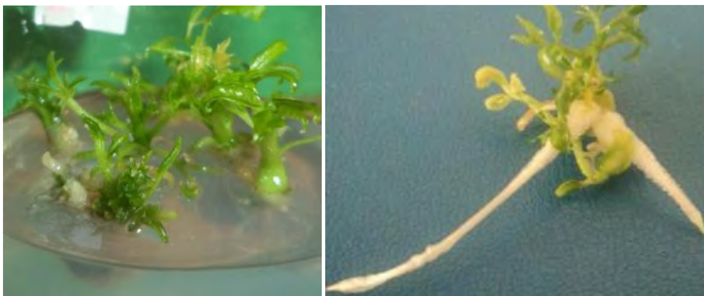
ADDIS ABABA UNIVERSITY

COLLEGE OF NATURAL SCIENCE

SCHOOL OF GRADUATE STUDIES

GENERAL BIOLOGY PROGRAM

Optimization of Growth Regulators on *In vitro* Propagation of *Moringa stenopetala* from Shoot Explants



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

By

ALELEGNE YESHAMEBEL ADUGNA

August 2016

Addis Ababa, Ethiopia

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LIST OF ABBREVIATIONS

2, 4-D = 2, 4-Dichlorophenoxyacetic acid

2-ip = Isopentenyl-adenine

BAP =6- Benzylamino purine

IAA= Indole acetic acid

IBA= Indole-3- butyric acid

Kin= Kinetin

MS = Murashige and Skoog

NAA= α -naphthalene acetic acid

PGR= Plant growth regulator

TDZ=Thidiazuron

CRD= Complete Random Design

ANOVA= Analysis of Variance

SPSS= Statistical Package for Social Science

LSD= Least Significance Difference

ABSTRACT

This thesis was conducted at Addis Ababa University entitled with Optimization of Growth Regulators on in vitro *Propagation of* M. stenopetala from shoot explant. Moringa stenopetala *belongs to flowering family Moringaceae and genus Moringa. It is often referred to as the East African Moringa tree because it is native only to southern Ethiopia and northern Kenya. The expansion of its cultivation and utilization throughout the world especially in Africa is becoming important. For such expansion, the existing propagation method is limiting, so it needs good propagation system to supply enough planting material with uniform genotype. Therefore, the main objective of this study was to optimize an* in vitro *shoot multiplication protocol for* M. stenopetala *by using shoot tip as explants. Shoots were sterilized and cultured on Muraghige and Skoog (MS) medium for* in vitro *shoot initiation. For multiple shoot induction, the explants were cultured on MS medium supplemented with different concentrations of kinetin (0.5, 1.0, 1.5, 2.0, 2.5 mg/l) along with Indole-3- butyric acid(IBA) or α -naphthalene acetic acid (NAA) (0.01, 0.1, 0.5mg/l) and maintained at 25 ± 2°C for four weeks. Rooting was achieved by culturing well developed shoots in half strength MS medium containing IBA (0.1, 0.5, 1.0, 1.5, 2.0 mg/l), NAA (0.1, 0.5, 1.0, 1.5, 2.0 mg/l) and 0.5 mg/l IBA in combination with NAA (0.1, 0.5, 1.0, 1.5, 2.0 mg/l). Statistical analysis revealed that there was significant difference among all treatments applied in both shoot multiplication and rooting experiments. Maximum number of shoots per explant (3.43±1.41) and 7.97±4.18 leaves per explant were obtained on MS medium containing 0.5 mg/l kinetin in combination with 0.01mg/l NAA. The highest mean number of roots per shoot (1.63±1.03) and mean root length (0.87±1.22 cm) were obtained on MS medium containing 1.0 mg/l NAA and 0.1 mg/l IBA alone respectively. After acclimatization, 76% plants survived in greenhouse. In general, using NAA along with kinetin for shoot multiplication was better than kinetin along with IBA and application of NAA alone at concentration of 1.0 mg/l and 1.0 mg/l NAA along with 0.5 mg/l IBA were more effective for root induction.*

***Key words**:* In vitro *propagation,* Moringa stenopetala, *rooting, shoot multiplication*

1. INTRODUCTION

Moringa stenopetala belongs to family *Moringaceae* that is represented by a single genus *Moringa*. The genus *Moringa* is represented by 14 species to which *M. stenopetala* belongs. The genus *Moringa* originated from sub-Himalayan tracts of Northern India, distributed worldwide in the tropics and sub-tropics, (Rajangam et al, 2001; Olson, 2002). *Moringa stenopetala* is often referred to as the East African Moringa tree because it is native only to southern Ethiopia and northern Kenya (Mark, 1998). Though it grows in many other parts of the tropics, it is not as widely known as its close relative, *Moringa oleifera* but often considered generally more desirable than *M. oleifera.* Mark (1998) and Edwards et al. (2000) stated that the taxonomic position of the family is not clear. It has some features similar to those of *Brassicaceae* and *Capparidaceae* but the seed structure does not agree with either of the above families. Pollen studies have not provided any other suggestions and recent molecular studies have pointed to relationship with the *Carricaceae.* This indicates that the taxonomic position of the family is not yet settled and is open for further studies. Its seed physiology is also yet studied in the tropics in general and Ethiopia in particular (Eyasu Seifu, 2014).

The habitat where the genus occur in Ethiopia includes: rocky areas along rivers, dry scrub land, Acacia-Commiphora woodland, water courses with some evergreens, open Acacia-Commiphora bush land on gray alluvial soil and in cultivation around village. It is cultivated in terraced fields, gardens and small towns (Edwards et al; 2000; Haile-Gebriel Endeshaw, 2003). It is dominantly found in well-drained soils of southern Ethiopia at an altitudinal range of about 1100-1600 m.a.s.l. with annual rainfall ranging from 500-1400 mm and annual temperature ranging from 24-30°C (Yalemtsehay Mekonnen and Amare Gessesse, 1998).

Due to its water storage capacity in the bottle-shaped stem, it is drought tolerant and remains green when the other forage is scarce during the dry season (Abera Melesse et al. 2008) and due to this character it is called „camel crop". Besides, one of the nicknames of the tree is "never die" due to its incredible ability to survive in harsh climate and drought condition (Singh, 2010). Cold temperatures are limiting factor for the cultivation of the species in Ethiopia because it does not tolerate frost or freeze may cause it to die back to ground level, where new sprouts may be produced. The species does not have any specific soil requirements, except it does not grow on waterlogged or swampy soils. The soil pH ranges from acidic to alkaline but mostly exhibit neutral reaction (Orwa et al., 2009).

Moringa stenopetala has the following features: it is fast growing evergreen perennial flowering plant or deciduous soft tree, well adapted to semi-arid areas with annual rainfall as little as 500 mm and poor soil (Steinmüller et al., 2002). It is more drought tolerant - but less frost-resistant than *M. oleifera* (Mughal et al., 1999). Even though, *Moringa stenopetala* is more resistant to insect pests than other species in its family. It is affected by a caterpillar of Noorda trimaculalis (Demeulenaere, 2001).

M. stenopetala is propagated both by direct sowing of the seeds without pretreatment and vegetatively using branch cuttings (ICRAF, 2006). The optimum temperature for the germination of *M. stenopetala* seeds was reported to be about 25°C (Demel,Teketay, 1995). Optimum light for germination of all *Moringa* species is half shade. When sown in the hotter weather of mid-April, germination percentages for *M. stenopetala* and *M. oleifera* were only 54 and 40 percent, compared to 92 and 94 percent in half shade. Seeds should be planted about 2 cm deep in soil that is moist but not too wet (Martin, 2007).The best suited season for sowing the seeds is March to August under Southern Ethiopian conditions. The time of

sowing has to be strictly adhered to because the flowering phase should not concede with rainy seasons, which results in heavy flower shedding (Kechero Yisehak et al., 2011).

M. stenopetala is one of the world‟s most nutritious crops: All parts of the tree except the wood are edible. The leaf of *Moringa* is very popular vegetable in southern Nation Nationalities and Peoples Regional State of Ethiopia and valued for its special flavor. (Steinmuller et al.,2002). The leaf is rich in carbohydrate, proteins, minerals and essential amino acids. It has more betacarotene than carrots, more protein than peas, more calcium than milk, more potassium than bananas, and more iron than spinach (Abera Melesse et al., 2008).

