of suffering from nutritional deficiencies.

Ethiopia has environment conduciveness and labor potential country. Many products can associate in market for economy. For example, *Moringa* leaf powder, *Moringa* leaf powder in

teabag, *Moringa* fortified fruit juice/honey, *Moringa* in capsule/tablets, *Moringa* fortified confectionaries and *Moringa* fresh leaf and so on. *Moringa* seeds are effective against skin-infecting bacteria. The leaf juice has a stabilizing effect on blood pressure. The leaf juice controls glucose levels in diabetic patients. Fresh leaves and leaf powder are recommended for tuberculosis patients because of the availability of vitamin A that boosts the immune system. If leaf juice is used as diuretic, it increases urine flow and cures gonorrhea. Leaf juice mixed with honey treats diarrhea, dysentery and colitis (colon inflammation). Fresh leaves are good for pregnant and lactating mothers; they improve milk production and are prescribed for anemia. Paste made from bark treats boils. Paste from ground bark can be applied to relieve pain caused by snake, scorpion and insect bites. Oil is sometimes applied externally for skin diseases. Markets for *Moringa* leaf exist at both the local and international levels. Thus, *Moringa* product has ample scope for economic development in Ethiopia (Hegde and Hegde, 2015).

2.2. Botanical description

It ranges in height from 5 to 10 m; has tuberous roots, a whitish bark, soft, spongy wood, a short trunk and slender branches. The leaves are alternate, twice- or thrice-pinnate leaves and spirally arranged. The flowers pleasantly fragrant and 2.5cm wide are white or cream-colored. The fruits (pod-like capsules) borne singly or in pairs, are light-green, slim, tender, firm and thinly woody.

The tree itself is rather slender, with dropping branches (Kassa Belay and Mesay Sisay, 2014). It is generally considered a small- to medium-size tree (Radovich, 2009).The stem is normally straight but occasionally is poorly formed. The tree grows with a short, straight stem that reaches a height of 1.5-2 m before it begins branching but can reach up to 3.0 m (Foidl *et al*., 2001).The

extended branches grow in a disorganized manner and the canopy is umbrella shaped. Trip innate compound leaves are feathery with green to dark green elliptical leaflets 1-2 cm long. The tree is often mistaken for a legume because of its leaves. The alternate, twice or thrice pinnate leaves grow mostly at the branch tips. They are 20-70 cm long, grayish-downy when young, long petiole with 8-10 pairs of pinnae each bearing two pairs of opposite, elliptic or obovate leaflets and one at the apex, 1-2 cm long (Morton, 1991).

The flowers, which are pleasantly fragrant and 2.5 cm wide are produced profusely in axillary, drooping panicles 10-25 cm long (Sachan *et al*., 2010). They are white or cream colored and yellow-dotted at the base. They surround the five stamens and five staminodes and are reflexed except for the lowest. The fruits are trilobed capsules, and are frequently referred to as pods. Immature pods are green and in some varieties have some reddish color. Pods are pendulous, brown, triangular, splitting length wise into 3 parts when dry, 30-120 cm long and 1.8 cm wide (Paliwal *et al*., 2011). The seeds are round with a brownish semi-permeable seed hull, with 3 papery wings. Seed hulls are generally brown to black, but can be white if kernels are of low viability. Viable seeds germinate within 2 weeks. The hull itself has three white wings. Each tree can produce between 15,000 and 25,000 seeds/year. The average weight per seed is 0.3 g.

Scientific (taxonomical) classification (*Moringa oleifera*)

Kingdom - Plantae

Division - Magnoliophyta

Class - Magnoliosida

Order - Capparales

Family - Moringaceace

Genus - *Moringa*

Species - *Moringa oleifera*

2.3. Distribution

Moringa is cultivated extensively in India and parts of Africa. *Moringa* is most commonly found in areas with South and Southeast Asian (particularly Filipino) populations. Today it is widely cultivated in Africa, Central and South America, Sri Lanka, India, Mexico, Malaysia, Indonesia and the Philippines (Mishara *et al*., 2011). It is considered one of the world's most useful trees, as almost every part of *Moringa* tree can be used for food or has some other beneficial properties (Paliwal *et al*., 2011).

A study on local uses and geographical distribution of *M.oleifera* that covers the major agro-ecological region in Nigeria, clearly established that "though considered a not indigenous species, *M. oleifera* has found wide acceptance among various ethnic Nigeria, who have exploited it for different uses (e.g., food, medicine, fodder *etc.*). Nowadays, *M. oleifera* and its derivatives are distributed mainly in Middle East, African and Asian countries and are still spreading to other areas.

2.4. Ecology: environmental preferences and tolerances

Climate: *Moringa* is widely adapted to the tropics and subtropics. Optimum leaf and pod production requires high average daily temperatures of 25-30°C (77-86°F), well-distributed annual rainfall of 1000-2000 mm. high radiation and well-drained soils (Odee, 1998). Growth slows significantly under temperatures below 20°C (68°F). *Moringa* is relatively tolerant of drought and poor soils and responds well to irrigation and fertilization.

Soils: *Moringa* tolerates a wide range of soil types and pH (4.5-9) but prefers well-drained soils at the neutral pH. It can grow well in heavy (clay) soils provided that they do not become saturated for prolonged periods of time. Light (sandy) soils are preferred for rooting branch cuttings directly in the ground (Radovich, 2009).

Growth and development: Plants from seed can grow very rapidly under ideal conditions. Selected early flowering varieties are sometimes called annual types because they produce vegetable pods for market within a year and may be removed and new plantings established. *Moringa* varieties generally tolerate the same climate. After coppicing, branches grow quickly and immature pods are harvested in 6 months (Paliwal *et al*., 2011).

Flowering and fruiting: *Moringa* is free flowering. Flowering generally occurs 4-12 months after planting, depending on the type. Some selections flower 4-5 months after planting.

Cultivation: The plant is propagated by planting limb cuttings 1-2 m long, from June to August. The plant starts bearing pods 6-8 months after planting, but regular bearing commences after the

second year. The tree bears for several years. It does not tolerate freeze or frost. It can also be propagated by seed. As with all plants, optimum cultivation depends on producing the right environment for the plant to thrive. *Moringa* is a sun and heat loving plant. Seeds are planted an inch below the surface and can be germinated year-round in well-draining soil. India is the largest producer of *Moringa*, where its triangular, ribbed pods with winged seeds are used as a vegetable crop. It is particularly suitable for dry regions and can be grown using rainwater without expensive irrigation techniques. The yield is good even if the water supply is not (Rajangam *et al*., 2001).

2.5. Economic importance

M. oleifera is one of the most useful tropical trees. The relative ease with which it propagates through both sexual and asexual means and its low demand for soil nutrients and water after being planted makes its production and management easy. Introduction of this plant into a farm, can be beneficial for both the owner of the farm and the surrounding eco-system (Foidl *et al*., 2001). *M. oleifera* was well known to the ancient world, but only recently has it been rediscovered as a multi-purpose tree with a tremendous variety of potential uses.

M. oleifera is certainly under-exploited at present. It's numerous uses as a vegetable, seed oil, gum, hedge tree, ornamental and medicinal plant, and its easy propagation and cultivation justify more intensive research into its biological and economic potential (Paliwal *et al*., 2011).

2.5.1. Medicinal uses and pharmacological properties

M.oleifera has more than 40 natural anti-oxidants and is used in more than 80 countries to relieve mineral and vitamin deficiencies and to neutralize free radicals (Ted and Fahey, 2005). *Moringa* plants produce special substances in their roots, leaves, flowers or seeds that help them to survive and healing with medicinal plants old as mankind itself. *M.oleifera* is considered to be effective in the treatment of many diseases. It is an exceptionally nutritious vegetable tree with a variety of potential uses.

