IN VITRO PROPAGATION OF AFRICAN MORINGA (*Moringa Stenopetala)* FROM SHOOT TIPS EXPLANTS

M.SC. THESIS

MITIKU MUANENDA ADULA

April, 2013
JIMMA UNIVERSITY

IN VITRO PROPAGATION OF AFRICAN MORINGA (*Moringa Stenopetala)* FROM SHOOT TIPS EXPLANTS

A Thesis

Submitted to School of Graduate Studies
Jimma University College of Agriculture and Veterinary Medicine

In Partial Fulfillment of the Requirements for the Degree of Master of Science in Plant Biotechnology

By

Mitiku Muanenda Adula

April, 2013

Jimma University

Jimma University College of Agriculture and Veterinary Medicine

Department of Horticulture and Plant Sciences

Thesis submission Request form (F-05)

Name of Student: **Mitiku Muanenda Adula** ID No. **05575/03**

Program: **Plant Biotechnology**

Tile; ***In vitro* Propagation of African Moringa** (*Moringa stenopetala)* **from Shoot Tips Explants**

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Major Advisor: **Kassahun Bante (PhD)** _____ _____

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Co-Advisor : **Tileye Feyissa (PhD)** _____ _____

Name signature date

Internal Examiner(If Depends on the verdict)

Name: **Temesgen Matiwos** (Mr) _____ _____

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I dedicated this thesis to the memory of my mother Girmayenesh Andeta

STATEMENT OF AUTHOR

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Date of Submission _____

BIOGRAPHICAL SKETCH

The author was born in 1985 in SNNPRS Gurage zone, Mhur and Aklil Woreda, Cheza kebele. He attended his elementary, junior and high school at Cheza sefer elementary, junior and high school respectively and preparatory at Goro preparatory at Wolkite. After completing preparatory education, he joined Jimma University College of agriculture and veterinary medicine in 2008 and he graduated in 2010 with B.Sc in Horticulture. The author was employed by Ministry of Education as graduated assistant in 2010 for Dilla University. After he served for two years in Dilla University, in September 2011, he joined the School of Graduate Studies of Jimma University to pursue his M.Sc Study in Plant Biotechnology. At the completion of his graduate study, he will lecture in the department of Horticulture at Dilla University.

ACKNOWLEDGEMENTS

Before and above all, I would like to thank the Almighty God for His unreserved support and follow up in my daily life. He has brought me on the stage at which I am today. I wish to express my deepest appreciation and gratitude to my respected advisors; Dr. Kassahun Bante and Dr. Tileye Feyissa for beneficial guidance, constructive comments and contributed experiences throughout my study and full write up of the thesis.

Special sincere thanks also go to Forest research center for their kind willingness to give the experimental material. Besides, I also express my gratitude to Dilla University for giving me the opportunity to pursue my master degree. I would like to extend my thanks to Jimma University College of Agriculture and Veterinary Medicine for allowing me to use its greenhouse and plant tissue culture laboratory where I freely used some of the precious resources and in which I developed the techniques and skills of plant tissue culture.

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ABBREVIATIONS

2,4, 5-T 2,4, 5-Trichlorophenoxyacetic acid

2,4-D 2,4-Dichlorophenoxyacetic acid

BAP 6- Benzylamino purine

GA_3 Gibberellins Acid

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

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IN VITRO PROPAGATION OF AFRICAN MORINGA (*Moringa stenopetala)* FROM SHOOT TIPS EXPLANTS

ABSTRACT

Moringa stenopetala *is belongs to the family Moringaceae. All parts of the tree except the wood are edible and have high medicinal value. It provides highly nutritious food for both humans and animals. In spite of incredible value for both medicine and nutrition,* it *is not known in most part of the world other than its area of cultivation (south Ethiopia and north Kenya) except recently which has attracted the attention of scientists across the globe for health management due to its' nutritional and medicinal properties. However, its expansion is limited by different factors like the propagation method and hence needs an efficient propagation system to supply enough planting material with uniform genotype. Therefore, this study was initiated to develop a protocol for* in vitro *propagation of this tree. In the study shoot tips from* in vitro *grown seedling were cultured in MS medium supplemented with different concentration of BAP, kinetin and BAP with NAA to see their effect on shoot initiation and multiplication. Micro shoots were then transferred into 1/2 MS medium supplemented with different combination of NAA and IBA for root induction. The experiments were carried out in completely randomized design (CRD) with ten replications per treatment and three explants per jar. The cultures were kept at a temperature of 25 ± 2^{o}C and light intensity of 2,000-Lux produced from cool white fluorescent tubes for 16 h photoperiod. Data like shoot number, shoot length and leaf number for shoot multiplication and root number and root length for root induction were collected, and analyzed using SAS software. Statistical analysis revealed that there was significant difference among all treatments applied in both shoot multiplication and rooting experiment. There was also 100% initiation of shoot after one week of culture in all applied treatment of shoot initiation and multiplication. Maximum numbers of shoots per explant (17.40±0.74) were obtained from MS medium containing 1.0 mg/l BAP. The highest numbers of induced roots per shoot (10.20±0.15) were obtained at 1.0 mg/l NAA. Finally well* rooted *shoots were transferred in to greenhouse for acclimatization and 78% of them survived. The results of this study indicate that large- scale propagation of this tree by tissue culture methods is feasible.*

1. INTRODUCTION

African moringa *(Moringa stenopetala,* Bak. f.) is small (up to 10 m tall) flowering plant which belongs to the family *Moringaceae*. The family consists of a single genus, *Moringa*, which has about 14 different species (Edwards *et al*., 2002; Shibiru, 2002). Among the 14 species, *M. stenopetala* and *M. oleifera* are common to Southern part of Ethiopia between altitude of 1100 and 1600 m.a.s.l. (Mekonnen and Gessesse, 1998). *M. stenopetala* is endemic to Southern Ethiopia and Northern Kenya hence named as African moringa. It is commonly called Shiferaw in Amharic and Cabbage tree or African Morniga in English. In the Southern Ethiopia, 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) (Mekonnen and Gessesse,1998; Shibru, 2002).

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 (Melesse *et al*., 2008), and due to this character it is called 'camel crop'. It can be grown even in the harshest and driest of soils, where barely anything else grows. In fact, one of the nicknames of the tree is "never die" due to its incredible ability to survive in harsh weather and drought condition (Singh, 2010). The geographical distribution in Ethiopia may be related to such nature of the plant. The habitat where the tree occur in Ethiopia includes: rocky areas along rivers, dry scrub land, acacia-commiphora woodland and water courses with some evergreens open acacia-commiphora bush land on grey alluvial soil. It is distributed in the rift valley of Southern Ethiopia, especially in Konso, Wollayta, Derashe, Gamogofa, Sidama, Bale and Borana areas (Edwards *et al*., 2002; Verdcourt, 1985).

According to Jahn (1991), annual production can reach 2000 fruits or 6 kg of seed per tree under ideal conditions. Productivity of other yield components like leaf, which is the main yield component, is not documented. Medium to high fruit and leaf yields are reported for the plains of the Rift Valley at about 1200 m altitude. At altitudes over 1650 m no fruits harvested at all and leaf production is poor (Jahn, 1991; Steinmüller *et al*, 2002).

The crop is a multipurpose and fast-growing tree and used for food, animal feed, medicine, oil extraction and water purification. Generally, it is used as animal forage (leaves and

treated seed-cake), biogas (from leaves), domestic cleaning agent (crushed leaves), blue dye (wood), fencing (living trees), fertilizer (seed-cake), foliar nutrient (juice extracted from the leaves), green manure (from leaves), medicine (all plant parts), ornamental plantings, biopesticide(soil incorporation of leaves to prevent seedling damping off), pulp (wood), rope (bark), tannin for tanning hides (bark and gum) and water purification (powdered seeds) (Singh, 2010).

M. stenopetala is one of the world's most nutritious crops; all parts of the tree except the wood are edible. 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). The leaf is rich in carbohydrate, proteins, minerals and essential amino acids. 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).

Moringa 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 and the leaves are renowned for their effectiveness against diarrhoea. In the Negelle and Wolayeta 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 (Mekonnen *et al*., 1999).

In spite of incredible 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). Recently it attracted the attention of scientists across the globe for health management due to its nutritional and medicinal properties. It has the potential to end malnutrition, starvation, as well as prevent and heal many diseases and maladies worldwide. It got a nick name as truly a miracle plant, and a divine 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 the third world like Africa is important. However, for such expansion, it needs good propagation system to supply adequate planting materials of superior genotypes.

The crop has different production constraints. One of the problems is associated with the attack by caterpillars of *Noorda trimaculalis*. 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. The other problem related to this tree is lack of alternative method of propagation. Unlike *Moringa oleifera* which is propagated using both seeds and stem cuttings, *M. stenopetala* is propagated by seeds only (Jahn, 1991; Demel, 1995; Beyene, 2005). It is easily propagated by seed (direct seeding), but it creates variability in yield. Moreover, flowering does not commence until a critical tree size is attained, and after some years the tree branches stop producing fruits/seed. The use of seeds for propagation also limits the utilization of seed for various other purposes such as for food, medication, water purification and oil extraction (Berger *et al*., 1984). Therefore, seed propagation for commercial/large scale/ production is limiting.