Many parts of the *M. stenopetala* plant have been used in medicinal preparations traditionally against different disease. The seed can be used for various purposes such as for food, medication, water purification and oil extraction (Berger et al., 1984). Besides, its vital value for human and livestock nutrition, this plant is largely uninvestigated (Bennett et al., 2003; Lalas et al., 2003). Due to this, *M. stenopetala* is not known in most part of the world other than its area of cultivation (Southern Ethiopia and Northern Kenya). Currently, it attracted the attention of scientists across the globe for health management due to its nutritional and medicinal properties as well as easy of propagation and ability to thrive under harsh environments. It has the potential to end malnutrition, starvation, as well as prevent and treat many diseases. It has got a nick name as „truly a miracle plant‟, „mothers‟ best friend‟ and a „God gift‟ for the nourishing and healing of man. Thus, it is emerging as a future crop considering its wider adaptability and tolerance to many abiotic stresses. Accordingly, it became essential to take stock of knowledge and develop a road map to harness its potential for the benefit of farmers as well as consumers (Singh, 2010).Therefore, expansion of cultivation and utilization of this tree throughout the world especially in Africa is important.

However, for such expansion, it needs good propagation system to supply adequate planting materials of superior genotypes.

Moringa species are associated with many production constraints, such as a relatively long period of fruit bearing, non availability of planting materials, lack of alternative method of propagation, requirement for long rainy period in regions where water is scarce and vulnerability to pests and diseases (Haile-Gebriel Endeshaw, 2003; Yitebitu Moges, 2004). Demeulenaere (2001) reported that the pest destroys the leaves of the entire plant population of a village within a week. This pest could be a drawback for in situ conservation of the plant. Moreover, flowering does not commence until a critical tree size is attained, and after some years the tree branches stop producing fruits/seed. Therefore, vegetative propagation like cutting is a necessity to obtain uniformity in yield and quality. However, it is less successful due to its slow regeneration and also requires large size cuttings (1-1.5m long) as well as trees grown from cuttings are known to have much shorter roots or a poor root system (Jahn, 1991). As a result, *in vitro* propagation methods are the best alternative for propagation of this plant with uniform genotypes within a relatively short period of time.

2. OBJECTIVES

2.1. General objective

- To optimize *in vitro* propagation protocol for *Moringa stenopetala*

2.2. Specific objectives

- To evaluate the combined effect of NAA and kinetin on shoot multiplication.
- To evaluate the combined effect of IBA and kinetin on shoot multiplication.
- To evaluate the effect of NAA and IBA on rooting of multiplied shoots.

3. LITERATURE REVIEW

3.1. Taxonomy, origin and distribution of Moringa stenopetala

M. stenopetala belongs to family *Moringaceae* that is represented only by a single genus *Moringa* and 14 different species. In fact, it is stated that the taxonomic position of the family is not clear. It has some morphological similarity to those of *Brassicaceae* and *Capparidaceae* but the seed structure does not agree with either of the above families (Edwards et al., 2000). Pollen studies have not provided any other suggestions and recent molecular studies have pointed a relationship with the *Carricaceae.* These indicate that the taxonomic position of the family is not yet settled and is open for further studies. Besides this, a study made by Olson (2002) on both morphological features such as leaf form, leaf glands, life form, woody anatomy, gum duct (articulated laticifers), flowers and fruit and test anatomy and molecular data on chloroplast genome (cpDNA), gene sequence of ribulose -1-5 bisphosphate carboxylase / oxygenase (rbcL) revealed that *Caricaceae* and *Moringacea*e are sister taxa.

The genus *Moringa* originated from sub-Himalayan tracts of Northern India, distributed worldwide in the tropics and sub-tropics, (Olson, 2002). *M. stenopetala* was cultivated in the east African lowlands and is native to Southern Ethiopia, North Kenya and Eastern Somalia and hence named as African moringa. It is distributed in the rift valley of Southern Ethiopia, especially in Konso, Wollayta, Dirashe, Gamo Gofa, Sidama, Bale and Borana areas (Edwards et al., 2002; Verdcourt, 1985).

It is commonly called Shiferaw in Amharic and Cabbage tree or African *Morniga* in English. In the Southern Ethiopia, it is known by different vernacular names such as Aleko, Aluko, Halako (Gamo Goffa), Kallanko (Benna), Haleko, Shalchada (Konso), Telahu (Tsemay),

Haleko (Derashe), and Halakwa (Wollayta) (Yalemtshay Mekonnen and Amare Gessesse 1998; Simon Shibru 2002).

The habitat where the tree occurs in Ethiopia includes: rocky areas along rivers, dry scrub land, Acacia-Commiphora woodland, water courses with some evergreens, Open Acacia-Commiphora bush land on grey alluvial soil and in cultivation around villages (Edwards et al., 2002; Verdcourt, 1985).

M. stenopetala is dominantly found in well-drained soils of southern Ethiopia at an altitudinal range of about 1100-1600 m.a.s.l. with annual rainfall ranging from 500-1400 mm and annual temperature ranging from 24-30°C (Yalemtsehay Mekonnen and Amare Gessesse, 1998). Due to its water storage capacity in the bottle-shaped stem, it is drought tolerant and remains green when the other forage is scarce during the dry season (Abera Melesse et al. 2008). It is more drought tolerant - but less frost-resistant than *M. oleifera*. Cold temperatures are limiting factor for the cultivation of the species in Ethiopia because it does not tolerate frost or freeze may cause it to die back to ground level, where new sprouts may be produced. The species does not have any specific soil requirements, except it does not grow on waterlogged or swampy soils. The soil pH ranges from acidic to alkaline but mostly exhibit neutral reaction (Mughal et al., 1999, Orwa et al., 2009). Even though, *M. stenopetala* is more resistant to insect pests than other species in its family, it is affected by a caterpillar (Demeulenaere, 2001).

3.2. Botanical Description of *M. stenopetala*

M. stenopetala is a small tree up to 10 m tall. Trunk is up to 100 cm in diameter, swollen, bottle-shaped. Bark is whitish, pale grey, silvery or blackish, smooth. Its crown strongly branched. Leaves are alternate, up to 55 cm long, 2–3-pinnate; with 5 pairs of pinnae; stipules

absent, but it contains petiole. Inflorescence is a dense, many-flowered panicle up to 60 cm long. Flowers are bisexual and regular with long hairs inside, white, pale yellow or yellow-green color. Sepals are free, creamy-pink color, 4–7 mm long. Petals are free, white or yellow color, oblong (circle) to linear-oblong, 8–10 mm long. White stamens contain yellow anthers and filaments that measures 4–6.5 mm long. Ovary is superior, stalked, ovoid, and 2 mm long, densely hairy. Style is narrowly cylindrical, without stigmatic lobes. Fruits are an elongate 20–50 cm long, grooved, twisted when young, later straight, and reddish with grayish bloom. Seeds elliptical-trigonous, 2.5–3.5 cm × 1.5–2 cm, with 3 thin wings 6–9 cm long. Its pods are elongated, reddish with grayish blooms and twisted when the fruit is fresh it has a white flower (Edwards et al., 2002; Bosch, 2004).

