ADDIS ABABA UNIVERSITY

INSTITUTE OF BIOTECHNOLOGY

In vitro regeneration of *Moringa oleifera (*moringa tree) Lam. from leaf explants

By:

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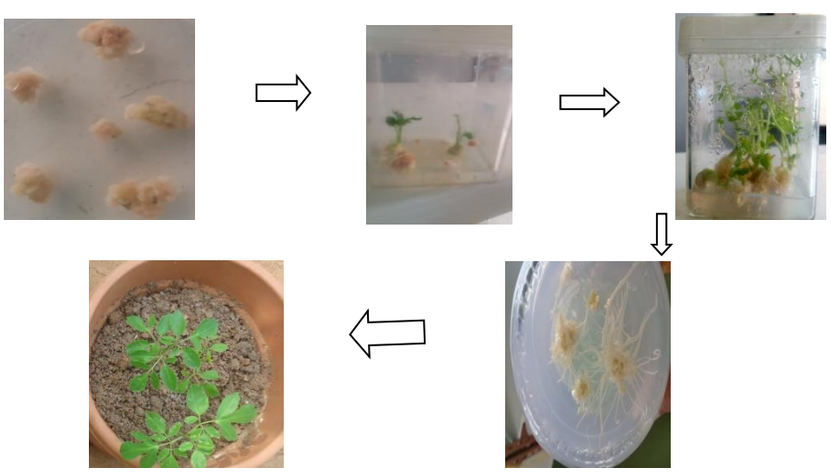

A Thesis Submitted to the School of Graduate Studies, Addis Ababa University, in Partial Fulfillment of the Requirement for the Degree of Master of Science in Biotechnology

January, 2017

ADDIS ABABA, ETHIOPIA

DECLARATION

I hereby declare that this is my original work and has not been submitted for a degree or any other award in any university.

Meskerem Mamo

Signature: ---------- Date: ----------

The work has been done under my supervision

Advisor: Tileye Feyissa (PhD)

Signature: ---------- Date: ----------

ADDIS ABABA UNIVERSITY
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This is to certify that the thesis prepared by **Meskerem Mamo** entitled ***in vitro* regeneration of moringa oliefera (moringa tree) lam.** and submitted in partial fulfillment of the requirements for the degree of Master of Science in Biotechnology complies with the regulations of the university and meets the accepted standards with respect to originality and quality.

Signed by the Examining Committee:-

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LIST OF ABBREVIATIONS

BAP	6-Benzyl Amino Purine
IBA	Indol-3-Butyric Acid
MS	Murashige and Skoog (1962) basal medium
NAA	Naphthalene acetic acid
ANOVA	Analysis of Variance
PGR	Plant Growth Regulator

ABSTRACT

Moringa oleifera is the most important tree because each and every part of the plant has nutritional and medicinal use. In Ethiopia studies related to this important plant are based on micropropagation. Therefore, the main objective of this study was to optimize an in vitro regeneration protocol for Moringa oleifera by using leaf as explants. Young leaves from in vitro multiplied shoots were excised, wounded perpendicular to midrib and cultured on MS medium supplemented with different concentrations of BAP in combination with NAA. For regeneration of shoot, the calli were cultured on MS medium supplemented with different concentrations of BAP (0.0, 0.5, 1.0, 1.5, 2.0, mg/l) in combination with NAA (0.0, 0.5, 1.0, 1.5, 2.0 mg/l). Multiplication of regenerated shoots was done on MS medium supplemented with different concentrations of BAP (0.0, 0.5, 1.0, 1.5,2.0) in combination with IBA (0.0,0.5, 1.5, 2.5, 3.5 mg/l). Rooting was achieved by culturing well developed shoots in half strength MS medium containing 0.5 1.5, 2, 2.5, mg/l NAA or 0, 0.5, 1.0, 2.0, 3.0, 4.0 mg/l IBA. The highest (73.3%) percentage of callus induction was obtained from the MS medium supplemented with 0.5mg/l BAP. When the callus transferred to the regeneration medium containing 0.5 NAA, it resulted in the maximum regeneration rate of shoot (33.3%). Statistical analysis revealed that there was significant difference among all treatments applied in both shoot multiplication and rooting experiments. Maximum number of shoots per explants (3.13±0.73) was obtained on MS medium containing 1.0 mg/l BAP. The highest mean number of roots per shoot (9.60±0.86) was obtained on MS medium containing 0.5mg/l IBA. After acclimatization, 90% plants survived in greenhouse. This protocol can be used for genetic improvement of this tree species.