Therefore vegetative propagation like cutting is a necessity to obtain uniformity in yield and quality. This method of propagation helps them to fix the desired variability. However, it is less successful due to its slow regeneration. The limitation of cutting is not only slow regeneration, but also requires large sized cuttings (1-1.5m long) (Jahn, 1991). Hence, *in vitro* propagation methods are the best alternative for propagation of this plant with uniform genotypes. *In vitro* propagation has many advantages for this plant as it helps to propagate in sufficient amount with uniform genotype from small amount of explants within a relatively short period of time.

In vitro propagation of *Moringa oleifera* was investigated well, but for this species there is no available report except one by Steintz *et al* (2009) for the purpose of biodiversity conservation (without the use of plant growth regulator). Therefore, this study was initiated with the following objectives;

General objective

- To develop *in vitro* propagation protocol for mass propagation of *Moringa stenopetala*

Specific objectives

- To determine the optimum concentrations of BAP and Kin for shoot initiation and multiplication.
- To determine the optimum combined concentrations of BAP and NAA for shoot initiation and multiplication.
- To determine the optimum concentrations of NAA and IBA for rooting of multiplied shoots.

2. LITERATURE REVIEW

2.1. Botanical Description

Moringa stenopetala belongs to family Moringaceae which is represented by a single genus *Moringa*, with 14 species. Edwards *et al* (2002) 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 a relationship with the Carricaceae. A study made by Olson (2002) on both morphological features (such as leaf form, leaf glands, woody anatomy, flowers and fruit anatomy) and molecular data (on chloroplast genome (cpDNA), gene sequence of ribulose -1-5- bisphosphate carboxylase / oxygenase (rbcL)) revealed that Caricaceae and Moringaceae are sister taxa.

Moringa stenopetala is small tree up to 10 m tall. The trunk is up to 100 cm in diameter, swollen and bottle-shaped. The bark is whitish, pale grey, silvery or blackish and smooth. Its crown strongly branched. Leaves are alternate, up to 55 cm long, 2–3-pinnate and with 5 pairs of pinnae. Inflorescence is a dense, many-flowered and with panicle up to 60 cm long. Flowers are bisexual, regular, 4–7 mm long with long hairs inside, white, pale yellow or yellow-green color. Fruit is an elongate 20–50 cm long, grooved, twisted when young, later straight, reddish with greyish bloom and many-seeded. Seeds is elliptical-trigonous, 2.5–3.5 cm × 1.5–2 cm, with 3 thin wings 6–9 cm long (Edwards *et al*., 2002).

It is more drought tolerant but less freeze-resistant than *M. oleifera*. Freeze may cause it to die back to ground level, where new sprouts may be produced. The geographical distribution in Ethiopia may be related to such nature of the plant. The major growing areas of *M. stenopetala* are Arbaminch and its surroundings, west Abaya and its surroundings, Goffa and its surroundings, Negelle, Keffa and Wollayta Soddo areas which are about 400 to 550 kms south of Addis Ababa. It also grows around Dessie (Mughal *et al.*, 1999).

The local people maintain the selection and propagation of this crop. Basically, the gene pool of *M. stenopetala* has been shaped and maintained predominantly by long time

selection and domestication experience of moringa farmers. Konso can be considered as the area where the tree was first domesticated (Engels and Gottsch, 1991).

2.2. Propagation of Moringa

M. stenopetala propagates from seeds unlike *M. oleifera* which propagates both from seeds and stem cuttings. It has a very low regeneration from stem cuttings (Jahn, 1991). The propagation of *M. stenopetala* has been established and maintained by local farmers. Information about its genetic diversity is scarce and pollen biology and patterns of mating is not documented. The most common method of propagating is by direct sowing without pre-treatment of seed (Muluvi *et al*., 1999; Muluvi *et al*., 2004).

2.2.1. Propagation by Seed

The seeds show 90-100% germination up to one year under ambient conditions. Afterwards, germination percent (51% in 440 days), plant height and number of branches decreased as seed age increased. Germination can be hastened by immersion of seeds in water for 24 h at room temperature. At higher altitude, seedling growth and survival were less than at lower altitude. Seed vigour decreases gradually during storage. The optimal temperature for germination of seeds is 25°C (Teketay, 1995). Jahn(1991) reported that 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. During the cool dry season, there was little difference.

Uses of seeds for propagation limit the utilization of seed for various purposes, since it is used for food, medication, water purification and other. Seeds are eaten green, roasted, powdered, steeped for tea or used in curries (Berger *et al*., 1984). Powdered seeds are highly effective for purifying drinking water by flocculation and sedimentation of particulate contaminants (Berger *et al*., 1984; Gassenschmidt *et al*., 1995; Olsen, 1987). *Moringa oleifera* seed oil (30-40% of seed weight) is sweet, non-sticking and non-drying oil that resists rancidity and has been used in salads, for fine machine lubrication, and in the manufacture of perfume and hair care products (Tsaknis *et al*., 1999).

2.2.2. Vegetative Propagation

Limb-cutting as vegetative method of propagation is generally followed for *Moringa* species. Cutting requires large cutting size (1-1.5 m long and 14-16 cm girth) and should be taken from the woody parts of the branches. The longer the cuttings are the better its survival rate. It is seasonal depended and should be taken in the rainy season (preferably during June to August) (Gassenschmidt *et al*., 1995).

Cuttings in *M. oleifera* are planted in pits of 1 m x 1 m x 1 m at spacing of 3 to 5 cm each way. It can be cured for three days in the shade and then planted in a nursery or in the field. One third of the cutting's length should be placed in the soil. The soil should be moist but not over watered. Cuttings planted in polybags will take a long time to develop roots and may be planted out after 2 or 3 months. It produces roots in moist soil in short period and grow to sizeable trees within a few months. However, the trees grown from cuttings are known to have much shorter roots. Where longer roots are an advantage for stabilization or access to water, seedlings are preferable. Rooting and survival are adversely affected by dehydration of cuttings. Lignified cutting are more resistant to dehydration than chlorophyllous shoots (Demeulenere, 2001).

2.3. *In Vitro* Propagation of Moringa

In vitro propagation (micropropagation) refers to the process of asexual reproduction by multiplication of genetically identical copies of individual plants. Micropropagation can also be described as the process of mass propagation of selected plants via *in vitro* techniques. Micropropagation is rapid and has been adopted for commercial propagation of important plants such as banana, apple, pears, sugarcane, strawberry, cardamom, many ornamentals (e.g. Orchids) and other plants (Omar and Aouine, 2007).

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 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).

Recently *in vitro* propagation was given due importance for *Moringa* species. Since flowering of a number of the large tree species of *Moringa* does not even commence until a critical size is attained. Additionally, 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 *Moringa stenopetala* which grow in specific place of the world. Amplification 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 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 axillary buds of either juvenile or adult origin of current year growth are commonly used as explants for *Moringa 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 *et al*., 2012). For example, stem segments from 10 days old seedling, as explant gave 100% regeneration of plantlets with profuse rooting in *Moringa oleifera* (Marfori, 2010).

Steintz *et al* (2009) propagated *in vitro Moringa oleifera*, *Moringa stenopetala* and *Moringa peregrine* using basal MS medium in the absence of plant growth regulators by following 3 steps; 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. His aim was not for micropropagation rather to conserve biodiversity by initiation and establishment (*in vitro*) of small vegetative clones that will serve subsequent vegetative and reproductive propagation.

Direct somatic embryogenesis was obtained in immature zygotic embryos of *Moringa oleifera* in media with GA_3, BAP and activated charcoal and cultured in continuous light. Long term fast growing callus cultures were established from rapidly elongated epicotyls (*in vitro* plantlets) in medium containing 2,4-D, NAA and coconut milk for *Moringa oleifera* (Kabir, 2003).

2.3.1. Culture Media

One of the most important factors governing the growth and morphogenesis of plant tissues in culture is the composition of the culture medium. The composition of plant tissue culture medium depends upon the type of plant tissues or cells that are used for culture. No single medium can be used for all types of plants and organs, so the composition of the culture medium for each plant material has to be worked out.

The basic nutrient requirements of cultured plant cells are very similar to those of whole plants. Plant tissue and cell culture media are generally made up of some or all of the following components: macronutrients, micronutrients, vitamins, amino acids or other nitrogen supplements, sugar(s), other undefined organic supplements, solidifying agents or support systems, and growth regulators. Several media formulations are commonly used for the majority of all cell and tissue culture work. These media formulations include White's medium – which is one of the earliest plant tissue culture media,(b) MS medium - formulated by Murashige and Skoog (MS) which is most widely used for many types of culture systems,(c) B_5 medium - developed by Gamborg for cell suspension and callus cultures and at present it's modified form used for protoplast culture ,(d) N_6 medium - formulated by Chu and used for cereal anther culture ,and (e)Nitsch's medium developed by Nitsch and Nitsch and used for anther culture.