3.3. Use of Moringa stenopetala

M. stenopetala is a multipurpose tree that is cultivated as agro-forestry in Southern Ethiopia. The plant is used as a living hedges and wind breaks to reduce the rate of erosion, vital nutritional, industrial, and medicinal applications. It is grown as a vegetable tree and medicinal plant in Southern Ethiopia and Njemb tribe, living in Kenya (Berger et al., 1984; Jahn, 1991). The entire plant parts, except the wood, are edible and provide large quantity of inorganic and organic nutrient material essential for maintenance of health care system of human and also other animals. Leaves are especially the most important part cooked and mixed with flour of cereal crops that would fulfill the nutritional requirement that a given complete diet should have since it contains significant amount of protein, carbohydrate, vitamins (A, B, C) and minerals (calcium, potassium, iron, phosphors, zinc) and essential amino acids that can be important in dry seasons in areas where there is lack of other vegetables (Abuye et al., 2003). During the dry season the average consumption of leaves by

adults in southern Ethiopia is 150 g/day, corresponding with 19% of the energy and 30% of the protein requirement (Steinmuller et al., 2002). It has more betacarotene than carrots, more protein than peas, more calcium than milk, more potassium than bananas, and more iron than spinach (Abera Melesse et al., 2008). The raw leaves contain (per 100 g dry matter) 1235 kJ (295 kcal) energy, 9.0 g protein, 5.8 g fat, 51.8 g carbohydrate, 20.8 g crude fibre, 793 mg Ca, 65.6 mg P, 0.53 mg Zn, 31 IU vitamin A and 28 mg ascorbic acid. Over 5 million people consume the leaf as a vegetable (Abuye et al., 2003). Reports indicated that given the high vitamin content, *M. stenopetala* leaves could be used to reduce child and maternal mortality rates in the country by 30-50% (Anon, 2003).

M. stenopetala has many other uses. The Turkana people of northern Kenya make an infusion of the leaves, which is used as a remedy against leprosy. The Njemp people in Kenya chew the bark as a treatment against coughs, and use it to make fortifying soups. In the Konso area of Ethiopia, the smoke from burning roots is used as a treatment for epilepsy. In the Negelle and Wollayta Sodo areas (Ethiopia) the leaves and roots are used as a cure for malaria, stomach problems and diabetes. The leaves are also used to treat hypertension, retained placenta, asthma, colds, as an anthelmintic, to induce vomiting and to promote wound healing (Yalemtsehay Mekonnen et al., 1999).

The seeds of *M. stenopetala* have natural flocculating and antimicrobial properties (Jahn, 1991). Dried and crushed seeds of the plant are important to clarify muddy and turbid water, to suit it for drinking analogous to the chemical coagulant Aluminum sulphate (alum) (Göttsch, 1984; Eilert et al., 1981 and Aschalew Hundie and Adinew Abebe, 1991). The active coagulating substances are found in the cotyledons of the seeds (ICRAF, 2006). Eilert et al. (1981) and Aschalew Hundie and Adinew Abebe (1991) have also further proved that it

lowers the concentration of bacteria (it has bactericidal effect) and fungi (it has fungicidal effect). Eilert et al. (1981) have also reported that water purification and its bactericidal and fungicidal effect over weigh that of *M. oleifera*. Therefore, water purified using crushed seeds of *M. oleifera* and *M. stenopetala* is safe for human consumption due to its non toxic effect and efficient coagulant (Berger et al., 1984). Besides, *M. stenopetala* seed powders have bioadsorbents properties. It could be used to remove heavy metals from water and industrial wastes (Mataka et al., 2006, Mataka et al. 2010, Shetie Gatew and Wassie Mersha, 2013). The seed of *M. stenopetala* is an important source of oil that could be used for cooking or for different industrial applications. The oil contains 78% mono-unsaturated fatty acid and 22% saturated fatty acid. Oleic acid is the dominant fatty acid and accounts for about 76% (Eyasu Mekete, 2008). Andinet Ejigu (2010) study showed that *M. stenopetala* seed oil could be used as a potential feedstock for biodiesel production. A research finding also indicated that *M. stenopetala* seed cake powder can be used for biogas production (Eyasu Mekete, 2008).

In addition to the seeds, the roots of *M. stenopetala* can also used to clarify dirty water. Nomadic peoples in the Omo Valley of Ethiopia apparently use the roots of wild *M. stenopetala* to clarify muddy water (Demeulenaere, 2001). The root is also used in traditional medicine to treat different aliments. Unlike *M. oleifera*, the *M. stenopetala* tree has soft wood and is not suitable for fuel but can be used for pulp production. In general, the plant has vast potential to benefit the local people and the country economy.

3.4. Propagation of *M. stenopetala*

M. stenopetala is propagated both by direct sowing of the seeds without pretreatment and vegetatively using branch cuttings (ICRAF, 2006). The optimum temperature for the germination of *M. stenopetala* seeds was reported to be about 25°C (Demel Teketay, 1995).

Optimum light for germination of all *Moringa* species is half shade. When sown in the hotter weather of mid-April, germination percentages for *M. stenopetala* and *M. oleifera* were only 54 and 40 percent, compared to 92 and 94 percent in half shade. Seeds should be planted about 2 cm deep in soil that is moist but not too wet (Martin, 2007).

Both *M. oleifera* and *M. stenopetala* can be started from cuttings. Cuttings of 45-100 cm long with stems 4-10 cm wide should be taken from the woody parts of the branches. Cuttings can be cured for three days in the shade and then planted in a nursery or in the field. However, trees grown from cuttings are known to have much shorter roots or a poor root system. Where longer roots are an advantage for stabilization or access to water, seedlings are clearly preferable (Martin, 2007). The most common practice in traditional cultivation is to transplant seedlings that have become established under old trees. Before transplanting, branches and roots are cut off and the seedlings are left to dry for a week, roots are covered with ash and upper parts with dung (Bosch, 2004).

3.4.1. *In vitro* propagation

Plant micropropagation is an integrated process in which cells, tissues or organs of selected plants are isolated, surface sterilized, and incubated in a growth-promoting aseptic environment to produce many clone plantlets (Altman, 2000). Micropropagation can also be described as the process of mass propagation of selected plants via *in vitro* techniques. *In vitro* propagation for mass production refers to the process of asexual reproduction by multiplication of genetically identical copies of individual plants (Omar and Aouine, 2007).

The micropropagation process can be divided in to five different stages:

Stage 0: Selection of mother plants.

Stage I: Initiation of culture. It involves the disinfestations and the cultivation under aseptic conditions.

Stage II: Rapid multiplication of numerous propagules (multiplication phase). Masses of tissues are repeatedly sub-cultured under aseptic conditions onto new culturing media that encourage propagule proliferation.

Stage III: Elongation and root induction or development (rooting phase). This phase is designed to induce the establishment of fully developed plantlets.

Stage IV: Transfer to ex vitro condition (acclimatization). Acclimatization is defined as the climatic or environmental adaptation of an organism, especially a plant that has been moved to a new environment (Zobayed et al., 2000).

In vitro propagation techniques are preferred over the conventional asexual propagation methods because of (a) a small amount of tissue is required to regenerate millions of clones of plants in a year. (b) rapid production of superior propagules in large scale (c) it produces disease free plantlets (d) it is season independent (e) for multiplication of sexually derived sterile hybrids (g) for long term storage of valuable germplasm (Neumann et al., 2009; Khanna, 2003).

Sexual propagation of some of *Moringa* species would be tedious and would not even be possible without having enough individual plants for cross-pollination. Since flowering of a number of the large tree species of *Moringa* does not even commence until a critical size is attained. Besides this, there is only one or a few *Moringa* species (*M. oleifera*) cultivated in the wide area, most of the other species of *Moringa* are rare including *M. stenopetala* which grow in specific places of the world. Multiplication of these rare species by tissue culture propagation would make them more widely available and less likely to become lost to

cultivation. Thus, developing tissue culture methods for this genus is extremely important (Stephenson and Fahey, 2004).

In vitro establishment of woody plants in general and tropical trees in particular is often difficult. The most frequently encountered problems are culture contamination by endogenous bacteria and browning of explants (Machado et al., 1991). Besides the age of the tree, the response of explants is primarily determined by genotype, physiological state of the tissue, and time of the year when the explants are collected and cultured. Only a few tree species with explants from mature trees have been propagated by tissue culture methods. In general juvenile tissues from forest trees are more responsive to *in vitro* manipulation than that of mature tissues. In general, success of *in vitro* regeneration depends on the control of morphogenesis, which is influenced by several factors namely genetic background, kinds of tissue or explants, nutritional components, growth regulators and culture environment (Anjaneyulu et al., 2003).