Key words: Moringa oleifera, Invitro regeneration, Callus induction, shoot multiplication

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1. INTRODUCTION

Moringa oleifera is the most widely cultivated species of the genus *Moringa*, which is the only genus in the family Moringaceae. It is commonly known in most of the world by the name of drumstick tree or horse-radish tree (Sabale *et al.,* 2008). *Moringa oleifera* is a drought-tolerant (Ashfaq *et al*, 2012), fast-growing evergreen or deciduous tree that mostly grows up to 10 to 12m in its height (Roloff *et al*, 2009). The leaves are bipinnate or more commonly tripinnate, up to 45 cm long, and are alternate and spirally arranged on the twigs (Roloff *et al*, 2009).

Moringa oleifera is multi-purpose and one of the most useful trees. Therefore, it is described as a 'miracle tree' (Ashfaq *et al.,* 2012). This tree has many uses including nutritional and medicinal values.

Every part of the moringa tree is edible .The tree has maximum leaves at the end of the dry season when other foods are typically scarce and it is a promising food source especially the leaves, which are rich in nutrients and minerals (Fuglie, 1999).

Almost every part of moringa has medicinal value and used to cure different diseases. Especially the leaves are the most commonly used part of the plant to treat different diseases like hyperglycemia, asthma, flu, heart burn, Dyslipidemia, malaria, syphilis, diarrhea, pneumonia, scurvy, headaches, bronchitis, skin diseases, and eye and ear infections. The leaves also reduce blood pressure and cholesterol, act as an anticancer, antioxidant, antimicrobial, anti-atherosclerotic, anti diabetic agents, and neuroprotectant (Ali *et al.,* 2015) and niaziminin. The alcoholic extract of leaves of *Moringa oleifera* was reported to have analgesic activity (Nitin *et al.,* 2008).

Root and stem of the plant also cure diseases due to the presence of different chemicals like an immune enhancing polysaccharide (Mondal *et al*, 2004). *Moringa oleifera* is used as a drug by many ayurvedic practitioners for the treatment of asthma and methanolic extract of *Moringa oleifera is also used to treat adult* Ethiopian earth worm's (*Pheretima posithuma)* (Iswar *et al*., 2010).

The ability of the tree to mitigate the effects of climate change is also impressive. According to the study of Villafuerte and Villafurte-Abonal (2009), *Moringa oleifera* has high ability to

absorb carbon dioxide from the environment. The rate of moringa tree to absorb CO_2 is twenty times higher than that of general vegetation. It has great contribution in reducing the global warming. *Moringa* tree is useful tool in the prevention of global warming; because it sequesters more carbon with its all parts. Therefore, planting such important tree in different parts of the country will mitigate the impacts of climate change. (Mekonin Deba, 2016)

Additionally, *Moringa oleifera* is also used for biogas production, food for animal, domestic cleaning agent, fertilizer, gum, manufacture of perfume and hair care products (Tsaknis *et al*, 1999).

The preservation of the *Moringa* species is thus of great concern from biodiversity, ethno botanical, dietary and pharmacological perspectives.

Establishment of an efficient *in vitro* regeneration protocol is an essential prerequisite for improving plant through different techniques like *in vitro* screening, genetic engineering, genome editing and etc. *Moringa oleifera* is important plant in the world. In Ethiopia tissue culture studies related to *Moringa oleifera* are depend on micropropagation. So this research is aiming to develop *in vitro* regeneration protocol for *Moringa oleifera Lam*. using leaf as explants. For the future, this protocol will be used to improving *moringa oleifera* and other plant by genetic transformation. Specifically, it increases capacity of research institutions, pharmaceutical industries to carry out tissue culture and genetic transformation research and develop genetically transformed medicinal plants of *Moringa oleifera*.