Water purification; Powdered seed act as a natural flocculent, able to clarify even the most turbid water. Seed powder can be used as a quick and simple method for cleaning dirty water. The powder joins with the solids in the water and sinks to the bottom. This treatment also removes 80-90% of bacteria contained in water. Using *Moringa* to purify water replaces chemicals such as aluminum sulphate, which are dangerous to people and the environment, and are expensive (Mohmood *et al*., 2010).

Animal feed fortification; *Moringa* leaves added to cattle feed increased their daily weight gain by up to 32 percent. Feed of milk cows was supplemented with 15 to 17 kilograms of fresh *Moringa* leaves daily, and the cattle's milk production increased by 43 percent (Mohmood *et al*., 2010).

2.5.1.1. Traditional medicine

Medicinal properties of plants have also been investigated in the light of recent scientific developments throughout the world, due to their potent pharmacological activities, low toxicity

and economic viability, when compared with synthetic drugs (Pracheta *et al*., 2011). All plant parts of *M. oleifera* are traditionally used for different purposes, but leaves are generally the most used.

M .oleifera possesses highly therapeutic and pharmacological values, so its consumption in regular diet could possibly reduce the risk of degenerative diseases (Paliwal *et al*., 2011). *M. oleifera* is believed to possess numerous medical properties and is being used for the treatment of ascites, venomous bites, rheumatism (Anwar *et al*., 2007), enhancing cardiac function, inflammation, liver disease, cancer, hematological and renal function (Mishara *et al*., 2011). Almost all the parts of this plant: root, bark, gum, leaf, fruit (pods), flowers, seed and seed oil have been used for various ailments in the indigenous medicine of South Asia, including the treatment of inflammation and infectious diseases along with cardiovascular, gastrointestinal and hematological disorders.

Moringa has been used in the traditional medicine passed down for centuries in many cultures around the world, for skin infections, anemia, anxiety, asthma, blackheads, blood impurities, bronchitis, chest congestion, cholera, conjunctivitis, cough, diarrhea, eye and ear infections, fever, glandular, swelling, headaches, abnormal blood pressure, hysteria, pain in joints, pimples, psoriasis, respiratory disorders, scurvy, semen deficiency, sore throat, sprain, tuberculosis, for intestinal worms, lactation and diabetes (Nikkon *et al*., 2003). The healing properties of *Moringa* oil have been documented by ancient cultures. *Moringa* oil has tremendous cosmetic value and is used in body and hair care as a moisturizer and skin conditioner. It is also helpful in curbing the infection in the body. It is very much effective in stimulating the nervous system. Due to pungent

taste it is effective in treating the digestive disorders, worm infestation, and constipation. It stimulates heart and also increases the blood density because of its hot potency. It is also a good antitussive and helps in resolving from extra mucus in the respiratory tract because of its bitter nature. Due to hot potency it is helpful in maintaining the proper menstrual cycle. It is also helpful in relieving from skin related problems as it generates sweat in the body (Paliwal *et al*., 2011).

Leaves are rich in protein, mineral, beta-carotene and antioxidant compounds, which are often lacking among the populations of underdeveloped or developing countries. *Moringa* leaves are added to food preparations as integrators of the diet (Leone *et al*., 2015). The extract of *Moringa* leaves shows antimicrobial and antifungal activity in addition to cancer preventive effect. According to ethno botanical studies, its roots are bitter, acrid, digestive, carminative, anthelmintic, constipating, diuretic, expectorant and stimulant. It is used in cough, asthma, bronchitis, pectoral diseases, splenomegaly and epilepsy (Devendra *et al*., 2012). Similarly, the use of seeds concerns both human nutrition and traditional medicine. Barks are boiled in water and soaked in alcohol to obtain drinks and infusions that can be used to treat stomach ailments. Finally, flowers are used to treat inflammations, muscle diseases, hysteria, tumors and enlargement of the spleen (Leone *et al*., 2015).

2.5.2. Nutrition

Moringa trees have been used to combat malnutrition, especially among infants and nursing mothers. Three non-governmental organizations in particular-Trees for Life, Church World Service and Educational Concerns for Hunger Organization have advocated *Moringa* as natural nutrition for the tropics. *M. oleifera* leaves have essential amino acids, including the sulfur-containing amino acids in higher levels than those recommended by the Food and Agriculture Organization (FAO), patterns similar to those of soybean seeds. The leaves are known to be great source of vitamins and minerals being served raw, cooked or dried (Fahey, 2005).

Moringa has long been considered a panacea for improving the nutrition of poor communities in the tropics and subtropics. Leaves can be eaten fresh, cooked, or stored as dried powder for many months without refrigeration, and reportedly without loss of nutritional value. Protein content of leaves is high (20-35% on a dry weight basis). Most important is that the protein is of high quality having significant quantities of all the essential amino acids (Foidl and Paull, 2008). This amino acid balance is very unusual in plant foods. Analyses of the proximate composition of *M. oleifera* seeds have showed high levels of lipids and proteins with minor variations (Anhwange *et al.*, 2004).

2.5.3. Commercial uses

M. oleifera is coming to the forefront as a result of scientific evidence that *Moringa* is an important source of naturally occurring phytochemicals and this provides a basis for future viable developments. *Moringa* seeds have specific protein fractions for skin and hair care. Two new active components for the cosmetic industry have been extracted from oil cake. Purisoft consists of peptides of the *Moringa* seed. It protects the human skin from environmental influences and combats premature skin aging. With dual activity, antipollution and conditioning/strengthening of hair, the *M. oleifera* seed extract is a globally acceptable innovative solution for hair care (Paliwal *et al*., 2011).

2.5.4 Other uses

Beyond the uses of *Moringa* as a food and for human health, other possible uses exist. It can be used as a natural plant growth enhancer; indeed leaves are rich in zeatin (a plant hormone belongs to the cytokinin group). Leaf extracts can stimulate plant growth and increasing crop yield. Researches performed using a spray based on leaf extracts of wheat, maize and rice support the wide range of beneficial effect on crops. Interestingly, leaf extracts and also seed extracts show biopesticide activity, effective against larvae and adults of *Trigoderma granarium* and can reduce the incidence of fungi on groundnut seeds (Leone *et al*., 2015).

Moringa seeds oil as a sustainable source of renewable energy for bio diesel production (Mathur, 2014). Due to the increasing energy demand and environmental problems associated with fossil fuels, the improvement of alternative fuels and renewable sources of energy is required.

Biodiesel can replace petroleum-derived oil (petrodiesel), without any sulphur or aromatic compound and with lower emission of monoxides, hydrocarbons and particulates. Furthermore, biodiesel can reduce dependence on imported fuels: a crucial problem in developing countries (Leone *et al*., 2015).

Moringa seeds have an oil content of 30%–40%, with a high-quality fatty acid composition *i.e.*, high oleic acid (>70%) (Rashid *et al*., 2008). In addition, they possess significant resistance to oxidative degradation. These proprieties make *Moringa* oil a good candidate to produce biodiesel. Since biodiesel production with *Moringa* seed oil is a second generation production (*i.e.*, not in direct competition with existing farmland and with food crops) and as *Moringa* can be grown on degraded land, studies suggest that *Moringa* biodiesel is an acceptable substitute to fossil fuels, even when compared against biodiesel derived from vegetable oil of other species (Leone *et al*., 2015).