In vitro propagation of *M. oleifera* is practiced by using explants such as axillary bud (Marfori, 2010)**,** nodal explants (Stephenson and Fahey, 2004), cotyledons (Nieves and Aspuria, 2011), shoot tip (Islam et al, 2005; Marfori, 2010) and other parts. Generally, shoot tips and nodal segments of either juvenile or adult origin of current year growth are commonly used as explants for *M. oleifera* micropropagation. The works done in this area indicate that most of explant source is from juvenile (laboratory grown seedlings) origin (Stephenson and Fahey, 2004; Marfori, 2010; Saini, 2012). For example, stem segments from 10 days old seedling, as explant gave 100% regeneration of plantlets with profuse rooting in *M. oleifera* (Marfori, 2010).

Steintz et al. (2009) successfully propagated *M. oleifera* Lamk. *M. stenopetala* (Baker F.) Cufod. and *Moringa peregrina Forssk*. using basal Murashige and Skoog (MS) medium in the absence of plant growth regulators by following 3 step procedure; 1) multiple shoot regeneration from cotyledonary node from decapitated seedlings, 2) axillary shoot growth from single node shoot segments and 3) rooting of excised shoots.

3.4.2. Factors affecting in vitro propagation

The most important factors in optimizing the tissue culture protocol includes media, types of explants, source of explants, genotype, mineral nutrition, growth regulators, carbon source, gelling agent and physical environment (humidity, light, pH, temperature). The most widely used culture medium is Murashige and Skoog (1962) (MS medium), because most of the plants respond favorably to MS medium, since it contains all the nutrients essential for plant growth *in vitro*. Selection, strength and combination of media are also one of important parameters for optimizing the regeneration protocol (Khan et al., 1988; Zukar et al., 1997; Prakash and Gurumurtthi, 2005). It is classified as a high salt medium in comparison to many other formulations, with high levels of nitrogen, potassium and some of the micronutrients, particularly boron and manganese (Cohen, 1995).

Growth and morphogenesis *in vitro* are regulated by the interaction and balance between the growth regulators supplied in the medium, and the growth substances produced endogenously (George, 1993). A balance between auxin and cytokinin is most often required for the formation of adventitious shoots and roots. The balance of growth regulators depends on the objective of the cultivation *in vitro* (as e.g. shoot, root, callus or suspension culture) and on the micropropagation phase considered (initiation, multiplication or rooting). In the multiplication phase, the level of cytokinins should be normally higher than of auxins. In the

rooting phase, in turn, the use of cytokinin is not necessary and higher levels of auxins can be supplemented to the culture medium (Taiz and Zeiger, 1991).

The most common cytokinins used are kinetin, 6- Benzylamino purine(BAP), Thidiazuron (TDZ) and Isopentenyl-adenine (2iP) (Pierik, 1997). One of the main functions of exogenous cytokinins in tissue culture is induction of adventitious shoots. They are also used to release axillary buds from apical dominance thus initiating shoot proliferation. Wareing and Phillips (1981) showed that BAP was more active than other cytokinins in shoot proliferation and it is also the only one that can be autoclaved. So in commercial micropropagation establishments, where lowering costs and ease of handling are major consideration, BAP is the most suitable cytokinin. It is most frequently used in *Moringa in vitro* propagation and it gave best shoot multiplication result. It was the effective cytokinin for shoot induction as well as shoot proliferation in *M. oleifera* (Islam et al., 2005). In general, cytokinin regulates cell division, stimulate axillary and adventitious shoot proliferation, regulate differentiation, and inhibit root formation.

Auxins (Indole acetic acid (IAA), Indole-3- butyric acid (IBA), α -naphthalene acetic acid(NAA) or2, 4-Dichlorophenoxyacetic acid (2, 4-D) are involved in the regulation of several physiological processes, as e.g. apical dominance and formation of lateral and adventitious roots. This growth regulator generally causes cell elongation and swelling of tissues, cell division (callus formation) and the formation of adventitious roots as well as the inhibition of adventitious and axillary shoot formation (Pierik, 1997). *In vitro* rooting of shoot mostly depends upon concentration and duration of auxin treatment and salt strength of basal medium or physical condition of cultures. Dilute media formulations has generally promoted better formation of roots, since high concentration of salts may inhibit root growth, even in

presence of auxins in the culture medium Grattapaglia and Machado,(1998) cited in Kumar and Reddy (2011). Micro shoots were cultured in ½ MS basal media with different types and concentrations of auxins to optimize the rooting of micro shoots of *Moringa* species specially *M. oleifera.* Maene and Debergh (1985) also reported the merits of reducing the concentration of macro and micronutrients to half of their normal concentrations during the rooting phase of most herbaceous plant species.

Type of explants like leaf, petiole, cotyledonary leaf, hypocotyle, epicotyle, embryo, internode and root explants significantly affect tissue culture process of plants (Khan et al., 1988; Sujatha and Mukta, 1996; Tyagi et al., 2001). This may be due to the different level of endogenous plant hormones present in the plants parts.

In vitro explant in general has better potential of organogenesis as compared to in vivo explant (Reddy et al., 2008). 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 (Teng, 1999; Tileye Feyissa et al., 2005) due to different level of plant hormones present in the plants.

4. MATRIALS AND METHODS

4.1. Source of explant and surface disinfection

Matured seeds of *M. stenopetala* were collected from Merab Abaya , Arba Minch area, in the Southern Nation Nationalities and peoples Regional State of Ethiopia during November and December of 2015. Fruits were cut open and the seeds were separated from pods and washed with local detergent (omo) for 10 min and rinsed in running tap water for five minutes then thoroughly washed and rinsed again in sterilized distilled water for about 24 hours to speed up seed germination.

Seeds were surface sterilized with 10 % NaOCl solution for 25 min followed by five washings with sterile distilled water and sown in culture jars containing 50 ml plant growth regulators-free MS (Muragshige and Skoog, 1962) medium under aseptic conditions. The MS medium was enriched with 30 g/l sucrose (w/v), 8 g/l agar (w/v), pH was adjusted to 5.8 and autoclaved at 121ºC with a pressure of 105 KPa for 15 min. The cultures were maintained in growth room under light intensity of 16 hour photoperiod provided by cool-white fluorescent lamps at a temperature of 25±2 ºC.

4.2. Preparation of Stock Solution and Culture Media

4.2.1. MS Stock Solution Preparation

The stock solution of macronutrients, micronutrients and vitamins were prepared separately by weighing the recommended amount of powder by dissolving in distilled water and stored them in refrigerator at a temperature of +4°C until used. The prepared stock solutions were stored at +4°C for a maximum of one month.

4.2.2. Growth regulators stock solution preparation

In this study, different plant growth regulators namely cytokinins (kinetin) and two auxins (IBA, NAA) were used with different concentrations and combinations. Each of these growth regulator stock solutions was prepared by weighing and dissolving the powder in distilled water at a concentration of 1.0 mg/ml. To begin the dissolving process 3-4 drops of 1M NaOH or 1M HCl were added based on the requirement of the growth regulators (NaOH for auxin, HCl for cytokinin). Then, the volume was adjusted by adding distilled water. Finally, growth regulators stock solutions were stored in a refrigerator at a temperature of +4°C until used.

4.2.3. Culture Media Preparation

Culture media were prepared by mixing the proper amount of MS stock solutions (50ml/L macro, 5 ml/L micro, 5 ml/L iron EDTA and 5 ml/L vitamin) then 30 g/l sucrose was added to the solution as energy and carbon source. After mixing all the components and adjusting the volume, growth regulators were added as required and pH was adjusted to 5.8 using 1N NaOH or 1N HCl. Finally, 8.0 g/l agar was added. After melting the agar by using hot plate, 50 ml medium was dispensed into each Magenta GA-7 culture vessel. The medium was sterilized by autoclaving at a temperature of 121°C at a pressure of 105 KPa for 15 min. Finally, the medium was allowed to cool in the laminar air flow cabinet.

The media compositions that were used for different experiments were as follows:

Media for shoot Multiplication

- MS + IBA and Kinetin + 30g/L sucrose + 8g/L agar
- MS + Kinetin + NAA + 30g/L sucrose + 8g/L agar

Media for Rooting

- 1/2 MS + IBA + 30g/L sucrose + 4g/L agar
- 1/2MS +NAA+30g/L sucrose + 4g/L agar
- 1/2MS +IBA+ NAA+30g/L sucrose + 4g/L agar

4.3. Culture Conditions

All types of cultures were kept in a growth room at a temperature of 25±2°C and under light intensity of 16 hour photoperiod provided by cool-white fluorescent lamps.