2. OBJECTIVES

2.1. General objective

- To develop efficient *in vitro* regeneration protocol for *Moringa oleifera* from leaf explants

2.2.Specific objective

- To optimize concentration and combination of plant growth hormone for callus induction and shoot regeneration from callus
- To optimize concentration and combination of plant growth hormone for shoot multiplication and for rooting

3. LITERATURE REVIEW

3.1. Overviews of *Moringa oleifera* Lam.

The *Moringaceae* family has a genus known as *Moringa*, which contains 13 species of shrubs and trees originated in tropics and subtropics (Steinitz *et al*., 2009). *Moringa oleifera* is the most widely cultivated species of *Moringaceae* family (Arora *et al.*, 2013; Fahey, 2005). It is a pan tropical multipurpose tree with a high biomass yield and capable of tolerating unfavorable environmental conditions (Foidl *et al*., 2001). The Moringa tree (*Moringa oleifera*) has many names throughout the world, likely due to its profligate uses, It is called the 'drumstick tree' due to the shape of its seed pods, the 'horseradish tree' because of the faint scent and flavor of horseradish that the tree's roots give off and the 'ben oil tree' drawn from the oil that is pressed from the seeds. The most explicit of all its names, though, is the 'miracle tree' which is inspired by this unassuming tree's has endless benefits (Garcia-Fayos *et al.*, 2010).

3.2. Geographical distribution of *Moringa oleifera*

In the monogeneric genus Moringa of Moringaceae family there are 13 species (namely, *M. arborea*, indigenous to Kenya; *M. rivae* indigenous to Kenya and Ethiopia; *M. borziana*, indigenous to Somalia and Kenia; *M. pygmaea* indigenous to Somalia; *M. longituba* indigenous to Kenia, Ethiopia and Somalia*; M. stenopetala* indigenous to Kenya and Ethiopia; *M ruspoliana* indigenous to Ethiopia; *M. ovalifolia* indigenous to Namibia and Angola; *M. drouhardii*, *M. hildebrandi* indigenous to Madagascar; *M. peregrine* indigenous o Red sea and Horn of Africa, *M. concanensis*, indigenous to sub-Himalayan tracts of Northern India (Paliwal *et al*., 2011).

Moringa oleifera is native to sub- Himalayan Mountains of northern India. Its cultivation extends in the Himalayan foothills of south Asia from north eastern Pakistan to north-western Bengal in India and north-eastern Bangladesh at an elevation of 1400 m above sea level. It is extensively grown near houses in Assam, Bengal and Peninsular India and also maintained as a coppice. Its cultivation and usage has gained momentum in South East Asia, West Asia, Arabian Peninsula, East and West Africa, West Indies and Southern Florida, Central and South America from Mexico to Peru including Brazil and Paraguay (Ganatra *et al*., 2012). It is considered one of the world's most useful trees, as almost every part of *Moringa* tree can be used for food or has some other beneficial properties (Paliwal *et al*., 2011).

Moringa tree originates from India and was introduced to Ethiopia long ago. The tree is now naturalized in many parts of southern Ethiopia. Konso people plant *Moringa tree* around their homesteads and also in the terraced fields (Padayachee and Bajnath, 2012).

3.3. Botanical description of *Moringa oleifera* Lam.

Moringa oleifera is a fast-growing evergreen and deciduous tree and attains a height of 10-12 meters. It bears drooping, fragile branches covered with thick, corky, whitish bark (Rollof, *et al*., 2009). The stem is brittle with a corky, whitish-gray bark, with drooping branches, pale green and bipinnate or more commonly tripinnate leaves (30–45 cm long) with opposite, ovate leaflets (Pandey *et al.*, 2011).

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

The stem is normally straight but occasionally poorly formed. The tree grows with a short, straight stem that reaches a height of 1.5-2 m before it begins branching but can reach up to 3.0 m (Foidl *et al*., 2001). The extended branches grow in a disorganized manner and the canopy is umbrella shaped.