2.6 Plant tissue culture and its application

Plant tissue culture and genetic transformation method offer an important option for effective multiplication and improvement of trees within a limited time frame. Interventions of biotechnological approaches for *in vitro* regeneration, mass propagation techniques and gene transfer studies in tree species have been encouraging, particularly in the last decade (Giri *et al*., 2003). Micropropagation of tree species offers a rapid means of producing clonal planting stock for forestation programs, woody biomass production and conservation of elite and rare germplasm. In general, tree propagation *in vitro* has been a difficult proposition compared to

other plants. Only a few tree species with explants from mature trees have been propagated by tissue culture methods (Giri *et al*., 2003).

Plant tissue culture can be defined as the culture of all types of plant cells, tissues and organs under aseptic condition to produce the clones of plants (Singh and Shetty, 2011).The controlled condition provides the culture an environment conductive for their growth and multiplication. These conditions include proper supply of nutrients, pH, adequate temperature and proper gaseous and liquid environment (Neumann *et al.,* 2009). This definition also extends to the culture of excised embryo and protoplast culture. Plant tissue culture has an important role to play in the manipulation of plants for improved agronomic performance. Plant tissue culture is an integral part of molecular approaches to plant improvement and act as an intermediary whereby advances made by the molecular biologists in gene isolation and modification are transferred to plant cells (Singh and Shetty, 2011).

Plant tissue culture technology is being widely used for large scale plant multiplication. Small pieces of tissue (explants) can be used to produce hundreds and thousands of plants in continuous process. Endangered, threatened and rare species have successfully been grown and conserved by micropropagation because of high coefficient of multiplication and small demands on number of initial plants and space (Hussain *et al.,* 2012). *In vitro* techniques were initially developed to demonstrate the totipotency of plant cell. Totipotency is the ability of plant cell to perform all the functions of development which are characteristic of zygote i.e. its ability to develop into a complete plant. Plant tissue culture is a direct result of development of methods for the regeneration of plants from individual cells. This is of much importance today and has

two fold benefits: In a single experiment, regeneration enables hundreds of plants to be produced; It is possible to isolate virus free cells that can be used to produce virus free crops, which will give an increased yield per acre (Sobti and Panchauri, 2009).

Tiny replicas of single parent are produced by growing small part of plant explants aseptically on a nutritional medium in a test tube. It happens in absence of microorganisms and presence of a balanced diet of chemicals. Plants multiplied by tissue culture generally have identical genetic make ups, they will, thus, prove to be true clones. Tissue culture techniques can be used for most plants, provided that right chemical solution and processes are used. Generally, via tissue culture procedures, diseases transmitted from parent to offspring can be eliminated. This also helps in saving of time and space and can be cheaper than other means of vegetative propagation. Plant tissue culture techniques are being used for the following purposes: To improve stress, salt, cold and chemical tolerance and to increase the nutrient contents in various crops; for production of virus, pesticide and herbicide resistant plants; to bypass the growing whole plants to get desired products (Sobti and Panchauri, 2009).

2.6.1 Micropropagation

Micropropagation is the application of tissue culture technique to the propagation of plants starting with very small parts grown aseptically in test tube or other suitable containers (Sidhu, 2010).

Micropropagationis one of the key tools of biotechnology that has been extensively exploited to meet the growing demands for elite planting material. There exists a large demand for disease

free clones of superior quality plants in ornamental, horticultural, floricultural and agro forestry sector, which form the core sectors of agriculture. This need has been successfully tapped through micropropagation by the application of techniques of plant tissue culture thereby effectively translating the concept technology for the commercial needs (Singh and Shetty, 2011). It is carried out in aseptic and favorable conditions on growth media, using various plant tissue culture techniques.

3. OBJECTIVES

3.1. General objective

To develop efficient micropropagation protocol for *Moringa oleifera* from shoot tip explants.

3.2. Specific objectives

- ✓ To determine and establish sterilization protocol for *in vitro* seed germination
- ✓ To optimize growth regulators concentration for shoot induction and multiplication
- ✓ To optimize concentration of auxins for rooting
- ✓ To acclimatize and evaluate the survival rate of *in vitro* grown *Moringa oleifera* plantlets in greenhouse.

4. MATERIALS AND METHODS

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

4.1. Plant material

M.oleifera seeds were collected from Karat, Konso, Southern Nations, Nationalities and People (SNNP), Ethiopia. The seed pod were removed by hand and stored at room temperature.

4.2. MS medium stock solution preparation

Murashige and Skoog (1962) (MS) medium was used throughout the research activity. Full strength stock solution of macronutrients, micronutrients, Fe-Na-EDTA and FeSO4 mixture and vitamins were prepared separately. To do so, appropriate amount of each nutrient was weighed in grams per litter (appendix 1) and dissolved in distilled water consecutively in such a way that the next nutrient was added after the first one was completely dissolved. After all the components were fully dissolved using magnetic stirrer, finally the solution was dispensed in to plastic bottles and stored at -20°C until used.

4.3. Plant growth regulators stock solution preparation

Plant growth regulators such as 6-Benzyl Amino-purine (BAP), Kinetin (KIN) and Indol-Butyric Acid (IBA) for shoot induction and Naphthalene Acetic Acid (NAA) for rooting were used in this study. All of the plant growth regulators stock solutions were prepared by weighing and dissolving the powder in double distilled water at a ratio of 1:1 (1mg/ml) and dissolved by 2-3 drops of NaOH based on the requirement of the plant growth regulators. Then, the volume was adjusted by adding double distilled water and stirred using magnetic stirrer. Finally, growth regulator stock solutions were stored in a refrigerator at +4°C for short term use.

4.4. Culture medium preparation

In the first category of studies, the culture medium for shoot initiation and multiplication contained full strength MS basal medium. The medium was prepared by taking 100 ml macronutrient, 10 ml micronutrient and 10 ml vitamin per liter. Then, 30 g/l of sucrose was dissolved and different concentrations of BAP, KIN and IBA for shoot induction and half strength MS basal medium with different concentrations of NAA and IBA for rooting were added. The pH of the culture medium was adjusted to5.8 using NaOH and/or HCl. Then 7 g/l agar was added. After adjusting the pH, then gently mixed medium was boiled in microwave oven until the agar was melted. Then 50 ml of the prepared medium was dispensed in to Magenta GA-7 culture vessels. The culture vessels were covered with caps immediately after dispensing the medium and autoclaved at a temperature of 121°C and pressure of 105 KPa for 15 minutes. Immediately after autoclaving, the medium was taken and kept in laminar air flow cabinet bench or kept in refrigerator for one or two days until used.

4.5. *In vitro* seed germination

Powder of calcium hypochlorite ($CaCl_20_2$) was weighted in different amounts of 5g, 10g and 15g. Each weighed powder was dissolved in 100ml distilled water and settled for few hours. Seeds of *M. oleifera* were removed from the seed coat and washed with tap water. Subsequently, seeds were surface sterilized in 70% ethanol for 9 min, then in 5%, 10%, 15%, solution of calcium hypochlorite for 20, 25 or 30, minutes and rinsed 3 to 4 times using distilled autoclaved water under a laminar flow hood. The sterilized seeds were germinated on MS medium supplemented with 7.0 g/l agar and 3% sucrose (w/v). The components of the media were dissolved; the pH was adjusted to 5.8with 0.1 HCl or 0.1 NaOH and autoclaved at 121^{o}C for 15 min. The media (50 mL each) were then dispensed in culture vessel (10 x 6 cm, height, width, respectively). Five seeds per vessel will be cultured with 6 replicates per treatment. Cultures will be incubated at 25 ± 2°C for 16 h photoperiod and light intensity of 22 μ mol m^{-2} s^{-1} under cool fluorescent. Seed germination indicated by at least radicle emergence was recorded at days 16, 20, and 24.