4.4. Shoot initiation

Shoot tips obtained from *in vitro* germinated seedlings were used for culture initiation. Shoot tips from *in vitro* germinated seedlings were excised and cultured on MS medium supplemented with BAP (0.5, 1.0, 1.5, 2.0, 2.5 mg/l) alone and in combination with NAA (0.1, 0.5 and 1.0 mg/l). The medium was supplemented with 30 g/l sucrose (w/v) and the pH was adjusted to 5.8 before addition of 8 g/l agar (w/v). Six shoots per Magenta culture vessel was considered as a unit of replication and there were five replications for each treatment. The cultures were maintained in culture room under light intensity of 16 hour photoperiod provided by cool-white fluorescent lamps at a temperature of 25±2 ℃.

4.5. Shoot Multiplication

The *in vitro* initiated shoots were cultured on MS medium supplemented with different concentrations of kinetin (0.0, 0.5, 1.0, 1.5, 2.0, 2.5 mg/l) in combination with IBA (0.0, 0.01, 0.1, 0.5) or kinetin (0.0, 0.5, 1.0, 1.5, 2.0, 2.5 mg/l) in combination with NAA (0.01, 0.1, 0.5 mg/l) to determine their effect on multiple axillary shoot formation. Growth regulators free MS basal medium was used as a control. Six shoots per Magenta culture vessel was considered as a unit of replication and there were five replications for each treatment. The cultures were maintained at 25°C± 2°C with 16 h photoperiod and subcultured every four

weeks. Shoot length, number of leaves and number of shoots per explant were recorded after four weeks.

4.6. Rooting

Micro shoots obtained from shoot multiplication medium were transferred to rooting medium. The rooting medium was half strength MS medium containing different concentrations of NAA and IBA. Six shoots were cultured in each culture vessel and a total of five replications were designed for each treatment. The cultures were maintained in growth room under the same condition as of shoot multiplication. Number of roots per shoot and root length were recorded after 4 weeks.

4.7. Acclimatization

Plantlets with well developed shoots and roots were taken out of the culture jars and the roots were washed thoroughly with running tap water and transferred into pots containing autoclaved (sterilized) sand, red soil and compost at a ratio of 2:1:1 respectively. The plantlets were covered with transparent plastic bag to maintain moisture for two weeks and placed them in the shade region of tissue culture room prior to their transfer to the greenhouse condition and watered within two days interval (morning and evening).The plastic cover was gradually removed after one month and the plantlets were successfully established in greenhouse.

4.8. Data Analyses

After four weeks of transferring explants into multiplication media, number of shoots per explant, mean shoot length and number of leaves were recorded. After four weeks of transferring well developed shoots into rooting media, number of roots per shoot, and mean root length were recorded. All data were subjected to analysis of variance (ANOVA) to

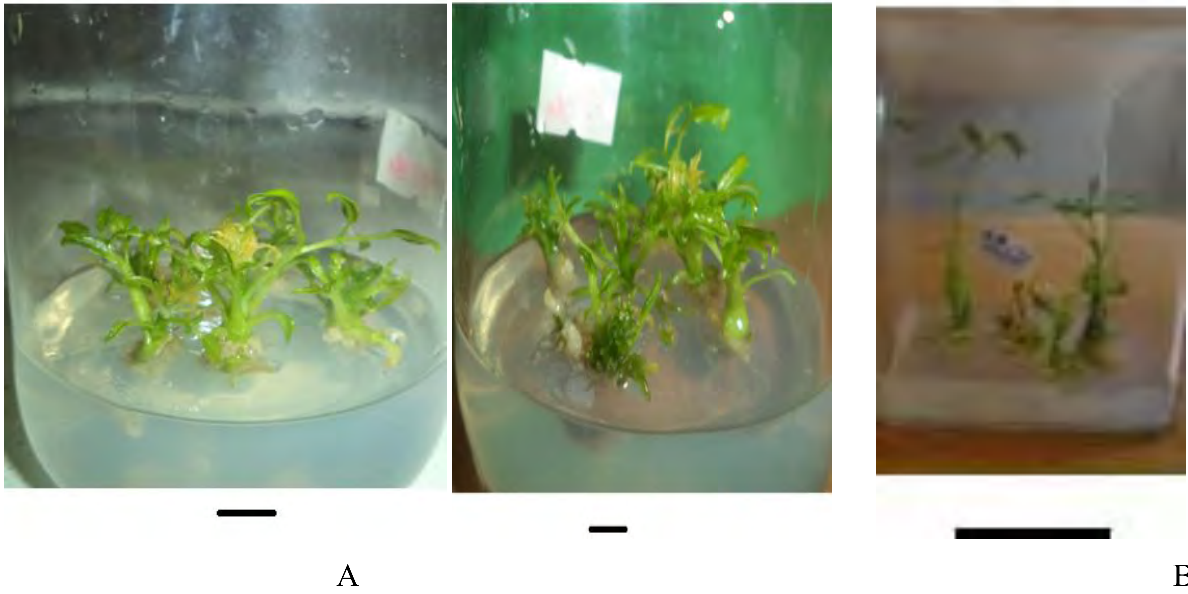
quantify the differences between applied treatments. Treatment means were separated using the least significance differences (LSD) at probability level of $p \leq 0.05$. ANOVA table was constructed using SPSS computer software of version 20 and Tukey‟s multiple range test was used.

5. RESULTS

5.1. Shoot Multiplication

The effect of NAA along with kinetin on shoot multiplication after four weeks of culture was highly significant ($P \leq 0.05$). The results of the present study showed that using NAA in combination with kinetin for shoot multiplication was better than kinetin in combination with IBA.

In all treatments, there were differences in the rate of shoot multiplication. The mean number of shoots per explant range from 1.00±0.48 to 3.43±1.41 (Table 1; Table 2; Fig.1). Among all the treatments, 0.5 mg/l kinetin in combination with 0.01 mg/l NAA resulted in the highest number of shoots per explant (3.43 ± 1.41) (Table 2). The MS medium supplemented with 0.5 mg/l kinetin combined with 0.1 mg/l NAA , 0.5 mg/l kinetin combined with 0.1 mg/IBA and 0.5 mg/l kinetin combined with 0.5 mg/l NAA gave the second (2.47±1.36) and the third (2.30±1.90), (2.33±1.85) maximum mean shoot number respectively. Shoot explants cultured on MS medium supplemented with 2.5 mg/l kinetin in combination with 0.5 mg/l NAA and 2.5 mg/l kinetin in combination with 0.5 mg/l IBA produced the lowest mean number of shoots per explant, 1.00±0.48 and 1.00±0.54 respectively. Shoot length was significantly higher on medium without any plant growth regulator (control) as compared to media containing IBA and Kinetin and NAA and kinetin but it produced the lowest mean number of shoots per explant (1.2±1.00). The maximum shoot length (1.10±0.97 cm) and number of leaves (9.33±10.56) were recorded on MS medium supplemented with 0.5 mg/l Kinetin combined with 0.01 mg/l IBA and 1.0 mg/l kinetin combined with 0.01 mg/l NAA respectively. With increasing the concentrations of IBA and NAA, the number of shoots per explant decreased.