Some efforts have made to standardize the media formulation for increasing the multiplication rate of moringa micropropagation. The most common basal media used are either full or half- strength MS salts(Murashige and skoog, 1962). However, there are also few reports on the use of the WPM(kantharajaj) and Dodd, 1991) medium.

2.3.2. Shoot Initiation and Multiplication

Different types and concentration of plant growth regulators (PGRs) such as BAP (1.0-4.0 mg/l), NAA (0.01-2.0 mg/l), and kinetin are used for *Moringa* species *(oleifera)* micropropagation. However, addition of BAP(1.0-2.0 mg/l) and NAA (0.01-1.0 mg/l) in the culture medium have suited well for shoot proliferation and growth of axillary buds, both for shoot initiation and subsequent multiplication (Stephenson and Fahey, 2004; Islam *et al.,* 2005; Marfori, 2010 and Saini *et al.,* 2012). BAP is most frequently used in *Moringa in vitro* propagation and it gave best shoot multiplication result. 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 the 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.

Induction of multiple shoots in *M. oleifera* has been characterized with different growth regulators. Marfori (2010) tested three cytokinins, namely BAP, Kin and TDZ, He reported that BAP at 0.5 mg/l was found to be optimal in inducing bud break, producing an average of 4.6 axillary shoots per explant after 2 weeks. Stephenson and Fahey (2004) obtained 4.7 shoots per cultured seed in MS medium containing 1.0 mg/l BAP with 1.0 mg/l GA3. According to Islam *et al* (2005) 1.0–1.5 mg/l BAP was found to be best for shoot response. Saini *et al* (2012) report similar result (BAP 1.0 mg/l is best) to Stephenson and Fahey (2004) and Islam *et al* (2005), but number of shoots per explant obtained in their investigation was higher when compared to earlier studies. They found that 1.0 mg/l BAP produced maximum average axillary shoots per explant (9.0 ± 1.0) after 15 days of culture. The effect of BAP in combination with other growth regulator like GA_3 and NAA was also reported. Xiang *et al* (2007) found that the combination of 0.5 mg/l BAP and 0.1 mg/l NAA was the best for shoot induction. Similarly, Zhang (2007) reported that MS medium supplemented with 2.0 mg/l BAP and 0.2 mg/l NAA is the appropriate medium for inducing buds, induction rate reached 86.7% and mean of axillary buds was 3.17.

2.3.3. Rooting

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. Micro shoots were cultured in ½ MS basal media with different types and concentrations of auxin to optimize the rooting of *Moringa* species specially *Moringa oleifera*. NAA (0.1-2.0 mg/l), IBA (0.1-2.0 mg/l) and IAA (0.1-2.0 mg/l) each alone or combination of two or three of them are most commonly used growth regulators in rooting of micro shoots.

The concentration of mineral salts in medium play an important role in root induction. High salt levels are inhibitory to root initiation. Tipirndamaz(2003) reported the induction of a greater number and length of roots on a combination of ½ MS medium and sucrose at 30 g/l for many plants. 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.

Stephenson and Fahey (2004) obtained maximum root numbers (8.1 ± 0.7) in 1/2 MS medium containing 0.5 mg/l NAA. Marfori (2010) obtained similar result as Stephenson and Fahey (2004), He optimal rooting at 0.5 mg/l NAA. Saini *et al* (2012) got the highest number of induced roots per shoot (7.0 ±1.3) after 7 days by application of 0.5 mg/l IAA along with IBA at 1 mg/l. They further investigated that IAA and IBA separately was found to be less effective, IAA was found less effective than IBA. According to Steinitz *et al* (2009) rooting of the three species of *Moringa* (*Moringa oleifera, Moringa stenopetala and Moringa peregrina*) also gave good result in MS medium without PGR. Saini *et al* (2012) also got best performing root in growth regurator free MS medium for *Moringa* oleifera, even if the number of shoots per explant was few in number. Again Islam *et al* (2005) stated that MS basal medium is efficient for rooting of micro shoots. He also reported that no shoot induced root in all media supplemented with different concentration NAA.

2.3.4. Acclimatization

Achievement of uniform plant growth and their high survival rare not only demands good greenhouse conditions, but also requires high modification of the internal micro-climate to match the local environmental need. Marfori (2010) transferred rooted 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 same author got 80% of survived plants. In terms of growth and morphology, no abnormality was observed when the tissue culture-derived plants were compared with the mother plant. Saini *et al* (2012) obtained similar result to Marfori (2010), 80% of the rooted plants survived after being transplanted in the soil, 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.

3. MATERIALS AND METHODS

The experiment was conducted in tissue culture laboratory of Jimma University, College of Agriculture and Veterinary Medicine (JUCAVM), Jimma, Ethiopia from June 2012 to January 2013.

3.1. Plant material

Seeds of *M. stenopetala* (accession 7) were used to establish seedling *in vitro* which is used as source of explant. The seeds were provided by Forest Research Center, Addis Ababa, Ethiopia.

3.2. Stock Solution and Media Preparation

MS basal medium (Murashige and Skoog, 1962) was used throughout the experiments. Table sugar was used for carbon source. Stock solutions of macronutrients, micronutrients, growth regulators and vitamins were prepared separately (Appendix 5) and stored at 4°C for immediate use. Then MS basal media was prepared by mixing the appropriate volumes of stock solutions and finally different concentrations and combinations of growth regulators were added accordingly. After dissolving all the components, the pH was adjusted to 5.8 using 1N NaOH and 1N HCl and then 0.8% agar was added. After melting the prepared media by using hot plate with magnetic stirrer, 40ml medium was dispensed into each culture jar that have a capacity of 200 ml. The culture jars were sealed with aluminum foil and autoclaved at 121^{o}C and 15psi for 15 minutes. The culture media was then stored at room temperature until used.

3.3. Surface Sterilization and Preparation of Explant

The seeds were removed from pods and washed under tap water with drop of liquid detergent thoroughly and then rinsed with distilled water. Then they were surface sterilized by washing with 70% ethanol for 2 min and then 0.1% mercuric chloride (w/v) for 15 min, followed by rinsing five to ten times in sterile distilled water.

After sterilization, seeds were cultured aseptically in Petri dish containing sterile distilled water with cotton support until sprouted out its radicle. Then seed coats were removed aseptically to permit direct contact of seed tissue with the culture medium and to reduce contamination, and the seeds were transferred into growth regulators free medium (MS +

3% table sugar + 0.8% agar). In this medium, the seedlings were kept until the first pair of leaves emerged and then shoot tip was taken as an explant.

3.4. Culturing and Culture Condition

The cultures were kept at a temperature of 25 ± 2^{o}C and light intensity of 2,000-Lux produced from cool white fluorescent tubes for 16 h photoperiod.

About 2cm shoot tips were taken after 10 days of germination and cultured in shoot multiplication medium (3 explants per jar). To avoid the carry over effect of the growth regulators used in shoot initiation and multiplication medium, the culture was maintained on growth regulators free MS medium for two weeks before transferring to the rooting media. The clumps of multiplied shoots were separated as individual shoot and well developed shoots were transferred to rooting medium (three explants per culture jar) and maintained on the rooting medium for 15 days. Each experiment was repeated twice to ensure reproducibility of the results.

3.5. Experimental Design and Treatments

In this study three different experiments were carried out in completely randomized design (CRD) with ten replications per treatment and three explants per jar. The explants were randomly culture with treatment combinations. Growth regulator free media was used as a control.

3.5.1. Effect of BAP and Kinetin on Shoot Initiation and Multiplication

Shoot tips taken from *in vitro* germinated seedlings were treated with six concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 mg/l) of both BAP and kinetin to see their effect on shoot initiation and multiplication.

3.5.2. The Combined Effect of BAP and NAA on Shoot Initiation and Multiplication

Shoot tips taken from *in vitro* germinated seedlings were treated with different combination of BAP and NAA. Six concentrations (0.5, 1.0, 1.5, 2.0, 2.5, 3.0 mg/l) of

BAP and two concentrations (0.1, 0.2 mg/l) of NAA with 6X2 treatment combinations were used.

3.5.3. The Effect of NAA and IBA on Rooting

In this experiment, half strength MS basal medium with different concentrations and combinations of NAA and IBA were investigated for rooting of micro-shoots. Accordingly five concentrations of NAA (0.0, 0.5, 1.0, 1.5, 2.0 mg/l) and IBA (0.0, 0.5, 1.0, 1.5, 2 mg/l) with 5x5 treatment combinations were used.