4.6. Shoot initiation

The shoot tips of the germinated seedlings were excised and cultured on full strength MS medium fortified with 3% sucrose (w/v), BAP (0.0, 0.25 0.5, 0.75, 1.0 mg /L) and KN (0.0, 0.25 0.5, 0.75, 1.0 mg /L) and 7.0 g/L agar was added after adjusting the pH to 5.8 before autoclaving. The explants were cultured in Magenta GA-7 culture vessels (10 × 6 mm) containing 50 ml medium. All cultures were maintained under white fluorescent light at light

intensity of 22 μ mol $m^{-2}s^{-1}$ under 16 hour's photoperiod at 25±2°C temperature. Number of initiated explants was recorded after four weeks.

4.7. Shoot multiplication

Shoots initiated from the germinated seedlings were used as explant in shoot multiplication. The shoots weretrimmed to about 1.5 cm length and cultured on 50 ml full strength MS medium in Magenta GA-7 culture vessels (10 x 6 cm) supplemented with different concentrations of BAP (0.0, 0.25, 0.5, 1.0, 1.5, 2.0 mg/L), BAP in combination with IBA (0.0, 0.1, 0.5, 1.0, mg/L) and BAP in combination with KIN (0.0, 0.1, 0.5, 1.0, mg/L). All experiments (total of 30 treatments) were performed by using five explants per culture vessel with 6 replications. The cultures were kept at 25 ± 2°C with 16 h photoperiod and light intensity of 22 μ mol $m^{-2}s^{-1}$ for 30 days.

4.8. Rooting

For root induction, half strength MS basal medium supplemented with IBA (0.0, 0.1, 0.25, 0.5, mg/L) in combination with NAA (0.0, 0.1, 0.25, 0.5, mg/L) were used. The media was fortified with 1.5% sucrose (w/v), and the pH was adjusted to 5.8 before addition of 6.0 g/L agar prior to autoclaving. Microshoots (2.5 to 3.5 cm long) were excised and transferred to the rooting media. Five explants per culture vessel in six replications were used. The cultures were maintained at 25 ± 2°C with 16 h photo period at light intensity of 22 μ mol $m^{-2}s^{-1}$.

4.9. Acclimatization

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

4.10. Experimental design and data analysis

A completely randomized design (CRD) was used for all experiments. Data were subjected to one-way analysis of variance (ANOVA) to detect if there are 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. *In vitro* seed germination

Among the different concentrations of calcium hypochlorite used, 10 % calcium hypochlorite and 30 min exposure time resulted in the highest (100%) contamination free seeds. The highest contamination (100%) of seeds was recorded from 5 % calcium hypochlorite and exposure time of 20, 25 and 30 min. In addition, the contamination percentage of 10 % calcium hypochlorite and 20 min exposure time was higher than10 % calcium hypochlorite and 25 min exposure time. Poor germinations were recorded from 5 % calcium hypochlorite and exposure time of 20, 25 and 30 min and 15% calcium hypochlorite solution for 30 min (Table 1). Although, 10% calcium hypochlorite and 20 min exposure time showed germination percentage (56.5%), it was less in disinfection efficiency (80%) and minimum in occurrence of clean explants (20%) availability than 10 % calcium hypochlorite and exposure time of 25 and 30 min. The data also revealed that the contamination percentage was dramatically decreased as the exposure time increased within the same level of calcium hypochlorite solution. However, disinfection with 15%calcium hypochlorite solution for 30 minute also resulted in least contamination and highest clean explants but no seed germinated. Comparing the interaction effect posed by the chemical and the duration of disinfection against the aim of sterilization, disinfection of *M. oleifera* seeds with 10% calcium hypochlorite solution for 30 min was the most effective sterilization treatment, which resulted in the highest germination percentage (100%) without any contamination.

Table1.Effect of calcium hypochlorite concentration and exposure time on seed sterilization and *in vitro* germination of *Moringa oleifera* seeds

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

5.2. Shoot initiation

Shoot tips cultured on the control and on MS medium supplemented with BAP and KN alone, showed a significant variation ($P > 0.05$) in terms of percentage of shoots induced. All shoot tips produced proliferated shoots on MS medium supplemented with 0.5 mg/l KN and BAP (Table 2). On the other hand, the lowest shoot induction percentage was observed on 1.0 mg/l KIN where 23.33% shoots only exhibited new growth.

The maximum mean number of shoots was 2.50±0.14 on MS medium containing 0.5 mg/l BAP followed by MS + 0.25 mg/l KN (2.37±0.15). Maximum mean shoot length of 5.76±0.15 was recorded in plant growth regulators-free MS medium and 5.01±0.081 were obtained on medium containing 0.5 mg/l KN (Table 4).

Table 2.Effect of BAP and kinetin on shoot proliferation on MS medium

Treatment				
BAP	KIN	Shoot induction (%)	No. of shoots/ explant Mean±SE	Shoot length(cm) Mean±SE
00	00	43.33	1.38±0.14^{b}	5.76±0.15^{a}
0.25	00	76.67	2.30±0.15^{a}	4.69±0.11^{bc}
0.5	00	100	2.50±0.14^{a}	4.85±0.05^{b}
1.0	00	36.67	1.18±0.12^{b}	2.72±0.07^{d}
00	0.25	80	2.37±0.15^{a}	4.22±0.15^{c}
00	0.5	100	2.36±0.18^{a}	5.01±0.81^{b}
00	1.0	23.33	1.42±0.20^{b}	2.35±0.14^{d}

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

5.3. Shoot Multiplication

The shoots on multiplication medium gave different responses based on the hormonal composition differences of medium (Table 3, 4). From all used PGRs MS medium supplemented with combination of BAP with IBA showed better result than BAP alone and combination of BAP with KN. The better shoot multiplication that provided highest numbers of shoots were obtained (Figure 1).

The maximum mean number of shoots from all used PGRs was 5.66±0.44 on MS multiplication medium containing 1.0 mg/l BAP + 0.5 mg/l IBA followed by MS + 0.25 mg/l BAP + 0.1 mg/l IBA (4.63±0.33). Maximum mean shoot length of4.51±0.21was recorded in MS medium supplemented with 0.25 mg/l BAP + 0.1 mg/l IBA and 4.30±0.11wereobtained on medium containing 1.0 mg/l BAP + 0.5 mg/l IBA (Table 4).

5.3.1. Effect of BAP and KN on Shoot Multiplication

Among the different concentration of BAP, medium containing 0.25 mg/l BAP showed maximum shoots per explants of 3.63±0.35, followed by 1.0 mg/l BAP (3.56±0. 37). Maximum shoot length of 3.98±0.18 was also recorded in MS medium supplemented with 0.25 mg/l BAP.

The response of explants cultured on MS medium supplemented with different concentrations of BAP in combination with KN is presented in (Table 3), 0.25 mg/l BAP + 0.5 mg/l KN resulted in maximum number of shoots per explants (2.36±0.29), followed by medium containing 0.25 mg/l BAP + 0.10 mg/l KN (1.93±0. 15). Maximum shoot length of 2.73±0.17 was recorded in MS medium supplemented with 0.25 mg/l BAP + 0.10 mg/l KN (Table 3).