A B

C

Figure 1.The effect of different growth regulators on shoot multiplication after four weeks of culture;

A) 0.5 mg/l kinetin + 0.01 mg/l NAA, B) 0.5 mg/l Kinetin + 0.1 mg/l IBA, C) PGR free. Bar =2cm

Table 1. The effect of different combination of kinetin and IBA on shoot multiplication

KN(mg/l)	IBA(mg/l)	Shoot no. per explant	leaf no. per explant	Shoot length (cm)
0.0	0.0	1.20^{bc}±1.00	2.33^{def}±1.15	2.67^{a}±1.13
0.5	0.01	2.27^{a}±1.62	6.60^{bcd}±8.30	1.10^{b}±0.97
0.5	0.1	2.30^{a}±1.90	7.73^{a}±7.57	1.08^{b}±1.07
0.5	0.5	2.03^{bc}±1.40	5.77^{bcde}±6.15	0.61^{bc}±0.31
1.0	0.01	1.83^{bc}±1.34	5.83^{bcde}±7.01	1.01^{bc}±0.72
1.0	0.1	2.20^{a}±1.21	6.77^{bc}±4.83	0.90^{de}±0.55
1.0	0.5	2.20^{a}±1.73	7.40^{a}±7.14	0.93^{bc}±0.98
1.5	0.01	2.03^{bc}±1.50	7.47^{a}±6.50	0.99^{bc}±0.84
1.5	0.1	1.23^{bc}±.94	3.83^{bcdef}±4.31	0.82^{bc}±0.67
1.5	0.5	1.50^{bc}±.94	2.15^{def}±1.45	0.89^{bc}±0.88
2.0	0.01	2.20^{a}±3.39	2.50^{cdef}±2.08	0.66^{bc}±0.36
2.0	0.1	1.23^{bc}±1.14	2.77^{cdef}±3.90	0.60^{bc}±0.43
2.0	0.5	1.67^{bc}±1.30	3.40^{bcdef}±3.77	0.71^{bc}±0.40
2.5	0.01	1.13^{bc}±.94	2.57^{cdef}±2.27	0.53^{bc}±0.40
2.5	0.1	1.00^{bc}±.64	1.53^{ef}±1.78	0.57^{bc}±0.46
2.5	0.5	1.00^{c}±.54	1.00^{f}±1.15	0.44^{c}±0.42
Over all means		1.70^{B}±1.58	4.49^{A}±5.55	0.79^{C}±0.70

Numbers within the same column with different letter(s) are significantly different from each other according to Tukey‟s multiple range test at P≤ 0.05. The upper case letters indicate the overall means. The values represent mean ± standard deviation.

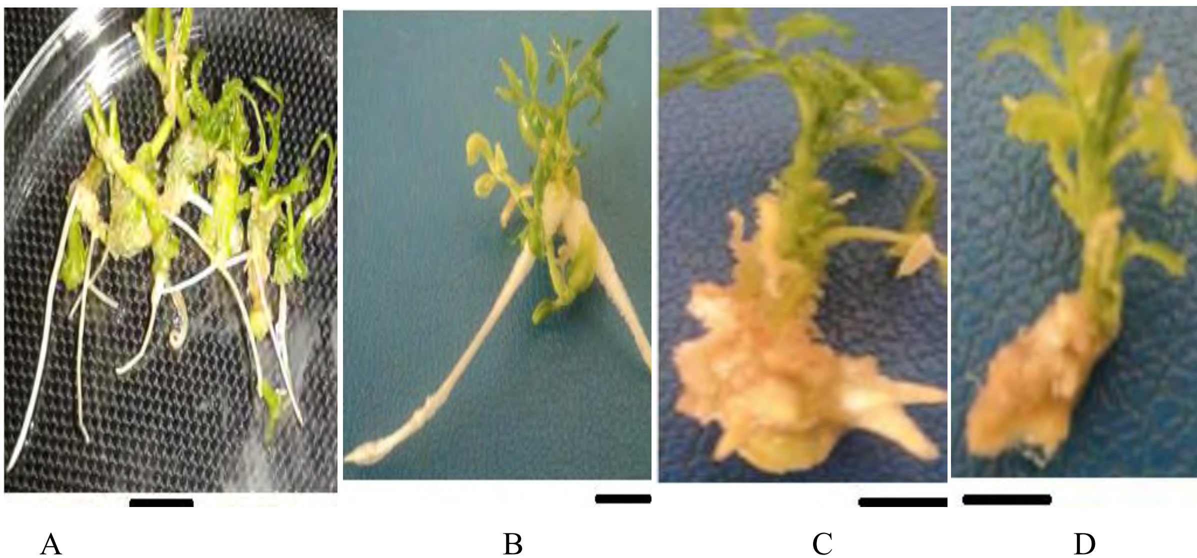
Table 2. Effect of different combination of kinetin and NAA on shoot multiplication

KN(mg/l)	NAA (mg/l)	Shoot no. per explant	Leaf no. per explant	Shoot length (cm) (no. ±SD)
0	0	1.20^{efg}±1.00	2.33^{f}±1.15	2.67^{a}±1.14
0.5	0.01	3.43^{a}±1.41	7.97^{bc}±4.18	0.68^{b}±0.26
0.5	0.1	2.47^{bc}±1.36	5.33^{bcde}±3.65	0.68^{b}±0.24
0.5	0.5	2.33^{cd}±1.85	7.20^{bcd}±7.44	0.50^{bc}±0.28
1.0	0.01	2.20^{cde}±1.81	9.33^{a}±10.56	0.59^{bc}±0.39
1.0	0.1	1.20^{efg}±.96	3.10^{df}±3.28	0.36^{c}±0.22
1.0	0.5	1.23^{efg}±1.04	3.67^{cde}±5.06	0.57^{bc}±0.40
1.5	0.01	1.57^{cdefg}±1.28	4.23^{cde}±6.63	0.46^{bc}±0.28
1.5	0.1	1.97^{cdef}±1.50	5.10^{bcde}±6.58	0.47^{bc}±0.20
1.5	0.5	1.43^{defg}±1.31	2.23^{f}±3.10	0.48^{bc}±0.40
2.0	0.01	1.23^{efg}±.73	1.90^{f}±1.03	0.50^{bc}±0.21
2.0	0.1	1.10^{fg}±0.96	2.40^{f}±4.42	0.47^{bc}±0.28
2.0	0.5	1.10^{fg}±0.183	2.57^{f}±2.08	0.53^{bc}±0.22
2.5	0.01	1.00f^{g}±0.31	1.63^{f}±1.03	0.55^{bc}±0.28
2.5	0.1	1.00f^{g}±0.38	1.53^{f}± .94	0.47^{bc}±0.27
2.5	0.5	1.00^{fg}±0.48	1.23^{o}±1.19	0.36^{c}±0.29
Overall means		1.57^{B}±1.36	3.96^{A}±5.40	0.51^{C}±0.30

Numbers within the same column with different letter(s) are significantly different from each other according to Tukey‟s multiple range test at P≤ 0.05. The upper case letters indicate the overall means. The values represent mean ± standard deviation.

5.2. Rooting

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 mean root number per shoot (1.63±1.03) and mean root length (0.87±1.22 cm) were obtained on medium containing 1.0 mg/l NAA and 0.1 mg/l IBA, respectively (Table 3). The second (1.50±0.38) and third (1.23) highest mean root number were obtained on medium containing 1.0 mg/l IBA alone and 0.5 mg/l IBA and NAA with 0.67±0.28 cm and 0.52±0.51 cm mean root length respectively. The lowest mean number of roots per explant was produced on growth regulator free medium (0.26±0.57), 2.0 mg/l NAA (0.27±0.45), and 2.0 mg/l NAA along with 0.5 mg/l IBA (0.27±0.58).