Taxonomy of *Moringa oleifera*

Moringa oleifera is the best known of the thirteen species in the genus Moringa of family *Moringaceae.* These are *Moringa oleifera*, *Moringa arborea*, *Moringa borziana*, *Moringa concanensis*, *Moringa drouhardii*, *Moringa hildebrandtii*, *Moringa longituba*, *Moringa ovalifolia*, *Moringa peregrine*, *Moringa pygmaea*, *Moringa rivae*, *Moringa ruspoliana* and *Moringa stenopetala* (Mahmood *et al*., 2010) the taxonomy of *moringa oliefera* is given below

Kingdom – Plantae

Division – Magnoliophyta

Class – Magnoliosida

Order – Capparales

Family – Moringaceace

Genus – *Moringa*

Species - Moringa oleifera

3.4. Ecology of *Moringa oleifera Lam.*

Optimum leaf and pod production requires high average daily temperature of 25-30 ˚C, well distributed annual rain fall of 1000- 2000 mm high solar radiation and well drained soils. Growth slows significantly under temperature below 20°C. Minimum annual rainfall requirement are estimated at 250 mm with maximum at over 3000mm, but in waterlogged soil the roots have a tendency to rot. It is usually found in areas with a temperature range of 25˚C to 40˚C but will tolerate 48˚C and light frosts. Moringa is relatively tolerant of drought and poor soils, and responds well to irrigation and fertilization. It grows in areas reaching 1400 meters above sea level with full sun exposure. Indeed, it has been reported to grow well in arid regions at average of annual rainfall less than 300 millimeters (Radovich, 2009).

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

Moringa oleifera seeds can be planted just after maturity, as the seeds do not undergo dormancy while retaining viability up to 1 year. The tree starts bearing fruits at an age between six and 8 months, with a low fruit set in the initial 1 to 2 years, however, the yield increases in the subsequent years (da Silva *et al*., 2010).

3.5. Uses of *Moringa oleifera*

Having value as a food item, a medicinal stock, a source of food oil, biofuel, and a water purifier, there is little wonder why it came to be known as the ‘miracle tree’. Some uses of the plants are given below

3.51. Nutritional value

The Moringa tree is often considered as important famine food because of its high tolerance to drought and arid conditions owing to their tuberous roots (Padayachee and Baijnath, 2012). It is especially promising as a food source in the tropics because the tree is in full leaf at the end of the dry season when other foods are typically scarce (Fahey, 2005).

Every part of the tree is edible. Different research reports show that the leaves are a storehouse of nutrients. It contains all the essential nutritional elements that are vital for human beings and livestock and it is a super food tree with a remarkable source of proteins, calcium, and Iron (Ali, *et al.*, 2015). Vitamins like beta-carotene of vitamin A, vitamin B such as folic acid, pyridoxine and nicotinic acid, vitamin C, D and E are also present in *Moringa oleifera* (Mbikay, 2012). On a dry matter basis, *Moringa oleifera* leaves contain 27.2% protein, 17.1% fat, 5.9% moisture and 38.6% carbohydrates (Yameogo *et al.*, 2011). The leaves also have considerable contents of trace minerals, beta-carotene, thiamin and riboflavin while the protein has significant quantities of all the essential amino acids, making it a complete protein (Parrotta, 2009). *Moringa oleifera* dry leaves contain 9 times proteins than yogurt, 10 times vitamin A than carrot, 25 times iron than spinach, 15 times potassium than bananas, 17 times calcium than milk and 7 times more vitamin C than orange. So *Moringa* leaves have been used to fight malnutrition, especially among infants and nursing mothers, for fast uterus contraction during child birth in pregnant women (Dangi *et al*., 2002). The leaves can be eaten fresh cooked or stored as dried powder for several months (Parrotta, 2009).

Folate is one of the most important water-soluble vitamins, plays an essential role in various cellular metabolisms, including oxidation and reduction of one-carbon units (Scotti *et al.*, 2013).

Folate deficiency causes severe chronic diseases and developmental disorders, including neural tube defects during pregnancy (Williams *et al*. 2015). Thus, a folate-sufficient diet is strongly recommended during pregnancy to prevent the neural tube defects and other chronic dysfunctions. *Moringa oleifera* contains folate on in the leaves (Saini *et al,* 2016). Thus, it is suggested that *Moringa oleifera* based food can be used as a significant source of folate (Scotti *et al.*, 2013).

According to Parrotta (2009) the root, with their horseradish flavor, are stripped of their bark because of its high alkaloid content, mixed with vinegar and used as a condiment.