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

BAP	KIN	No. of shoots/ explants Mean±SE	Shoot length(cm) Mean±SE
00	00	1.71±0.17 ^{cd}	2.77±0.14^{b}
0.25	00	3.63±0. 35^{a}	3.98±0. 18^{a}
0.5	00	3.10±0. 25^{ab}	3.45±0. 20^{a}
1.0	00	3.56±0. 37^{a}	3.43±0. 17^{a}
1.5	00	1.80±0. 17^{cd}	2.16±0. 11^{bc}
2.0	00	1.73±0. 23 ^{cd}	2.58±0. 21^{b}
0.25	0.5	2.36± 0.29^{bc}	2.59±0.14^{b}
1.0	0.5	1.40±0.14 ^{cd}	2.40±0.11^{bc}
1.5	0.5	1.50±0. 16 ^{cd}	2.22±0. 10^{bc}
0.5	1.0	1.67±0. 13 ^{cd}	2.05±0. 08^{bc}
1.0	1.0	1.20±0. 07^{d}	2.06±0. 08^{bc}
1.5	1.0	1.43±0. 11 ^{cd}	1.78±0. 07^{c}
0.25	0.1	1.93±0. 15^{cd}	2.73±0. 17^{b}
0.5	0.1	1.16±0. 06^{d}	2.47±0. 12^{b}
1.0	0.1	1.73±0.15 ^{cd}	2.30±0. 11^{bc}
1.5	0.1	1.47±0.14 ^{cd}	2.28±0. 11^{bc}

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

5.3.2. Effect of BAP and IBA on Shoot Multiplication

The response of explants cultured on MS medium supplemented with different concentrations of BAP in combination with IBA is presented in Table 4. After two weeks of culture on MS medium containing different concentrations of BAP in combination with IBA, the shoots emerged from the explants (Figure 1). The MS medium containing various concentrations of

BAP in combination with low level of IBA produced shoots that are significantly different at P = 0.05 after 4 weeks (Table 4). Maximum number of shoots per explants (5.66±0.44 and 4.63±0.33) were induced in MS medium supplemented with 1.0 mg/l BAP and 0.5 mg/l IBA and 0.25 mg/l BAP + 0.1 mg/l IBA respectively. Maximum shoot length per explants (4.51±0.21 cm) was induced in MS medium supplemented with 0.25 mg/l BAP + 0.1 mg/l IBA. Lowest number of shoots per explants (1.23±0.09) was induced in MS medium supplemented with 2.0 mg/l BAP and 0.1 mg/l IBA.

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

BAP	IBA	No. of shoots/explants	Shoot length(cm)
00	00	1.71 ± 0.17^{def}	2.77 ± 0.14^{b}
0.25	0.1	4.63 ± 0.33^{ab}	4.51 ± 0.21^{a}
0.5	0.1	1.36 ± 0.10^{ef}	2.34 ± 0.12^{c}
1.0	0.1	3.10 ± 0.32^{cd}	3.32 ± 0.14^{b}
1.5	0.1	2.63 ± 0.35^{cde}	2.19 ± 0.14^{c}
2.0	0.1	1.23 ± 0.09^{f}	1.92 ± 0.08^{c}
0.25	0.5	3.73 ± 0.36^{bc}	3.07 ± 0.11^{b}
0.5	0.5	3.10 ± 0.32^{cd}	3.15 ± 0.14^{b}
1.0	0.5	5.66 ± 0.44^{a}	4.30 ± 0.11^{a}
1.5	0.5	1.50 ± 0.11^{ef}	2.15 ± 0.14^{c}
2.0	0.5	1.33 ± 0.09^{ef}	1.88 ± 0.05^{c}
0.25	1.0	3.60 ± 0.35^{bc}	3.16 ± 0.14^{b}
0.5	1.0	1.40 ± 0.14^{ef}	2.30 ± 0.11^{c}
1.0	1.0	1.90 ± 0.16^{def}	2.18 ± 0.08^{c}
1.5	1.0	1.93 ± 0.30^{def}	2.03 ± 0.11^{c}
2.0	1.0	1.40 ± 0.13^{ef}	2.09 ± 0.11^{c}

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

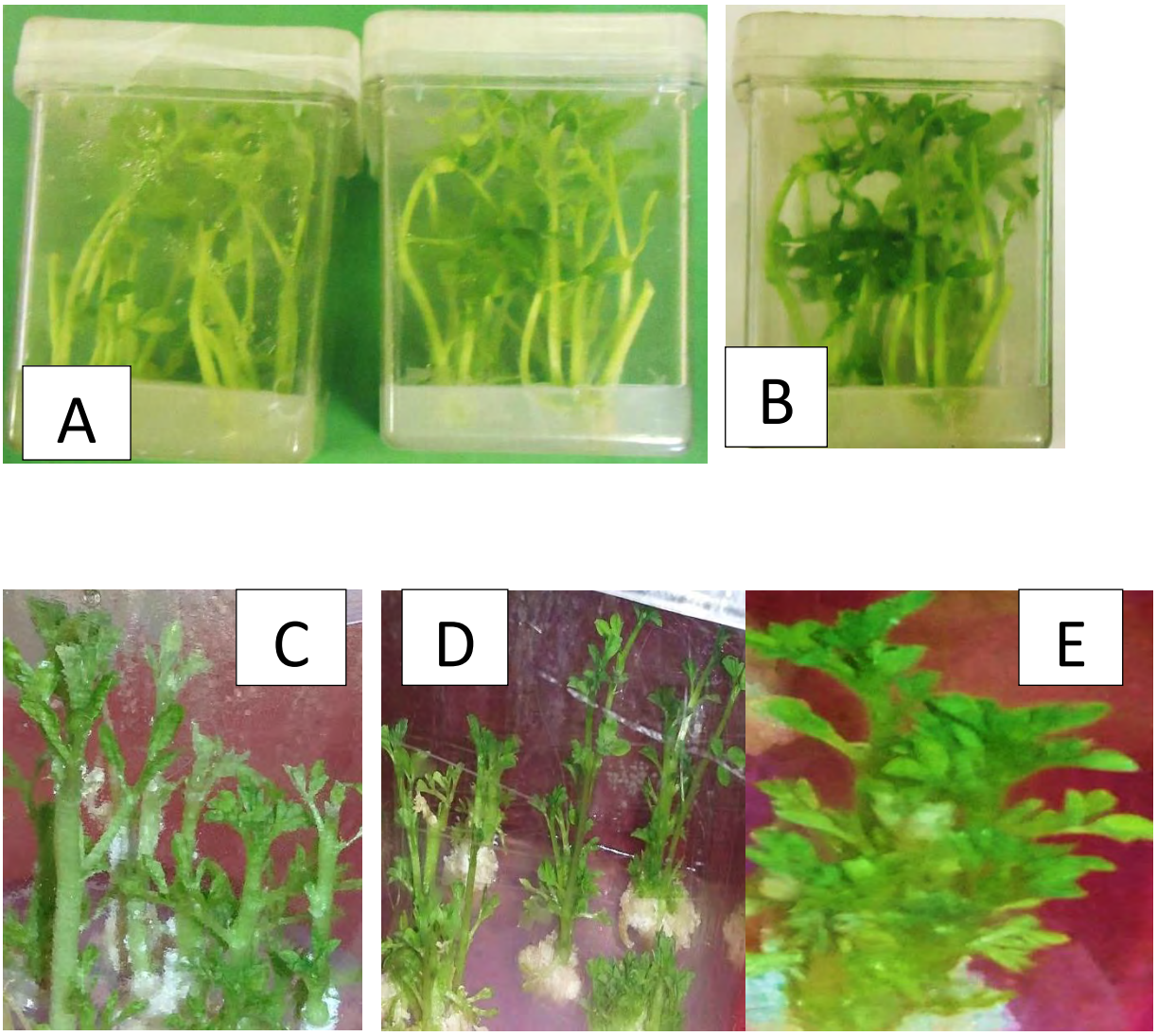

Figure 1: Shoot multiplication from shoot tip explants of *Moringa oleifera* on MS medium containing BAP alone and in combination with IBA (A) 1.0mg/l BAP + 0.5 mg/l IBA; (B) 0.25 mg/l BAP + 0.1 mg/l IBA; (C) 1.0 mg/l BAP; (D) 0.25 mg/l BAP and (E) 0.25 mg/l BAP.