3.6. Acclimatization

Fifty plantlets with well developed shoots and roots were taken out of the culture jars and thoroughly washed with water to remove all traces of the medium. The plantlets were planted in plastic pot containing a mixture of sun sterilized soil; sand; compost (2:1:1) ratio and acclimatized to greenhouse condition. Then, the plantlets were placed under plastic cover (as a shade) for two weeks and after another two weeks by removing the plastic cover. In first week of transfer, Plantlets was watered frequently and after that watering frequency was reduced and at the fourth week of acclimatization watered only two times per day (morning and evening).

3.7. Data Collection

After 15 days of transferring shoot tips into shoot initiation and multiplication media, date of shoot initiated, percent of shoot initiation, number of shoots per explant, shoot length (cm), and number of leaves were recorded. After 15 days of transferring well developed shoots into rooting media number of shoots that produced root, number of roots per shoot, and length of roots (cm) were recorded.

3.8. Data Analysis

Data analysis was done using SAS software version 9.2 (SAS, 2008). The collected data was subjected to one and two way analysis of variance (ANOVA) test at 1% level of significance. Mean separation was done using REGWQ (Ryan, Elinot, Gabriel, and Welsh).

4. RESULTS AND DISCUSSION

4.1. Effect of BAP and Kinetin on Shoot Initiation and Multiplication

The analysis of variance indicated that the effect of BAP and kin were significantly different (P<0.001) in shoot number, shoot length and leaf number (Appendix 1; Appendix 2; Fig. 1). There was 100% initiation of shoot after first week culture of the shoot tips in shoot initiation and multiplication media in all six concentrations of both BAP and kin (the data was not included). 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 *Moringa oleifera*. Initiation of the culture started after a week in first culture (shoot tip culture taken from seedlings). In second culture (initiation of micro shoot) it started after third day of culturing.

Table 1 . The effect of BAP and Kinetin on shoot multiplication

Effect of BAP on shoot Multiplication				**Effect of kinetin on shoot multiplication**			
B	**Shoot number**	**Shoot length(cm)**	**Leaf number**	**K**	**Shoot number**	**Shoot length(cm)**	**Leaf number**
0.0	3.00^{e}±0.15	1.41^{e}±0.12	5.20^{b}±0.80	0.0	3.00^{f}±0.15	1.41^{c}±0.12	5.20^{bc}±0.80
0.5	8.14^{d}±0.46	1.57^{d}±0.16	6.30^{a}±0.35	0.5	6.85^{e}±0.58	1.40^{c}±0.11	5.80^{a}±0.42
1.0	17.40^{a}±0.74	1.90^{a}±0.12	6.50^{a}±0.53	1.0	13.60^{a}±1.50	1.52^{bc}±0.12	5.20^{bc}±0.18
1.5	16.60^{ab}±1.01	1.79^{ab}±0.09	6.20^{a}±0.38	1.5	12.40^{b}±0.90	1.52^{bc} ±0.15	5.10^{bc}±0.23
2.0	16.50^{ab}±1.36	1.73^{bc}±0.95	5.90^{a}±0.66	2.0	12.31^{b} ±0.64	1.65^{ab}±0.07	4.70^{c}±0.44
2.5	15.30^{c}±0.84	1.61^{cd}±0.14	6.00^{a}±0.26	2.5	11.11^{c}±0.91	1.69^{a}±0.73	5.70^{ab}±0.55
3.0	15.90^{bc}±0.92	1.47^{de}±0.11	5.90^{a}±0.54	3.0	10.00^{d}±1.16	1.40^{c}±0.10	5.60^{ab}±0.54
CV	6.55	7.33	8.82	CV	9.33	7.22	9.14

B= BAP (mg/l), K= Kinetin (mg/l), Numbers within the same column with different letter(s) are significantly different from each other according to REFGQ at P≤ 0.5. The values represent mean ± standard deviation.

BAP at concentration level of 1.0mg/l exhibited the maximum mean shoot number per explant (17.40±0.74), mean shoot length (1.90±0.12) and mean leaf number (6.50±0.53). At similar concentrations (1.0 mg/l), 13.60±1.50 mean shoot numbers per explant and 1.52±0.12 mean shoot length were recorded for kin, which was the maximum from the six concentrations of kin (Table 1; Fig. 2).

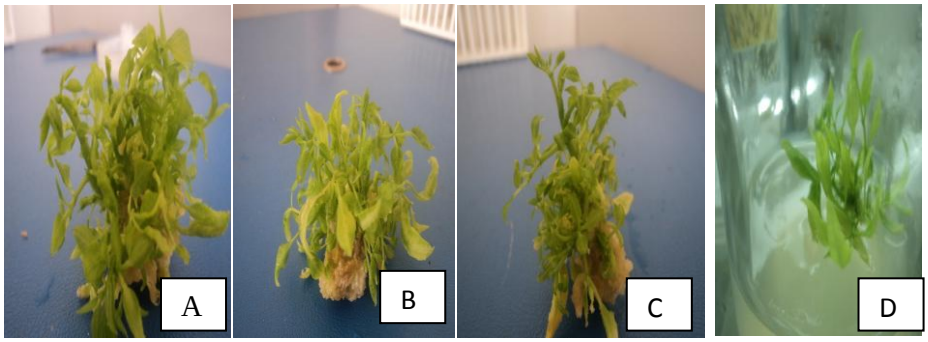

Figure 1. The Effect of Different Growth Regulators on Shoot Multiplication (after two weeks); A) 1.0 mg/l BAP , B) 1.0 mg/l Kinetin, C) 1.0 mg/l BAP combined with 0.1mg/l NAA, E) PGR free

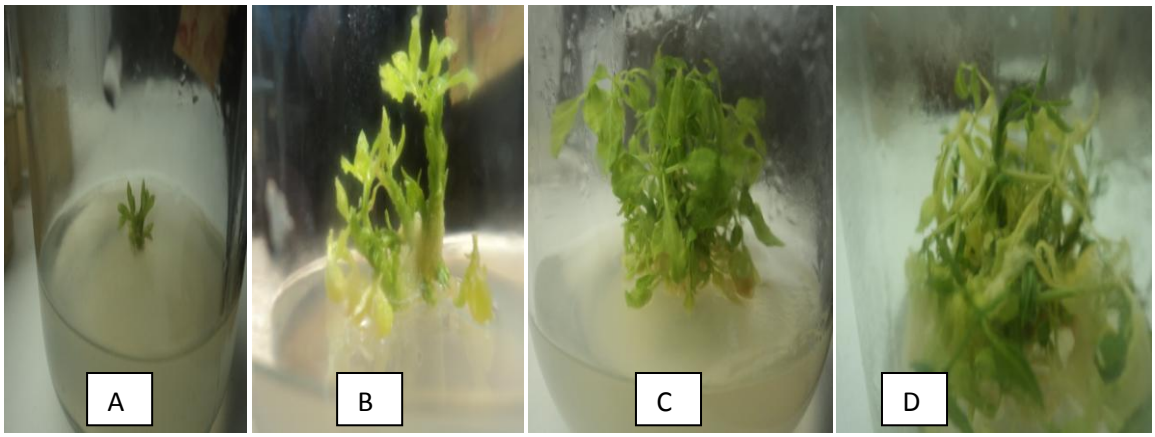

Figure 2. The effect of 1.0 mg/l BAP on shoot multiplication; A) the explant during culturing, B) after 1 week C) after 2 weeks and D) after three weeks

Increasing the concentration level from 0.0 to 1.0 mg/l has increased shoot number and shoot length in both BAP and kin. Further increasing BAP from 1.0 mg/l to 3.0 mg/l decreased shoot number from 17.40±0.74 to 15.30±0.92 and shoot length from 1.90±0.12 to 1.47±0.11. Similarly, increasing Kin from 1.0 mg/l to 3.0 mg/l, decreased shoot number from 13.60±1.50 to 10.00±1.16 and shoot length from 1.52±0.12 to 1.40±0.10 cm. Increasing the concentration of BAP from 0.5 to 3.0 mg/l decreased leaf number from

6.30±0.35 to 5.90±0.54. When the concentration of kin increases from 0.5 to 2.0 mg/l, it decreased leaf number from 5.80±0.4 to 4.70±0.44. But, at 2.5 mg/l kin it was increased to 5.70±0.55 and then decreased to 5.60±0.54 at 3.0 mg/l. This indicates that higher concentrations of both BAP and kin inhibit shoot multiplication. High concentrations of cytokinins inhibits cell division and thereby influences shoot multiplication. The inhibitory effect of BAP and kin at high concentration level (>2.0 mg/l) was reported in *Moringa oleifera* (Saini et al., 2012).