The seeds contain 30-35% oil that is high in palmetic, stearic, behmic, and oleic acids and has similar flavor and properties to olive oil making it a highly nutritive alternative to other vegetable oils (Garcia-Fayos *et al.*, 2010). Young green seed pods which are high in ascorbic acid are boiled, steamed or pickled like string beans or asparagus and are a common addition to soups and stews in the tree's native areas (Radovich, 2009). This simple tree could therefore pose at least part of the solution to the nutritional famine present in much of the developing world.

3.5.2. Health benefit

The products derived from several herbs and plants, being a source of multifunctional curing agents and bioactive compounds, are relatively considered safe for consumption. According to the Food and Agriculture Organization's (FAO) report, about 70–80 % of the world's population, especially in developing countries, relies on herbal medicine to prevent and cure diseases (Ekor, 2014). *Moringa oleifera* has an impressive range of medicinal uses. Almost all parts of this plant: root, bark, gum, leaf, fruit (pods), flowers, seed have been used for treating various ailments (Kesharwani *et al*., 2014) such as skin infections, anemia, coughs, diarrhea, swelling, headaches, gout, acute rheumatism, hysteria, cholera, heart complaints, fever, respiratory disorders, inflammation, digestive disorders, asthma, intestinal complaints, diabetes and rheumatism (Padayachee and Bajnath, 2012). *Moringa oleifera* contains isothiocyanates which have been shown to have antitumor and anti-carcinogenic properties Radovich (2009). The leaf of *Moringa oleifera* have pharmacologically important chemical compounds such as Carbohydrates, Saponins, Tannins, Steroids, Flavonoids, Coumarins ,Quinine, phenolicn compound and Alkaloids (*Kassa Belay and Mesay Sisay* 2014) In addition, several low molecular weight bioactive compounds from moringa seeds with bactericidal, fungicidal and immunosuppressive activities (Mahajan and Mehta, 2010). The flowers, leaves, and roots are used in folk remedies for treatment of tumors and the seeds for abdominal tumors. Bark regarded as antiscorbic and exudes a reddish gum with properties of tragacanth is sometimes used for treating diarrhea. Roots are bitter and act as a tonic to the body and lungs. Moreover, bioactive nitrile glycosides niaziridin and niazirin in the leaves, pods and bark (Shanker *et al*., 2007) and coagulant lectin as bio insecticide (Oliveira *et al*., 2010). Protease isolated from *moringa oleifera* has a potential for use as seafood preservative against proteolysis in Penaeus monodon on storage was also evaluated. According to ethnobotanical studies, its roots are bitter, acrid, thermogenic, digestive, carminative, anthelmintic, constipating, anti inflammatory,

emmenagogue, diuretic, ophthalmic, expectorant and stimulant. They are useful in dyspepsia, anorexia, verminosis, diarrhea, colic, flatulence, paralysis, inflammations, amenorrhea, dysmenorrheal fever, strangury, vesicle and renal calculi. It is used in cough, asthma, bronchitis, pectoral diseases, splenomegaly, epilepsy and cardiopathy (Nepolean *et al.*, 2009).

The different parts of *Moringa oleifera* tree, including roots, bark, leaves, flowers, fruits, and seeds are traditionally used in various therapeutic applications, including, abdominal tumors, hysteria (a psychological disorder), scurvy paralysis, helminthic bladder, prostate problems, sores and other skin infections (Farooq *et al.*, 2012)**.**

The highest content of glucosinolate is found in the leaves and seeds. The enzymatic catabolism of glucosinolates by the endogenous plant enzyme myrosinase produces isothiocyanates, nitriles, and thiocarbamates that are known for strong hypotensive (blood pressure lowering) and spasmolytic (muscle relaxant) effects (Anwar *et al*., 2007).

3.5.3. Water purification property

Moringa oleifera has been shown to contain water-soluble proteins that act as coagulants. Several studies have demonstrated that, when the seeds of *Moringa oleifera* are crushed and dissolved into the water, they attracts dominant negatively charged particles such as clay, silk, and other toxic particles (Schwarz, 2000).