5.4. Rooting and acclimatization

5.4.1. Effect of NAA and IBA on rooting

The shoots cultured on half strength MS basal media supplemented with different concentrations of IBA, NAA and combination of IBA and NAA resulted in different rooting responses including the control (Figure 2).The highest mean number of roots per shoot (7.96±0.69) was obtained on 1/2 strength MS medium containing 0.25 mg/l NAA followed by 0.5 mg/l NAA (6.16±0.84) (Table 5). The highest mean length of root per explants (2.44±0. 04 cm) was obtained on growth regulators free half strength MS medium which was used as control.

After one month of acclimatization, the survival rate of total plants transferred to greenhouse was 90% (Figure 3).

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

IBA (mg/l)	NAA (mg/l)	Rooting (%)	No. of roots/ explants Mean±SE	Root length (cm) Mean±SE
00	00	73.33	3.04±0. 36^{b}	2.44±0. 04^{a}
0.25	00	33.33	3.30±0. 85^{b}	1.42±0. 07^{c}
0.5	00	63.33	2.94±0. 44^{b}	1.57±0. 06^{c}
00	0.1	73.33	3.08±0. 36^{b}	1.44±0. 05^{c}
00	0.25	94.00	7.96±0. 69^{a}	2.11±0. 08^{b}
00	0.5	86.67	6.16±0. 84^{ab}	1.55±0. 04^{c}
0.1	0.1	70.00	4.23±0. 62^{b}	1.48±0. 03^{c}
0.25	0.1	66.67	4.85±0. 73^{ab}	1.53±0. 04^{c}
0.5	0.1	60.00	3.55±0. 42^{b}	1.44±0. 03^{c}
0.1	0.25	93.33	4.21±0. 43^{b}	1.52±0. 05^{c}
0.25	0.25	60.00	5.76±0. 88^{ab}	1.57±0. 06^{c}
0.5	0.25	20.00	3.00±0. 1.0^{b}	1.51±0. 06^{c}

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

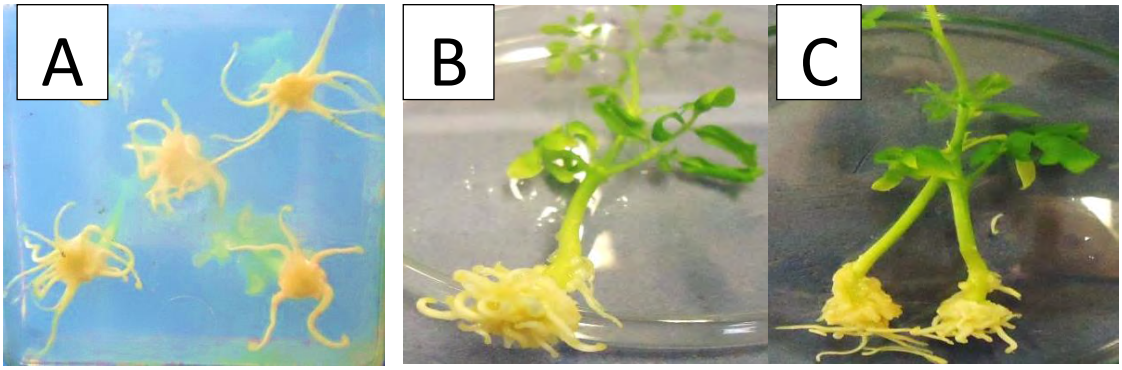

Figure 2: *In vitro* rooting of *Moringa oleifera* shoots on half strength MS medium containing NAA alone (A and B) 0.25 mg/l NAA and (C) 0.5 mg/l NAA

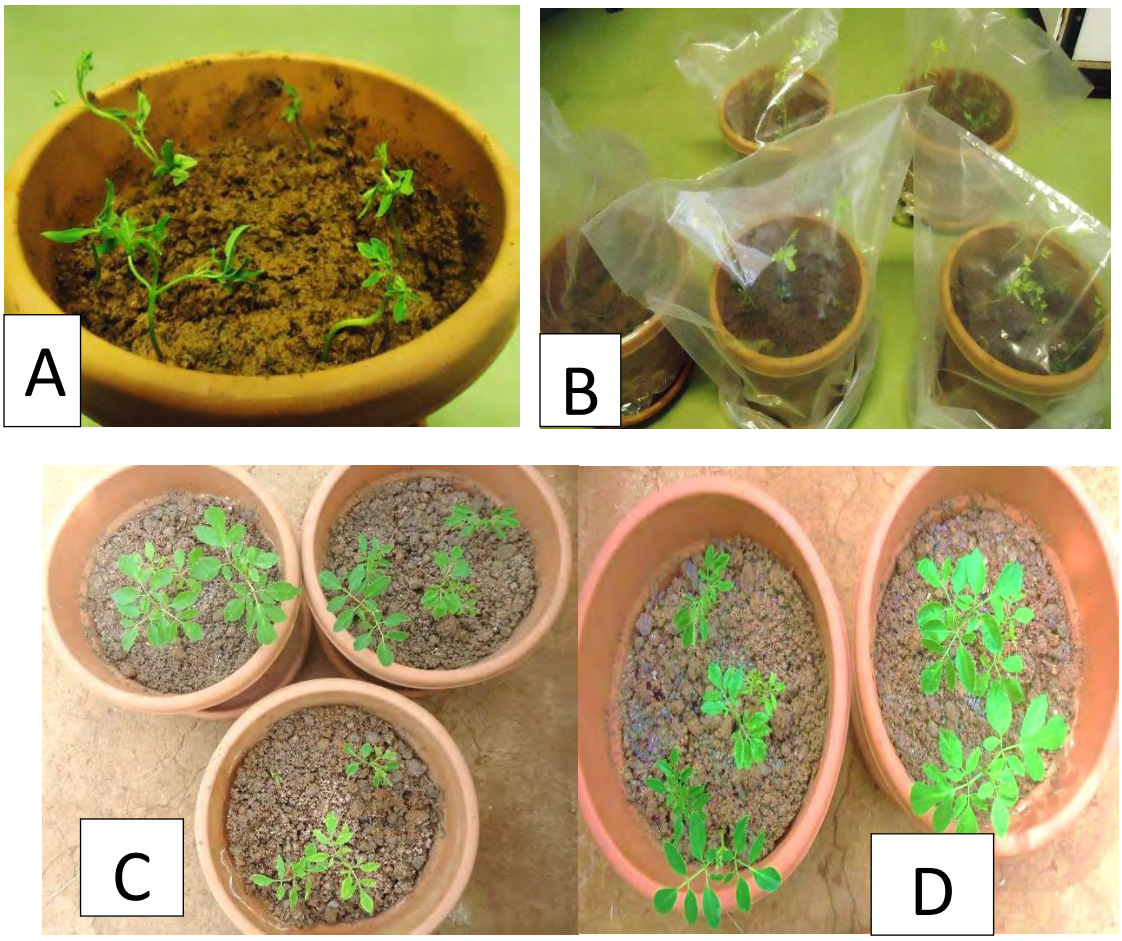

Figure 3: Acclimatization of *in vitro* rooted shoots of *Moringa oleifera* in greenhouse. (A) Plants transferred from the medium to the pots; (B) Plants covered with plastic bags and (C and D) after 30 days of acclimatization

6. DISCUSSION

6.1. Sterilization of the Seeds

Analysis of variance (ANOVA) revealed that concentration of local bleach, exposure time, and their interaction had highly significant difference (P>0.05) on overcoming contamination of growth media and improving survival and germination level of *Moringa oleifera in vitro* seed culture.