This finding is in agreement with the work done on *M. oleifera* that BAP gave better results than kin for shoot multiplication. Saini *et al* (2012) reported maximum mean shoot number of 9.0 ± 1.0 on MS media supplemented with 1.0 mg/l BAP in *M. oleifera*. However, the number of shoots per explant (17.40±0.74) reported in the present study was higher than those obtained by the Saini *et al* (2012) from *M. oleifera*. Marfori (2010) tested three cytokinins(BAP, Kin and TDZ). This author reported that MS media supplemented with BAP at 0.5 mg/l was found to be optimal, producing an average of 4.6 axillary shoots per explant after 2 weeks. In present study, 8.14±0.46 for BAP and 6.85±0.58 mean shoot per explant for kin was obtained at BAP (0.5 mg/l, indicating better response of the genotypes even if it is not the optimum concentration in the present study. Islam *et al* (2005) also reported that 1.0 to1.5 mg/l BAP was found to be best for shoot response. The performance of BAP in shoot multiplication is further supported by works done on plant in sister families (Brassicaceae and Capparidaceae) of *Moringa*. The studies in papaya (Capparidaceae family) showed that shoot multiplication in MS Media supplemented with BAP was better than kinetin (Drew, 1988; Winnar, 1988).

4.2. Effect of BAP and NAA on Shoot Initiation and Multiplication

The analysis of variance indicated that the combined effects of BAP and NAA were significantly different ($P<0.001$) in all tested parameters of shoot multiplication; shoot number, shoot length and leaf number (Appendix 3). Shoot were initiated in all treatment combinations started from a week in first culture (shoot tip culture taken from seedlings) and after third day in second culture (initiation of micro shoot).

The highest mean shoot number per explant (16.80±0.35) and mean shoot length (1.74±0.048) were obtained at a combination of 1.0 mg/l BAP and 0.1 mg/l NAA. The

minimum shoot number (6.62±0.25) was recorded at the combination of 1.0 mg/l BAP and 0.2 NAA. There was a continuous increase in number of shoots (7.60±0.39 to 14.60±0.28) when concentration of BAP increased from 0.5 to 2.0 mg/l. In contrast, there was a continuous decrease in number of shoots when the concentration of BAP was greater than 2.0 mg/l. From the two concentrations of NAA (0.1 and 0.2 mg/l), the combination of 0.1 mg/l NAA with all levels of BAP gave relatively better results. This indicates that combination of BAP with low concentration NAA was more effective.

A low concentration of auxin is often beneficial in conjunction with higher levels of cytokinin during shoot multiplication. Exogenous auxin does not promote auxiliary shoot proliferation; however, their presence in culture medium may improve the culture growth. Although cytokinins are known to stimulate cell division, they do not induce DNA synthesis. The presence of auxin promotes DNA synthesis. Hence, the presence of auxin together with cytokinin stimulates cell division and control morphogenesis there by influencing shoot multiplication (George et al., 2008).

Table 2. Effect of BAP and NAA on shoot multiplication

Level of BAP	Level of NAA	Shoot number (cm)	Shoot length	Leaf number
0.0	0.0	2.91^{h}±1.46	1.37^{c}±0.56	5.60^{bc}±1.51
0.5	0.1	14.20^{b}±0.36	1.67^{ba}±0.05	5.60^{bc}±0.15
0.5	0.2	7.60^{f}±0.39	1.40^{c}±0.13	4.30^{e}±0.32
1.0	0.1	16.80^{a}±0.35	1.74^{a}±0.048	6.41^{a}±0.43
1.0	0.2	6.62^{g}±0.25	1.51^{bac}±0.145	4.41^{e}±0.26
1.5	0.1	14.34^{b}±0.46	1.71^{a}±0.029	6.01^{ba}±0.36
1.5	0.2	11.01^{d}±0.45	1.40^{c}±0.039	4.70^{de}±0.27
2.0	0.1	14.60^{b}±0.28	1.69^{ba}±0.04	5.56^{bc}±0.34
2.0	0.2	12.00^{c}±0.28	1.55^{bac}±0.13	5.15^{dc}±0.36
2.5	0.1	12.03^{c}±0.39	1.50^{bac}±0.12	5.00^{dce}±0.29
2.5	0.2	9.77^{e}±0.32	1.43^{bc}±0.03	5.55^{bc}±0.29
3.0	0.1	10.91^{d}±0.51	1.50^{bac}±0.17	4.41^{e}±0.16
3.0	0.2	10.39^{ed}±0.48	1.54^{bac}±0.03	4.88^{dce}±0.39
CV		4.98	11.83	9.88

Numbers within the same column with different letter(s) are significantly different from each other according to REFGQ at $P \leq 0.5$. The values represent mean ± standard deviation.

The optimum combination of BAP and NAA in present study was not in agreement with the previous work done on *M. oleifera*. Xiang *et al* (2007) found that MS media supplemented with 0.5 mg/l BAP and 0.1 mg/l NAA was best for shoot induction and multiplication in *M. oleifera*. Similarly, Zhang (2007) reported that MS medium supplemented with 2.0mg/l BAP and 0.2mg/l NAA was the best medium for inducing buds, induction rate reaching 86.7%.

Callus formation on the leaf (especially at the margin of the leaf), abnormal growth structures such as curling of leaf and formation of callus outside of the curl; and etiolated seedling and some micro shoot were seen as problem during study (Fig. 4).

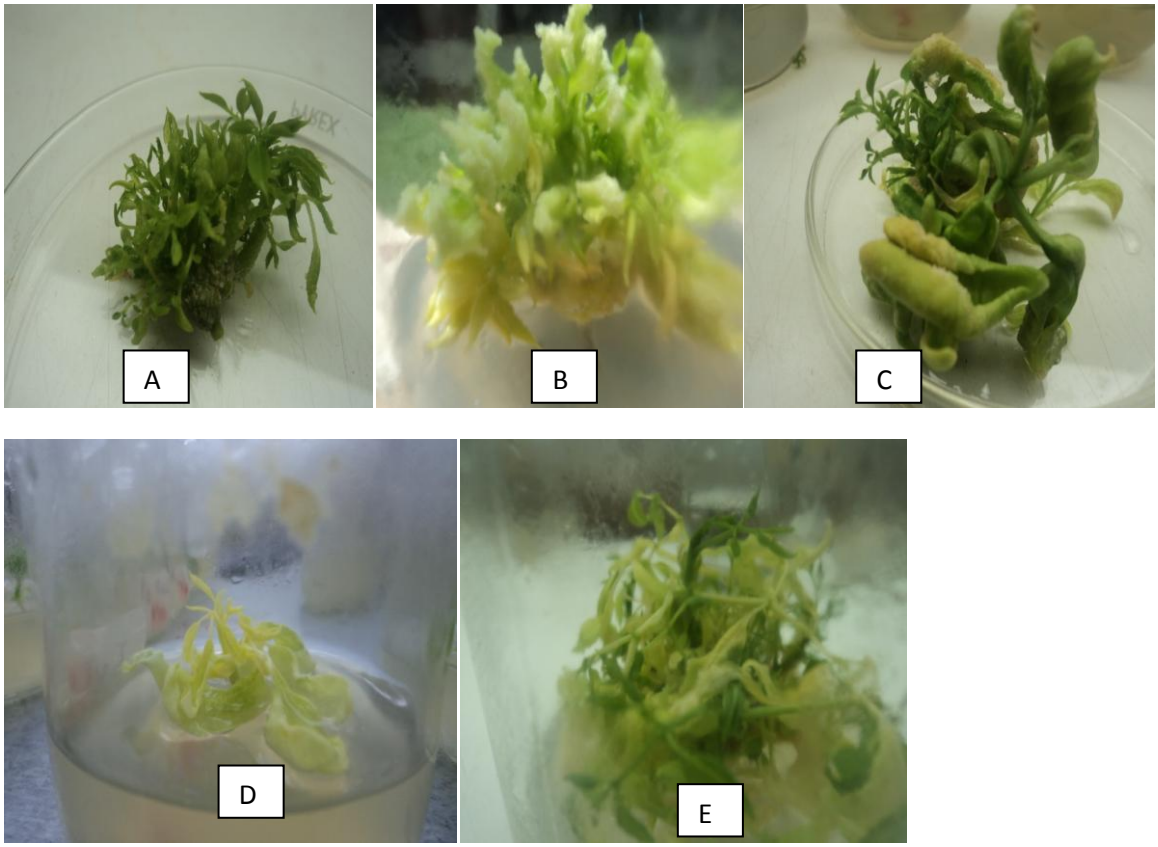

Figure 3. Abnormal morphology of shoots; A) burning of the shoot tip, B) callus formation on the leaf(especially at the margin of the leaves), C) curling of leaf and formation of callus outside of the curl, D) etiolated seedling and micro shoot, E) colour change, senescence and defoliation after three weeks.

The formation of moderate friable callus was observed at the cut surface and leaf margin in some cultures (Fig 4a and 4b). However, this problem was occurred randomly across treatments and it was not associated with a specific treatment combination. It is also reported in previous studies of M. oleifera (Steintz et al., 2009; Stephenson and Fahey, 2004).