A B C D

Figure 2. The effect of NAA and IBA on rooting of micro shoots after four weeks of culture;

A) 1.0 mg/l NAA, B) 1.0 mg/l IBA, C) 1.0 mg/l NAA in combination with 0.5 mg/l IBA, D) PGR free medium. Bar = 2 cm

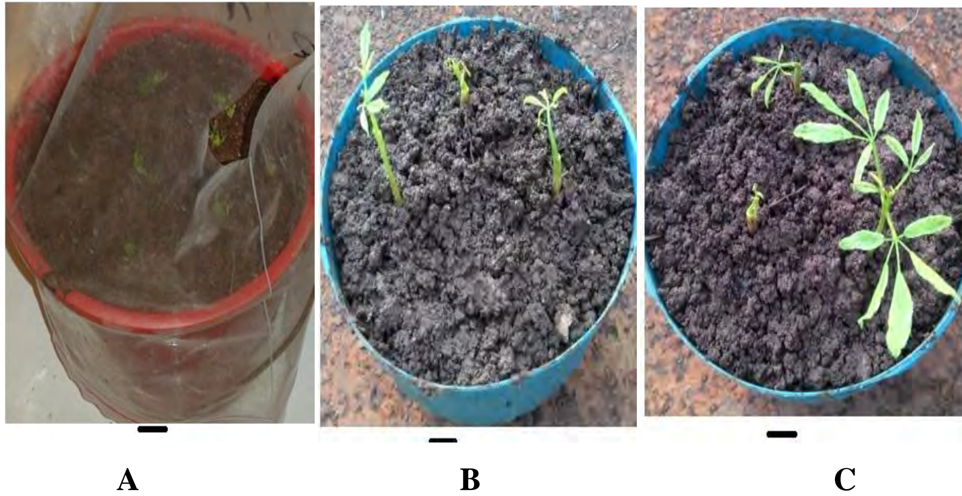
Table 3. Effect of NAA and IBA on rooting of micro shoots

NAA (mg/l)	IBA (mg/l)	Root no. per explant	Root length (cm)
0	0	0.26^{bc}±0.57	0.12^{bc}±0.21
0	0.1	1.03^{c}±1.47	0.87^{a}±1.22
0	0.5	1.23^{b}±0.70	0.43^{b}±0.43
0	1.0	1.50^{a}±0.38	0.67^{c}±0.28
0	1.5	0.13^{bc}±0.35	0.15^{bc}±0.48
0	2.0	0.07^{bc}±0.25	0.05^{bc}±0.20
0.1	0	1.07^{bcd}±1.86	0.34^{cd}±0.55
0.5	0	1.23^{bc}±1.19	0.52^{bc}±0.51
1.0	0	1.63^{a}±1.03	0.84^{a}±0.54
1.5	0	0.67^{cd}±0.84	0.23^{cd}±0.28
2.0	0	0.27^{d}±0.45	0.14^{d}±0.24
0.1	0.5	0.50^{bc}±0.97	0.13^{c}±0 .21
0.5	0.5	0.67^{bc}±1.16	0.24^{bc}± 0.37
1.0	0.5	1.20^{a}±1.16	0.43^{a}± 0.40
1.5	0.5	1.03^{a}±1.13	0.41^{a}± 0.46
2.0	0.5	0.27^{f}±0.58	0.09^{c}±0 .19

Numbers within the same column with different letter(s) are significantly different from each other according to Tukey"s test at $P \leq 0.05$. The values represent mean ± standard deviation.

5.3. Acclimatization

Among 50 plantlets acclimatized in the green house, 38 (76%) survived.



A B C

Figure 3. Acclimatization; A) During transplanting, B) After three weeks, D) After four weeks. Bar = 2 cm

6. DISCUSSION

6.1. Effect of kinetin, IBA and NAA on shoot multiplication

In vitro propagation of *M. oleifera* was investigated well, but for M. stenopetala there are few available reports. Therefore, in the present study efforts have been made to optimize *in vitro* propagation protocol for *M. stenopetala*. Surface sterilization of seeds with 10% NaOCl solution for 25 minute was effective to reduce microbial contaminants. In this study, there was 100% initiation of shoot after four week of culture of the shoot tip in shoot initiation media supplemented with BAP alone. 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*.

In this study the effect of cytokinin in combination with auxin were compared. Application of 0.5 mg/l kinetin along with 0.01 mg/l NAA resulted in the maximum mean numbers of shoots (3.43±1.41) with 7.97±4.18 mean number of leaf and 0.67±0.26 cm mean shoot length per explant. In addition, there was continuous decrease in the number of shoots when the concentration of NAA increased from 0.01 to 0.1 mg/l combined with kinetin. Moreover, a high cytokinin to auxin ratio favours shoot formation.

Induction of multiple shoots in *Moringa concanensis* has been previously characterized with different growth regulators. Fatima et al. (2016) on nodal explants of *Moringa concanensis* obtained 11.00 ± 1.15 mean shoot number with 5.00±1.95cm mean length on MS medium supplemented with 0.1 mg/l kinetin along with 0.05 mg/l NAA. The finding of these authors is contrasting to the present one. In the present study, the maximum mean number of shoots per explant (3.43±1.41) with mean shoot length of 0.68±0.26 cm was recorded at the concentration of 0.5 mg/l kinetin along with 0.01mg/l NAA. Even though, the result reported

by this author is greater than the present study, the trend was similar. In both studies relatively at low concentration of auxin (NAA) and high concentration of cytokinin (kin) best result was obtained. The difference is due to the genotype difference, source of explants, and difference in concentration of cytokinin (kinetin) and auxin (NAA). In addition, the performance of kinetin in shoot length is further supported by sister family (*Brassicaceae)* of *Moringa*. The study in *Matthiola incana (Brassicaceae*) showed that MS medium supplemented with 2.0 mg/l kinetin without NAA resulted in the best shoot length (1.20 cm) (Kaviani et al., 2013). In the present study the medium containing 2.0 mg/l kinetin along with 0.01 NAA produced 1.23±0.73 mean number of shoot with 0.50±0.21 cm mean shoot length. Even if the concentration of kinetin is the same in both studies, the length of shoot reported by these authors is greater than the present study. This is may be due to the genotype difference of the explants, source of explants and the authors used kinetin alone.

Shoot explants cultured on MS medium supplemented with 0.5 mg/l kinetin in combination with 0.1 mg/l IBA exhibited both the maximum number of mean shoot number (2.30±1. 90) and mean number of leaves (7.73±7.57) per explant. The result from combined effect of IBA and kinetin was found to vary with the concentration of IBA and kinetin in all three parameters of shoot growth (mean number of shoot, number of leaf and shoot length). When the concentration of kinetin was greater than that of IBA, relatively better result was recorded. This is due to the effect of cytokinin as it promotes the axilliary branching or axilliary bud proliferation (Vieitez and Vieitez, 1980). Although both auxin and cytokinin are usually required for growth or morphogenesis, auxin inhibits cytokinin accumulation (Hansen et al., 1985) while cytokinin can inhibit at least some of the action of auxin. There was continuous

decrease in number of shoots when concentration of IBA increased from 0.01 to 0.5 mg/l combined with kinetin.

The finding of Alkhateeb (2013) on *Moringa peregrina* (Forsk) showed that MS medium supplemented with 1.0 mg/l kin resulted in 2.6 mean numbers of shoots per explant. 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. The finding of this author agrees with the present study except the result obtained in hormone free medium. In the present study, the MS medium supplemented with 0.5 mg/l kin along with 0.1 mg/l IBA resulted in both maximum number of shoot (2.30±1.90) and mean number of leaves (7.73±7.57). In addition, when the concentration of kin raised from 0.5 mg/l to 2.5 mg/l along with increasing IBA concentration from 0.01 mg/l to 0.5 mg/l lower number of shoot per explant (1.0±0.54) with 0.44±0.42 cm shoot length were obtained. The explants on MS medium without growth regulators (as a control) were elongated compared to MS medium supplemented with growth regulator hormones. This finding is in agreement with the finding of Hesar (2011), who got simultaneous increase number of nodes from 3.61 to 4.64 when the concentration of kinetin increased from 0.5 to 2.0 mg/l in *Matthiola incana (Brassicaceae)*. In conclusion, type and concentration of growth regulators and species (genotype) are the most important factors in *in vitro* shoot multiplication.

In general, the combined effect of kinetin and NAA exhibited more effect as compared to the combined effect of IBA and kinetin.

6.2. The effect of NAA and IBA on rooting

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 (1.63±1.03) and mean root length (0.87±1.22 cm) were obtained at 1.0 mg/l NAA and 0.1 mg/l IBA respectively.