From this study, among the different disinfectant treatments investigated, 10% calcium hypochlorite treatment for 30 min resulted in the highest percentage of contamination free explants (100%), with 100% seed germination percentage. The second seed germination percentage (87.4%) was obtained at 10% calcium hypochlorite concentration and 25 min exposure. The highest contamination (100%) was recorded from 5 % calcium hypochlorite and exposure time of 20, 25 and 30 min. In addition, the contamination percentage of 10 % calcium hypochlorite for 20 min was higher than 10% calcium hypochlorite for 25 min. This result indicates that the concentration of active calcium hypochlorite solution and exposure time used for disinfectant were interrelated factors to obtain contamination free explants. This is similar to the finding of Saini *et al*. (2013) who reported the best surface sterilization for the seeds of *Moringa oleifera* were 30% Clorox solution for 30 minutes of exposure time. However, in contrast with this study, Saini *et al*. (2012) reported that seeds were surface sterilized by immersion in 0.1% mercury chloride for 2 min and 20% sodium hypochlorite for 10 min, followed by rinsing three times in sterile distilled water. Seed coats were removed aseptically and seeds were again surface sterilized by immersion in 20% sodium hypochlorite for 5 min and this was found to be effective for sterilization of seeds of *Moringa oleifera*.

In this study, no and poor seed germination was recorded from 5% calcium hypochlorite and exposure time of 20, 25, 30 min and 15% calcium hypochlorite and exposure time of 30 min. Although 10% calcium hypochlorite and 20 min exposure time showed germination percentage (56.5 %), it was less in disinfection efficiency (80%) than 10 % calcium hypochlorite for 25 and 30 min. These results indicate that the chemical concentration and the duration at which *M.oleifera* seeds exposed to the disinfectant time affected the seed germination percentage significantly. The data also revealed that the contamination percentage was dramatically decreased as the exposure time increased within the same level of calcium hypochlorite solution. However, disinfection with higher concentration (15%) calcium hypochlorite solution for 30 minute also resulted in least contamination but no seeds germinated. This observation is also in agreement with the work of Birhanu Kahsay (2015) that the concentration of active chlorinated local bleach and exposure time used for disinfectant were interrelated factors to obtain microorganism free explants of *Oxytenanthera abyssinica* from seed culture. Comparing the interaction effect posed by the chemical concentration and the duration of disinfection against the aim of sterilization of *M. oleifera* seed with 10% calcium hypochlorite solution for 30 min was the most effective sterilization treatment, which gave highest germination percentage, no contamination, and moderately clean explants.

6.2. Shoot Induction from Shoot Tip Explants

In culture initiation experiment, shoot tips cultured on control and MS medium supplemented with 0.25, 0.5, 0.75, 1.0 mg/l BAP and KN separately, showed a significant variation ($P > 0.05$). The result thus conclusively showed that cytokinins were essential for shoot induction and proliferation of shoot tip explants of *M.oleifera.* Kinetin and BAP at 0.5 mg/l concentration resulted in 100% shoots initiation. This is similar to the finding of Islam *et al*. (2005) who got

100% initiation of shoots from stem segment explants taken from 10-day-old seedlings of *M. oleifera.*

The initiation percentages were found to vary in the different concentrations of cytokinins and control treatment. The minimum shoot initiation percentage was 23.33% which was observed on MS medium containing 1.0 mg/l KN and 36.67% which was recorded on MS medium containing 1.0 mg/l BAP. This showed that the shoot initiation percentage was greatly influenced by type and concentrations of cytokinin. Arya *et al*. (1999; 2012) has already reported the capacity of BAP on shoot induction of *Dendrocalamus asper* and *Dendrocalamus hamiltonii* seeds respectively.

Due to their ability of enhancing shoot initiation investigations have been revealed that cytokinins were a key factor for multiple shoot proliferation (Riyathong *et al*., 2008). The present study also showed that the ability of shoot induction of *Moringa oleifera* is dependent on the concentration of cytokinins (BAP and KN). This is in contrast to the report by Fatima *et al*. (2016) on nodal explants of *Moringa concanens is* who obtained the combination of 0.1 mg/l BAP and 0.5 mg/l NAA were considered as the best hormone for better proliferation with 19.66 shoots with an average length of 8.16 cm. The control medium also showed multiple shoot development but they were smaller in size.

Previous works on the tissue culture of *M. oleifera* showed different results regarding the best growth regulators for induction of multiple shoots. Stephenson and Fahey (2004) reported 1.0 mg/l BAP to be the best for shoot induction from nodal explants obtained seedlings. Islam *et al*.

(2005) also pointed out 1.0-1.5 mg/l BAP to be the best cytokinin for induction of shoots from shoot explants obtained from filed-grown plants. These variable responses may be attributed to differences in the plant source and the type of explants used to establish tissue cultures.

The present result is in agreement with the findings of other workers who have noted the effectiveness of BAP for the shoot induction of *Moringa oleifera* shoot explants are cultured on MS medium supplemented with different concentrations of BAP, it was found that all the media induced 100% shoot induction (Riyathong *et al*., 2008).

6.3. Shoot Multiplication

There was a significant difference (P >0.05) among the different treatment effects both in terms of shoot number per explants and shoot length. In this study, 31 different concentrations of KIN in combination with BAP and BAP in combination with IBA were used for shoot multiplication. BAP alone or in combination with IBA were found to be the most suitable plant growth regulators for micropropagation of *Moringa oleifera* rather than combination of KN and BAP. This result is in contrast with the findings of Shahzad *et al.* (2014) who reported the maximum shoot length (4.31±0.49 cm) was attained when the hypocotyl explants were cultured on the MS medium supplemented with Kinetin. The lowest shoot length (2.18±0.31cm) was attained when these hypocotyls were cultured on the MS medium supplemented with BAP. Lower concentration of kinetin (0.1 mg/l and 0.5 mg/l) resulted in the maximum shoot length of 7.0±0.58 cm and 6.27±0.41 cm, respectively. Abdellatef and Khalafalla (2010) used nodal explants and reported that BA was more effective as compared to Kinetin for shoot induction. Low concentration of BA and Kinetin also produced more number of shoots in other studies conducted on *Moringa* by Riyathong (2010) and Abdellatef and Khalafallah (2010).

Alkhateeb (2013) reported that MS medium supplemented with 1.0 mg/l kin resulted in 2.6 mean numbers of shoots per explants of *Moringa peregrine* (Forsk). Higher levels of cytokinin (2.0 mg/l kinetin) significantly reduced the number of leaves per microshoot. Microshoots grown on growth regulators free MS medium and that supplemented with kinetin were similar in length. Maximum shoot multiplication (5.66±0. 44) was recorded on MS medium augmented with 1.0 mg/l BAP and 0.5 mg/l IBA followed by 4.63±0.33 shoot per explants on MS medium supplemented with 0.25 mg/l of BAP and 0.1mg/l of IBA. The result from combined effect of IBA and BAP was found to vary with the concentration of IBA and BAP in all two parameters of shoot growth (mean number of shoot and shoot length).