After three weeks, there were no signs of additional shoot formation instead shoot vigor and quality declined rapidly almost in all but the plant growth regulator-free medium. After three weeks, shoots in shoot multiplication media, abruptly began to show colour change, signs of senescence and final defoliation (Fig 4E). Such phenomenon was observed in previous studies of *Moringa oleifera* (Kantharajah and Dodd, 1991; Mohan *et al.*, 1995; Saini *et al.*, 2012). Release of ethylene and high level growth regulator were mentioned as the cause for such problem. In the present study, such problem was solved by transferring the culture in to growth regulator free fresh basal medium after three weeks. In previous works on *Moringa oleifera*, it was solved by the addition of low concentration of $AgN0_3$ to bind to the tissue's ethylene receptors (Leonhardt, 1987; Mohiuddin, 1997) or passage through growth regulators free fresh media (Kantharajah and Dodd, 1991; Saini *et al.,* 2012).

In some cultures very small, narrow leaves and shortened internodes were also observed in some treatments. This is may be due to low-level bacterial or fungal contamination of explants and/or cultures which generate excessive ethylene gas leading to rapid tissue ripening, leaf narrowing and abscission (Jackson 1991; Marino 1996; Weingart 1997).

4.3. The Effect of NAA and IBA on Rooting of Micro Shoots

The analysis of variance revealed that both root number and root length varied significantly with concentration of NAA, IBA and their combinations (Appendix 4; Fig.4). When the concentration of NAA is greater than that of IBA, relatively better result was recorded. Both the highest mean root numbers per shoot (10.20±0.15) and mean root length (6.30±0.15 cm) were obtained at 1.0 mg/l NAA combined with 0.0 mg/l IBA. The second highest mean root number (9.60±0.15) and mean root length (5.70±0.15) were obtained at 1.0 mg/l NAA in combination with 0.5 mg/l IBA (Table 3).

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

NAA (mg/l)	IBA (mg/l)	Root number	Root length (cm)
0.0	0.0	1.05^{k}±0.83	0.70^{m}±0.62
0.0	0.5	4.22^{i}±0.23	2.66^{kl}±0.14
0.0	1.0	8.70^{cd}±0.12	4.19^{fg}±0.18
0.0	1.5	6.10^{g}±0.15	3.50^{hi}±0.15
0.0	2.0	5.95^{g}±0.11	3.28^{ij}±0.16
0.5	0.0	6.19^{g}±0.14	4.40^{f}±0.15
0.5	0.5	6.19^{g}±0.12	4.30^{fg}±0.15
0.5	1.0	4.07^{ij}±0.16	2.81^{k}±0.17
0.5	1.5	3.90^{j}±0.13	2.70^{kl}±0.15
0.5	2.0	3.80^{j}±0.15	2.60^{kl}±0.15
1.0	0.0	10.20^{a}±0.15	6.30^{a}±0.15
1.0	0.5	9.60^{b}±0.15	5.70^{bc}±0.15
1.0	1.0	9.40^{b}±0.15	5.60^{cd}±0.15
1.0	1.5	8.30^{e}±0.15	5.65^{bcd}±0.15
1.0	2.0	8.10^{e}±0.15	4.30^{fg}±0.15
1.5	0.0	9.40^{b}±0.15	5.90^{b}±0.15
1.5	0.5	9.40^{b}±0.15	5.60^{cd}±0.15
1.5	1.0	8.90^{c}±0.15	5.60^{cd}±0.15
1.5	1.5	8.70^{cd}±0.15	5.40^{d}±0.15
1.5	2.0	8.40^{de}±0.15	4.30^{fg}±0.15
2.0	0.0	7.10^{f}±0.15	4.10^{g}±0.15
2.0	0.5	8.10^{e}±0.15	4.70^{e}±0.15
2.0	1.0	7.10^{f}±0.15	4.30^{fg}±0.15
2.0	1.5	6.90^{f}±0.15	3.70^{h}±0.15
2.0	2.0	5.40^{h}±0.15	3.10^{j}±0.15
CV		3.63	4.67

Numbers within the same column with different letter(s) are significantly different from each other according to REFGQ at $P \leq 0.5$. The values represent mean ± standard deviation

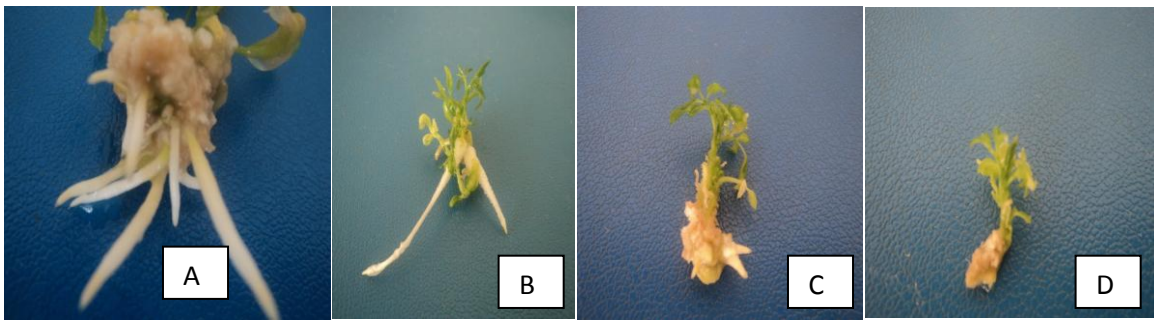

Figure 4. The effect of NAA and IBA on rooting (**after two weeks of culture)***;* A) NAA (1.0 mg/l), B) IBA (1.0 mg/l), C) the combined effect of NAA and IBA, D) rooting condition in PGR free medium.

When, the concentration of the two auxins increased from 0.0 to 1.0, it increased both root number and root length. Increasing NAA from 0.0 mg/l to 1.0 mg/l by keeping IBA concentration at 0 mg/l showed a significant increase in the number of roots per shoot from 1.05 ± 0.83 to 10.20 ± 0.15 and mean root length from 0.70±0.62 to 6.30 ± 0.15 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 to 7.10±0.15 and 4.10±0.15 cm respectively. Increasing IBA from 0.0 mg/l to 1.0 mg/l increased both number of roots per shoot from 1.05±0.83 to 8.70±0.12 and mean root length from 0.70±0.62 to 4.19±0.18 cm. Further increase in the concentration of IBA from 1.0 to 2.0 mg/l reduced mean root number and root length to 5.95 ± 0.11 and 3.28 ± 0.16 cm, respectively.

Increasing IBA from 0.5 mg/l to 2.0 mg/l by keeping NAA concentration to 1.0 mg/l showed a continuous decrease in the number of roots per shoot from 9.60±0.15 to 8.10±0.15 and mean root length from 5.70±0.15 to 4.30±0.15 cm. Similarly, increased NAA from 1.0 mg/l to 2.0 mg/l by keeping IBA concentration at 1.0 mg/l decreased the number of roots per shoot from 9.40±0.15 to 7.10±0.15 and mean root length from 5.60±0.15 to 4.30±0.15 cm.

In all other higher combinations of the two auxins, lower root number and root length were recorded. The combination of 2.0 mg/l concentration of the two cytokinins (the highest concentration) gave 5.40±0.15 mean root number and 3.10±0.15 root length. The combination of 2.0 mg/l NAA and 1.5 mg/l IBA result in 6.90±0.15 mean root number and 3.70±0.15 mean root length. This indicates inhibitor effect of high concentration of auxins.

Rooting has been previously induced with different growth regulators in *Moringa oleifera*. Stephenson and Fahey (2004) obtained 4.7 roots per explant in 1/2 MS medium containing 0.5 mg/l NAA. In the present study, higher mean root numbers (6.19 ± 0.14) were obtained in this concentration (0.5mg/l) than those reported by these authors. However, it was not optimum level and the highest number of roots per explant obtained in the present study was 10.20±0.15 on MS medium containing 1.0mg/l NAA. According to Islam *et al*. (2005) rooting was efficient on MS basal medium which is contrast to the present one. Even if there were rooting of shoots on MS basal medium, the root number recorded was

the lowest (1.05±0.83) in present study. He also stated that medium supplemented with NAA has not induced rooting at all, which provided high root number in present study.

The number and length of roots increased in proportion to the concentration of NAA and IBA applied. However, when the concentration of both of the growth regulators was greater than 2.0 mg/l, both root numbers and root length were decreased. In agreement with the present observation, George and Sherrington (1984) reported decreasing of root number when the concentration of IBA is great than 2.0 mg/l. They indicated the inhibitory effect of high concentration of auxin on root formation of plants as a cause for such decrease. 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 this author, the other reason for reduced response of root number and root length at higher concentration of auxin may be due to poor vascular connection of the root with the stem because of the interventions of callus.

4.4. Acclimatization

Among the acclimatized plantlets, 78% survived(Fig 5), which is similar to the results 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), that the potted plantlets were covered with clear polythene bags and kept in a shaded greenhouse for 15 days before exposure to ambient conditions.