Furthermore, increasing NAA from 0.0 mg/l to 1.0 mg/l by keeping IBA concentration to 0.0 mg/l showed a significant increase in the mean number of roots per shoot from 0.26±0.57 to 1.63±1.03 and mean root length from 0.12±0.21 to 0.84± 0. 54 cm. However, further increase in the concentration of NAA from 1.0 to 2.0 mg/l showed a reduction in the mean root number per shoot and mean root length from 1.63±1.03 to 0.27±0.45 and 0.84±0.54 to 0.14±0.24 cm respectively. The same trend was observed in both treatments (the effect of different concentration of IBA alone and IBA in combination with NAA). Increasing IBA from 0.0 mg/l to 1.0 mg/l increased both the number of roots per shoot from 0.26±0.57 to 1.50±0.38 and mean root length from 0.12±0.21 to 0.67±0.28 cm. Further increase in the concentration of IBA from 1.0 mg/l to 2.0 mg/l, mean root number were reduced from1.50±0.38 to 0.07±0.25 and mean root length from 0.67±0.28 to 0.05±0.20 cm, respectively. This result revealed that when the concentration of both growth regulators (NAA and IBA) was greater than 2.0 mg/l, mean root numbers and mean root length were decreased. In agreement with the finding, George and Sherrington (1984) reported the decreasing of root number when the concentration of IBA is greater than 2.0 mg/l. They stated the inhibitory effect of high concentration of auxin on root formation of plants as a cause for such

decreasing. However, the concentration varies with genotype. Moreover, the optimum concentration is between1.0 and 2.0 mg/l as the present result indicated.

The combined effect of NAA and IBA on root induction was intermediate effect of the separate effect of each of them. Increasing NAA from 0.1 mg/l to 1.0 mg/l by keeping IBA concentration to 0.5 mg/l showed a significant increase in the mean number of roots per shoot from 0.50±0.97 to 1.20±1.16 and mean length of root from 0.13± 0.21cm to 0.43± 0.40 cm. Further increase in the concentration of NAA above 1.0 mg/l by keeping IBA concentration to 0.5 mg/l reduced mean root number from 1.20±1.16 to 0.27±0.58.

In this combination, the maximum result was obtained at 1.0 mg/l NAA in combination with 0.5 mg/l IBA, it resulted in 1.20±1.16 mean number of roots per shoot and 0.43± 0.40 cm mean root length. The minimum result was observed at 2.0 mg/l NAA in combination with 0.5 mg/l IBA, which resulted in 0.27±0.58 mean numbers of roots per shoot and 0.09± 0.19 cm mean root length.

Stephenson and Fahey (2004) obtained 4.7 roots per explant in ½ strength MS medium supplemented with 0.5 mg/l NAA in *M. oleifera*. In contrast, Islam et al. (2005) found that hormone-free medium was the best rooting medium. In the present study, less mean root numbers (1.23±1.19) were obtained in this concentration (0.5mg/l) and growth regulator free medium (0.26±0.57) as compared to the result reported by these authors. But the highest mean number of root and mean root length per explant obtained in the present study was 1.63±1.03 cm and 0.87±1.22 cm in MS medium containing 1.0 mg/l NAA and 0.1 mg/l IBA alone respectively. Islam et al. (2005) stated that medium supplemented with 1.0 and 2.0 mg/l NAA was not inducing rooting at all. In addition, these authors also stated that the medium supplemented with 0.05 mg/l, 1.0 mg/l and 2.0 mg/l IBA produced 0.0, 2.0 and 2.8 mean

numbers of roots per explant respectively. In the present study, the first highest mean root number were produced at concentration of 1.0 mg/l NAA and the second highest mean number of root per explant (1.50±0.38) and the least mean number of root (0.07±0.25) were obtained in these concentrations (1.0 mg/l and 2.0 mg/l IBA) respectively. When the concentrations of IBA increased from 0.05 mg/l to 2.0 mg/l, mean number of root per explant also increased. The trend reported by these authors agrees with the present study particularly in the effect of IBA rather than NAA. The result of the present study indicated that when the concentration of IBA increased from 0.1 mg/l to 1.0 mg/l, mean number of roots per explant continuously increased but further increasing of IBA concentration beyond 1.0 mg/l led to decreasing mean number of roots per explant. Islam et al. (2005) found the highest number of roots per explant at 2.0 mg/l IBA which showed the least mean number of root in the present study.

In agreement with the finding of Alkhateeb (2013) on *Moringa peregrina (*Forsk) who got the maximum number of roots (44.0) per explant in MS medium supplemented with 1.0 mg/l IBA and MS medium containing 0.5 mg/l IBA produced longer roots than control or medium supplemented with different levels of NAA. Here, medium supplemented with1.0 mg/l IBA alone showed highest rate of root induction (1.50±0.38) with longer roots at 0.1 mg/l IBA (0.87±1.22) compared to control medium. As the present data shows that using lower levels of auxin (either NAA or IBA) is significantly better than higher levels for root induction and elongation. Weiler (1984) also reported the inhibition of root elongation by higher concentration of growth regulators and stated ethylene deposition as the reason. Auxins of all types stimulate plant cell to produce ethylene, especially when high amount of synthetic auxins are used. Ethylene retard root elongation. According to this author, the other reason for

reduced response of root number and root length at higher concentration of auxin may be poor vascular connection of the root with the stem because of the interventions of callus.

In an experiment for *in vitro* rooting of *M. oleifera*, when microshoots were cultured on a medium containing different levels of IAA and IBA, medium supplemented with 0.5 mg/l IAA along with IBA at 1.0 mg/l resulted in the highest number of induced 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).

6.3. Acclimatization

Among 50 plantlets acclimatized in the green house, 38 (76%) survived. which is similar to the result of Saini *et al*. (2012) and Marfori (2010) on *Moringa oleifera*. Marfori (2010) transferred plantlets of *Moringa oleifera* to small plastic pots containing fumigate soil and by covering potted plantlets with clear polythene bags and kept in a shaded greenhouse for 2–4 weeks before exposure to ambient conditions. The author 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. CONCLUSION

Results of this study indicate that large- scale propagation of *M. stenopetala* by tissue culture methods is feasible and several plantlets can be regenerated from a single shoot tip explant. The analysis of variance revealed that the effect of NAA along with kinetin on shoot multiplication after four weeks of culture was highly significant ($P \leq 0.05$). Results indicate that using NAA along with kinetin for shoot multiplication was better than kin along with IBA. Therefore, 0.5 mg/l kinentin combined with 0.01 mg/l NAA was found to be optimal in producing maximum number of shoots per explant (3.43±1.41). Application of NAA alone at concentration of 1.0 mg/l (1.63±1.03) and 1.0mg/l IBA (1.20±1.16) were more effective for root induction. This implies that this protocol enables mass propagation of *M. stenopetala* from shoot tip explant.

8. RECOMMENDATIONS

- According to the result obtained in this study, MS medium supplemented with 0.5 mg/l kinetin along with 0.01 mg/l NAA for shoot multiplication and ½ MS medium supplemented with 1.0 mg/l NAA alone and 1.0 mg/l NAA combined with 0.5 mg/l IBA for rooting is recommended for *in vitro* propagation of *M. stenopetala* from shoot tip explant.
- Therefore, further optimization of this protocol may be required for mass propagation of this plant.
- Further optimization of this protocol may also require low cost mass propagation of this plant by reducing the media components used in this protocol or substituting with cheaper components and methods.
- It is also recommended to practice other techniques like culturing of explants in liquid medium reduces the cost of agar for mass propagation of *M. stenopetala.*

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Appendix 1.

Table 4. Nutrient composition and concentration of full strength MS basal medium.

Components	Concentration (gm/L)
Macronutrients	
NH_4NO_3	16.5
KNO_3	19.0
$CaCl_2.2H_2O$	4.4
$MgSO_4.7H_2O$	3.7
KH_2PO_4	1.7
Micronutrients	
Fe-Na-EDTA	4.0
$ZnSO_4.7H_2O$	0.86
H_3BO_3	0.62
$MnSO_4.4H_2O$	2.23
$CuSO_4.5H_2O$	0.0025
KI	0.083
$Na_2MoO_4.2H_2O$	0.025
$COCl_2.6H_2O$	0.0025
Organic supplements	
Myo-inositol	1.0
Glycin (Glycocoll)	0.2
Nicotinic acid	0.05
Pyridoxin (B6)	0.05
Thiamin (B1)	0.01

Declaration

I declare that this thesis is my original work and has not been submitted to any other institution anywhere for the award of any academic degree, diploma, or certificate. All sources of materials used for the thesis have been duly acknowledged.

Alelegne Yeshamebel Signature___________ Date__________________

This Thesis has been submitted for examination with my approval as advisor:

Dr. Tileye Feyissa Signature_____________ Date__________________