The present study showed when the concentration of BAP raised from 0.25 mg/l to 2.0 mg/l, lower number of shoots per explants were obtained. In contrast with Saini *et al.*(2013) who reported MS medium containing 2.0 mg/l BAP produced the highest mean number (10.8) of shoots per explants followed by those treated with 1.0 and 3.0 mg/l of BAP, respectively. In the present study, using different concentration of BAP alone resulted in formation of callus at the cut ends of the shoots. This is in agreement with the findings of Islam *et al*. (2005) who reported that higher concentration of the cytokinin increases the callus initiation at the cut ends of the shoots. Most of the shoots were observed with the callus development at the base and this phenomenon was most prominent when the concentration of the growth regulator was increased.

Tileye Feyissa *et al.,* (2005) reported that highest number of shoots per explants of *Hagenia abyssinica* was obtained on MS medium supplemented with 1 mg/l of BAP and 0.01 mg/l of IBA. In another study, when the concentration of BAP was greater than that of IBA, relatively better result was recorded which is similar to present study. This is due to the effect of cytokinin as it

promotes the axillary branching or axillary bud proliferation. Induction of multiple shoots in *Moringa concanensis* has been previously characterized with different growth regulators. Fatima *et al*. (2016) obtained 11.00 ± 1.15 mean shoot number with 5.00±1.95cm mean length from nodal explants of *Moringa concanensis* on MS medium supplemented with 0.1 mg/l KN along with 0.05 mg/l NAA. The finding of these authors is contrasting to the present one. On the other hand, BAP alone resulted in maximum shoot proliferation (3.63±0. 35) on MS medium supplemented with 0.25 mg/l BAP when compared to BAP and KIN combinations that gave significantly ($P > 0.05$) less number of shoots. Pflanzen, (2013) also reported similar result while the highest multiplication ability of the different plant parts, especially the base parts of *M. oleifera in vitro* plants, was observed after 3 weeks of cultivation on MS medium with 0.5 mg/l BAP.

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

6.4. Rooting and acclimatization

Roots were not spontaneously induced during culture initiation and shoot multiplication. The analysis of variance revealed that root number and root length varied significantly with half strength MS medium supplemented with NAA, IBA and the combination of both. Application of NAA alone exhibited the maximum mean root number per shoot as compared to IBA alone and

IBA in combination with NAA. The highest number of mean roots per shoot (7.96±0. 69) was recorded from 0.25 mg/l NAA and mean root length (2.44±0. 04 cm) was observed on growth regulators free basal MS medium. This is similar to finding of Stephenson and Fahey (2004) who obtained 4.7 roots per explants in ½ strength MS medium supplemented with 0.5 mg/l NAA in *M. oleifera*. This result is also in agreement with other literature (Riyathong *et al*., 2008) who reported the explants on MS medium without growth regulator were elongated and grown to be new drumstick tree plantlets with healthy roots. Islam *et al.* (2005) also found that hormone-free medium was the best rooting medium. In contrast with the finding of Alkhateeb (2013), who got the maximum number of roots (44.0) per explants of *Moringa peregrina (*Forsk) in MS medium supplemented with 1.0 mg/l IBA and MS medium containing 0.5 mg/l IBA, it produced longer roots than control or medium supplemented with different levels of NAA. In the present study, lowest mean root number (2.94±0.44) was obtained on medium containing 0.5 mg/l IBA and lowest shoot length (1.42±0.07) was obtained at 0.25 mg/l IBA.

The number of roots produced per shoot increased when higher concentrations of NAA were applied. However, further increase in the concentration of NAA showed a reduction in the mean root number per shoot which agrees with Tileye Feyissa *et al.* (2005) and Vijaya *et al.* (2012). The same trend was observed in both treatments (the effect of different concentrations of IBA alone and IBA in combination with NAA). Also the same with finding of Bayoudh *et al.* (2015).

In the present study, however, from the comparison made between the rooting hormones NAA and IBA, NAA was more effective to increase root number and length. IBA relatively increased

the root length. ANOVA showed NAA significantly affected length and number of roots, but not IBA, at 5% probability level.

Another report on *in vitro* rooting of *M. oleifera* showed that when microshoots were cultured on medium containing different levels of IAA and IBA, the medium supplemented with 0.5 mg/l IAA along with IBA at 1.0 mg/l resulted in the highest number of roots (Saini *et al*., 2012). These variable responses could be due to different factors including genetic differences, differences in the explant source, the concentration difference of growth regulators and the type and/or age of explants used to establish the cultures (Marfori, 2010)

Acclimatization of *in vitro* rooted plantlets was successful, where 90% plants survived and established as healthy plant. This result was better than previous report by Saini *et al*. (2012) and Marfori (2010) who got 80% of survived plants. Saini *et al.* (2012) also obtained the same result as Marfori (2010), provided that the potted plantlets were covered with clear polythene bags and kept in a shaded greenhouse for 15 days before exposure to ambient conditions.

7. CONCLUSIONS

- Results of this study indicate that large scale propagation of *M. oleifera* by tissue culture methods is economically feasible and several plantlets can be regenerated from a single shoot tip explant.
- The analysis of variance revealed 10 % calcium hypochlorite and 30 min duration is optimal for seed sterilization and germination of M. *oleifera*.
- Shoot induction on 0.5 mg/l Kinetin and BAP alone found to be optimalin producing maximum number of shoots per explant
- The effect of IBA along with BAP on shoot multiplication after four weeks of culture was highly significant ($P \geq 0.05$). Results indicate that using IBA along with BAP for shoot multiplication was better than KIN along with BAP. Therefore, 1.0 mg/l BAP combined with 0.1 mg/l IBA was found to be optimal in producing maximum number of shoots per explant.
- Application of NAA alone at concentration of 0.25 mg/l was found to be effective for root induction.
- The protocol developed in this study enables mass propagation of *M. oleifera* from shoot tip explants.

8. RECOMMENDATIONS

- ✓ Explants other than shoot tips culture for *in vitro* propagation of *M. oleifera* should be considered to investigate if they can offer better and efficient responses.
- ✓ Further studies on the performance of the tested cytokinins in combination with other type of hormone (cytokinins and/or auxins) should be carried out for better result on shoot multiplication.
- ✓ Further studies on identifying an optimum sub-culture of the microshoots should be carried out to obtain an increased shoot number from multiplication period.
- ✓ The effect of different concentrations of auxins on induction of roots should be further studied.
- ✓ It is also recommended to practice other techniques like culturing of explants in liquid medium for mass propagation of *M. oleifera.*

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10. APPENDIX

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

Components	Concentration (g/l)	ml/l during media preparation
Micronutrients		
ZnSO4.7H2O	1.72	5ml/l
H3BO3	1.124	
* MnSO2.4H2O	3.38	
* 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	50ml/l
KNO3	38	
Cacl2.2H2O	8.8	
MgSO4.7H2O	7.4	
KH2PO4	3.4	
Vitamins		
Myo-inositol	20	5ml/l
Glycin (glycocoll)	0.4	
Nicotinic acid (NaOH)	0.1	
Pyridoxine (B6)	0.1	
Thiamin (B1)	0.02	