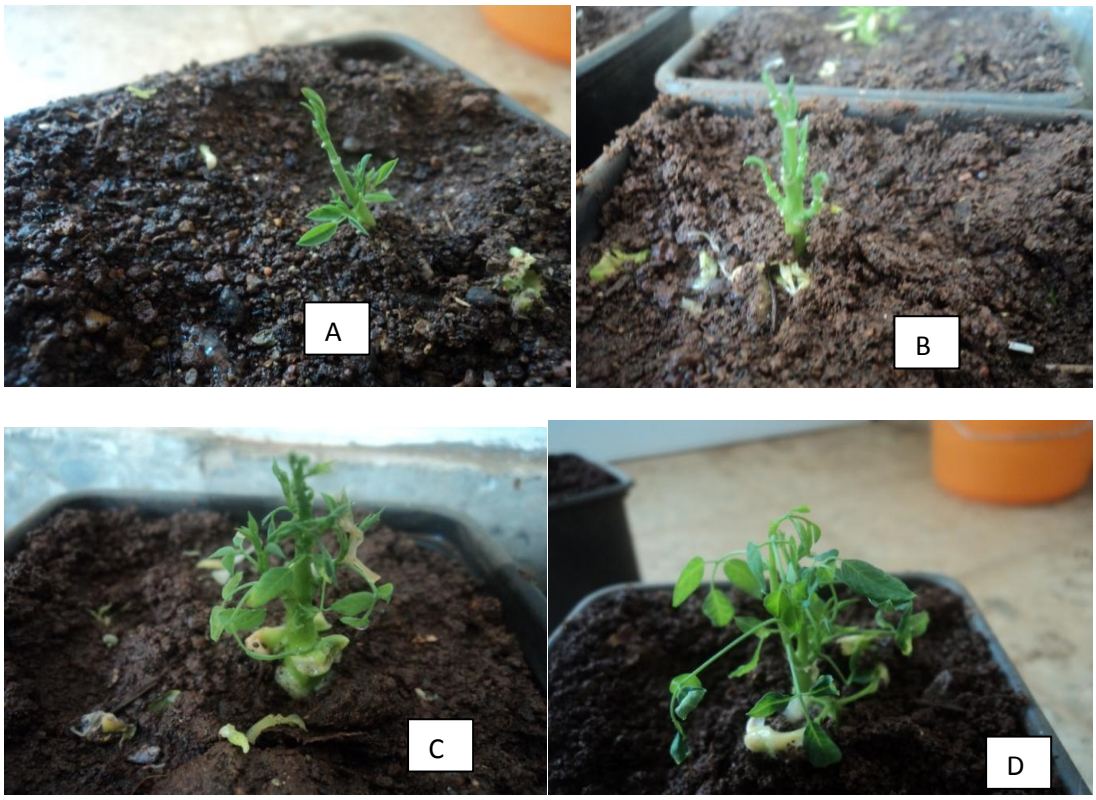

Figure 5. Acclimatization; A) during transplanting, B) after one week, C) after two weeks, D) after three weeks.

5. SUMMARY AND CONCLUSION

Moringa stenopetala is small flowering plant which is belongs to the family Moringaceae. All parts of the tree except the wood are edible and have high medicinal value. It provides highly nutritious food for both humans and animals. In spite of incredible value for both medicine and nutrition, it is not known in most part of the world other than its area of cultivation (south Ethiopia and north Kenya) except recently which has attracted the attention of scientists across the globe for health management due to its' nutritional and medicinal properties. However, its expansion is limited by different factors like the propagation method and hence needs an efficient propagation system to supply enough planting material with uniform genotype. Therefore, this study was initiated to develop a protocol for in vitro propagation of this tree.

Seeds were used to establish in vitro seedling which was used as source of explant. After the pods were removed, seeds were sterilized by 70% ethanol for 2 min followed by 0.1% mercuric chloride for 15 min. The sterilized seeds were cultured aseptically in Petri dishes contained autoclaved water with cotton support. After the seed sprouted out its radicle, it was transferred into growth hormone free MS medium by removing the seed coat aseptically. Shoot tips from seedlings were taken after 10 days of germination and cultured in MS medium supplemented with different concentration of BAP, kinetin and BAP with NAA to see their effect on shoot initiation and multiplication. The multiple clums of shoots were separated as individual shoot and well developed shoots transferred in to 1/2 MS medium supplemented with different combination of NAA and IBA for root induction. The experiments were carried out in completely randomized design (CRD) with ten replications per treatment and three explants per jar. The cultures were kept at a temperature of 25 ± 2^{o}C and light intensity of 2,000-Lux produced from cool white fluorescent tubes for 16 h photoperiod. Data like shoot number, shoot length and leaf number for shoot multiplication and root number and root length for root induction were collected, and analyzed using SAS software.

The analysis of variance revealed that the effect of the growth regulators were highly significant (P<0.001) in all tested parameters of shoot growth (shoot number, shoot length and leaf number) and root growth (root number and root length). In all applied treatments

there was 100% initiation of shoots after one week of culture of the shoot tips into shoot multiplication and initiation media.

The effect of BAP was more effective than kinetin for shoot multiplication. BAP at concentration level of 1.0mg/l exhibited the maximum mean shoot number per explant (17.40±0.74), mean shoot length (1.90±0.12) and mean leaf number (6.50±0.53). At similar concentrations (1.0 mg/l), 13.60±1.50 mean shoot numbers per explant and 1.52±0.12 mean shoot length were recorded for kin, which was the maximum from the six concentrations of kin. Increasing the concentration level from 0.0 to 1.0 mg/l has increased shoot number and shoot length in both BAP and kin. Further increased from 1.0 mg/l to 3.0 mg/l decreased shoot number in both cytokinins. In combination of BAP with NAA, the highest mean shoot number per explant (16.80±0.35) and mean shoot length (1.74±0.048) were obtained at combination of 1.0 mg/l BAP and 0.1 mg/l NAA.

When the concentration of NAA is greater than that of IBA, relatively better rooting results was recorded. Both the highest mean root numbers per shoot (10.20±0.15) and mean root length (6.30±0.15 cm) were obtained at 1.0 mg/l NAA combined with 0.0 mg/l IBA. The second highest mean root number (9.60±0.15) and mean root length (5.70±0.15) were obtained at 1.0 mg/l NAA in combination with 0.5 mg/l IBA.

Callus formation on the leaf (especially at the margin of the leaf); abnormal growth like curling of leaf and formation of callus outside of the curl; and etiolated seedling and some micro shoots were some of the problems observed during the study.

The results of this study indicate that large- scale propagation of this tree by tissue culture methods is feasible. BAP at concentration of 1.0 mg/l was found to be optimal concentration in producing maximum number of shoots per explant (17.40±0.74). Application of NAA alone was more effective than IBA for root induction. Efficient *in vitro* rooting of individual shoot culture was obtained in 1.0 mg/l NAA which resulted in both the highest number of mean roots per shoot (10.20±0.15) and mean root length (6.30±0.15).

According to the result obtained in this study MS medium supplemented with 1.0 mg/l BAP for shoot initiation and multiplication and ½ MS medium supplemented 1.0 mg/l

NAA for rooting is recommended for *in vitro* propagation *M. stenopetala* from shoot tip explants. However, further optimization of this protocol may be required for mass propagation of this tree. It is also recommended to practice other techniques like Bio-reactor for mass propagation.

6. REFERENCES

Abuye C., Urga K., Knapp H., Selmar D., Omwega A.M., Imungi J.K., Winterhalter P. 2003. A compositional study of *Moringa stenopetala* leaves. East African Medical Journal, 80 (5): 247-252

Bennett R. N., Mellon F. A., Foidl N., Pratt J. H., Dupont M. S., Perkins L. and Kroon P. A. 2003. Profiling glucosinolates and phenolics in vegetative and reproductive tissues of the multi-purpose trees Moringa oleifera L; (horseradish tree) and Moringa stenopetala L. J. Agric. Food Chem. 51: 3546–3553

Berger M. R. M., Habs S. A. A., Jahn and Schmahl D. 1984. Toxicological assessment of seeds from *Moringa oleifera* and *Moringa stenopetala*, two highly efficient primary coagulants for domestic water treatment of tropical raw waters. East African Medical Journal, 61:712-716.

Bhojwani S. S. and Razdan M. K. 1996. Plant Tissue Culture, Theory and Practice. A Revised Edition. Elsevier, Amsterdam, P. 467.

Demeulenere E. 2001. *Moringa stenopetala*, a subsistence resource in Konso district, In: proceedings: International work shop "Development potential of Moringa products" 29/10/01-2/11/01. Dareselaam, Tanzania

Edwards S., Tadesse M., Demissew S. and Hedberg I. 2002. Flora of Ethiopia and Eritrea, Vol. 2, part 1: Magnoliaceae to Flacourtiaceae. Addis Ababa, Ethiopia.

Fuglie L.J. 1999. The Miracle Tree: *Moringa oleifera*. Natural Nutrition for the Tropics, Church World Service, Dakar, p. 68.

Gassenschmidt U. K. D., Jany B., Tauscher H. and Niebergall. 1995. Isolation and characterization of a flocculating protein from *Moringa oleifera* Lam. Biochimical and Biophysica Acta 1243:477-481.

George E. and Sherington P. 1984. Plant propagation by tissue culture, hand book and dictionary of commercial laboratories. Exegenetics Ltd. Basing Stoke, UK.

George E.F., Marchakova I. and Zazimalova E. 2008. Plant propagation by tissue culture, 3rd edit. Springer, London, Pp.175-205.

Islam S., Jahan M.A.A. and Khatun R. 2005. *In vitro* regeneration and multiplication of year-round fruit bearing *Moringa oleifera* L. J Biol Sci 5:145–148

Jahn S.A.A. 1991. The traditional domestication of a multipurpose tree *Moringa stenopetala* (Bak.f.) Cuf. in the Ethiopian rift valley. Ambio 20(6): 244–247.

Kantharajah A. S. and Dodd W. A. 1991. Rapid clonal propagation of *Moringa oleifera*. South Indian Horticulture 39:224-228.

Neumann K.H., Kumar A. and Imani J. 2009. Plant Cell and Tissue Culture: A Tool in Biotechnology Basics and Application Principles. Springer, Verlag Berlin Heidelberg, Germany, P.45

Khanna V.K. 2003. Plant Tissue Culture and Practice. Kalyani Publishers, New Delhi, India, p-12-15

Lalas S., Tsaknis J., and Sflomos K. 2003. Characterisation of *Moringa stenopetala* seed oil from island Kokwa. Euro. J. Lipid Sci. Technol. 105: 123–31.

Marfori E.C. 2010. Clonal micropropagation of *Moringa oleifera* L. Philipp Agric Sci 93:454–457

Mekonnen Y. and Gessesse A. 1998. Documentation on the uses of *Moringa stenopetala* and its possible antileishmanial effect. SINET: Ethipi. J. Sci, 21(2): 287-295.

Mekonnen Y., Yardley V., Rock P., and Croft S. 1999. *In vitro* antitrypanosomal activity of *Moringa stenopetala* leaves and roots. Phototherapy Research, 13: 538–539.

Melesse A., Bulang M. and Kluth H. 2008. Evaluating the nutritive values and *in vitro* degradability characteristics of leaves, seeds and seedpods from *Moringa stenopetala*. Journal of the Science of Food and Agriculture, 89: 281–287.

Muluvi G.M., Sprent J.I., Odee D. and Powell W. 2004. Estimates of out crossing rates in Moringa *oleifera* Amplified Fragment Length Polymorphism (AFLP). African Journal of Biotechnology, 3: 146-151.

Murashige T. and Skoog F. 1962. A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiologia Plantarum, 15:473-497.

Nieves M.C. and Aspuria E.T. 2011. Callus Induction in Cotyledons of Moringa oleifera. Lam, 94(3): 239-247

Olsen A. 1987. Low technology water purification by bentonite clay and Moringa oleifera seed flocculation as performed in Sudanese villages: Effects on Schistosoma mansoni cercariae. Water Research, 21:517-522.

Olson M. E. 2002. Combining data from DNA sequences and morphology for a phylogeny of Moringaceae (Brassicales). Systematic Botany, 27:5573.

Omar M.S. and Aouine M. 2007.Commercial *in vitro* Mass Propagation of Plants: Current Status and Future Investment Prospects. J Agric. Inv. (JAI special issue).

Saini R.K., Shetty N.P., Giridhar P. and Ravishankar G.A. 2012. Rapid in vitro regeneration method for *Moringa oleifera* and performance evaluation of field grown nutritionally enriched tissue cultured plants. Food Technological Research Institute, CFTRI, Mysore 570 020, India

Shibru S. 2002. A Wonderful but neglected tree species in Ethiopia: Cabbage tree. Biological Newsletter.1 (2): 6-8.

Singh H.P. 2010. Morniga; A crop of future, brain storming session on Moringa. Tamil Nadu Agticultural University, Coimbatore.

Steinitz B., Tabib Y., Gaba V., Gefen T. and Vaknin Y. 2009. Vegetative micro-cloning to sustain biodiversity of threatened Moringa species. In Vitro Cell. Dev. Biol.-Plant, 45: 65-71.

Steinmuller N., Sonder K. and Kroschel J. 2002. Fodder tree research with *Moringa stenopetala*: a daily leafy vegetable of Konso People, Ethiopia. Journal of the Science of Food and Agriculture, 89: 281 - 287.

Stephenson K.K, and Fahey J.W. 2004. Development of tissue culture methods for the rescue and propagation of endangered Moringa spp. germplasm. Econ Bot 58:116–124

Demel T. 1995. The effect of temperature on the Germination of Moringa stenopetala, A multipurpose tree. Tropical Ecology, 36:49-57.

Verdcourt B .1985. A synopsis of the Moringaceae. Kew Bull, 40:1–23

Weiler E.W. 1984. Immunoassay of plant growth regulators. Am. Review of plant phsiol, 35: 85-95.

Wareing P.F. and Phillips I.D.J. 1981. Growth and Differentiation in plants, 3rd edu. Pergamon press, Oxford. New York.

Zhang J. 2007. Studies on Tissue Culture and Induction of Moringa Oleifera Lam. Southwestern University, 565.9

APPENDICES

Appendix Table 1: ANOVA summary for the effect of BAP on shoot multiplication

Source of freedom	Degree of	Mean Square		
		Shoot number	**Shoot length**	**Leaf number**
BAP	6	302.34***	0.31***	1.73***
CV		6.55	7.33	8.82

***=very significant at $P \leq 0.0001$, P= probability value at $P \leq 0.05$, CV= coefficient of variance

Appendix Table 2: ANOVA summary for the effect of Kin on shoot multiplication

Source of freedom	Degree of	Mean Square		
		Shoot number	**Shoot length**	**Leaf number**
Kin	6	140.12***	0.15***	1.52***
CV		9.33	7.22	9.14

***=very significant at $P \leq 0.0001$, P= probability value at $P \leq 0.05$, CV= coefficient of variance

Appendix Table 3: ANOVA summary for the effect of BAP and NAA on shoot multiplication

Source	Degree of freedom	Mean Square		
		Shoot number	**Shoot length**	**Leaf number**
BAP*NAA	12	140.12***	0.16***	4.29***
CV		4.98	11.83	9.88

***=very significant at $P \leq 0.0001$, P= probability value at $P \leq 0.05$, CV= coefficient of variance

Appendix Table 4: ANOVA summary for the effect of NAA and IBA on rooting of shoots

Source	Degree of freedom	Mean Square	
		Root number	**Root length**
NAA	4	192.31***	64.48***
IBA	4	16.12***	9.53***
NAA * IBA	16	23.68***	7.48***
CV		3.63	4.69

***=very significant at $P \leq 0.0001$, P= probability value at $P \leq 0.05$, CV= coefficient of variance

Appendix Table 5: Modified MS Media components and stock solutions (prepared during the study time)

Component	Concentration in one litter (g/l)	Concentration in stock solution(g)	Amount taken for 1litrer medium
Macroelements			
Stock solution 1		10X (g/1000 mL)	
NH4N03	1.650	16.5	
KNO_3	1.900	19	100ml
$MgS0_4.7H_20$	0.370	3.7	
KH_2P0_4	0.170	1.7	
Sock solution 2		10X (g/100 mL)	10ml
$CaCl_2.2H_20$	0.440	4.4	
Microelements			
Stock solution 3		1000x(g/100ml)	
$CuS0_4.5H_20$	0.000025	0.025	0.1ml
$CoC1_2.6H_20$	0.000025	0.025	
Stock solution 4		100X (g/500 mL)	
H_3B0_3	0.00620	0.62	
$MnSO_4.4H_20$	0.02230	2.230	5ml
$ZnS0_4.7H_20$	0.008600	0.860	
KI	0.000830	0.083	
$Na_2Mo0_4.2H_20$	0.000250	0.025	
Stock solution 5		100X (g/500 mL)	
$FeSO_4 \cdot 7H_2O$	0.02780	2.78	5ml
$Na_2EDTA \cdot 2H_2O$	0.03720	3.72	
vitamins(mg/L)			
Stock solution 6		1000X (g/100 mL)	
Nicotmic acid	0.0005	0.5	
Pyrrdoxine-HCl	0.0005	0.5	0.1ml
Thiamine-HCl	0.0001	0.1	
Glycine	0.002	2.0	
Myo-inositol	100mg	-	100mg
Table sugar	30 g/l	-	30 g/l
Agar	8.5g/l	-	8.5g/l
Plant Growth Regulator		g/100 mL	
BAP	1	0.1	
IBA	1	0.1	
NAA	1	0.1	