Then effectively precipitates mineral particulate and various organics out of solution. The mechanism of action that facilitates precipitation is attributed to the ability of charged protein molecules of *Moringa oleifera*. Protein produces a positive charge that acts like a magnet and has a potential of organic pollutant absorber in solution (Akhtar *et al*., 2007), bind and flocculate soluble particulate matter (Suarez *et al.*, 2003)

3.5.4. Other uses of *Moringa oleifera*

Beyond the uses of moringa as a food, water purifier and for human health, other possible uses exist. From the moringa fresh leaves juice can be extracted and used as a growth hormone that can increase yields of crop by 25-35% (Foidl *et al*., 2001). Amazingly, leaf extracts and also seed extracts of moringa show biopesticide activity, effective against larvae and adults of *Trigoderma granarium* and can reduce the incidence of fungi on groundnut seeds (Leone *et al*., 2015).

Moringa seed oil is a potential candidate for biodiesel production, as it meets all the main specifications of the biodiesel standards of US, Germany, and Europe (Mofijur *et al.*, 2014). The seeds also contain 30 to 40% oil which is highly valued in the cosmetic industry for its unique property beside having exceptional anti ageing and anti cancer properties due to the antioxidants and the nutrients present in it that help to curb the activity of the free radicals on the skin. (Palada,1996). *Moringa oleifera* is also used as animal food. When Moringa leaves are added to cattle feed, it increased their daily weight gain by up to 32 percent. Feed of milk cows was supplemented with 15 to 17 kilograms of fresh *Moringa* leaves daily, and the cattle's milk production increased by 43 percent (Mohmood *et al*.,2010).

3.6. Plant tissue culture and its application

Tissue culture is the *in vitro* aseptic culture of cells, tissues, organs or whole plant under controlled conditions. The controlled condition provides the culture an environment conductive for their growth and multiplication. These conditions include proper supply of nutrients, pH, adequate temperature and proper gaseous and liquid environment (Neumann *et al.*, 2009). The theoretical basis for plant tissue culture was proposed by Gottlieb Haberlandt in 1902. He predicted that eventually a complete and functional plant could be regenerated from a single cell.

Along with the totipotent potential of plant cell, the capacity of cells to alter their metabolism, growth and development is also equally important and crucial to regenerate the entire plant (Thorpe, 2007). Tissue culture techniques are being increasingly exploited for clonal multiplication and *in vitro* conservation of valuable indigenous germplasm threatened to extinction. Greater demand for plants especially for the purpose of food and medicine is one of the causes of their rapid depletion from primary habitats (Boro *et al*., 1998**).** In addition, plant tissue culture plays an important role in rapid micro propagation of many agronomically important plants (Iktena and Reada, 2010).

It also represents an efficient method of plant regeneration and rapid propagation through organogenesis and embryogenesis of any valuable genotype obtained by non conventional methods. Therefore, regeneration of whole plant by somatic embryogenesis and organogenesis result in formation of plantlet from determined tissue in order to form complete plant (Aazami *et al.*, 2010).

3.7.1. *In vitro* regeneration

Depending on the plant species and cultural condition, tissue culture may enable the mass production of genetically homogenous population from elite (e.g. high yielding or disease resistant) individuals. There by introduction of foreign genes by genetic engineering technique as a means of plant improvement required the development of an efficient regeneration system for the desired plant species. Such a system must be rapid, reliable and applicable to a broad range of genotypes.

Until the early 1980s, efficient regeneration of plants from cultured cell and tissues of most of the important food crops had proven to be very difficult. The problem was solved by the culture of explants from immature tissues which retain their morphogenetic potential, on nutrient media containing potent plant growth regulator (Deore and Johonson, 2008). However, the lack of efficient tissue culture system generally applicable to agriculturally important crop is the major obstacle in the application of genetic engineering technology. Similarly, this problem was also the problem of *Moringa oleifera*. Thus, an establishment of such efficient protocol for high frequency direct regeneration of plantlets from leaf explants *of Moringa oleifera* has a vital role for the application of genetic engineering technology, for analyses of genetic material and mass propagation of plants in short period of time.

Different studies done related to moringa oliefera for example Devendra *et al* ,2010 done on callus induction and somatic embryogenesis of *moringa olifera* in india and Riyathong *et al* 2010 done on shoot multiplication and and plant regeneration from *in vitro* culture of moringa tree in Thailand, this study is aiming to develop *in vitro* regeneration protocol for this multipurpose tree

4. MATERIALS AND METHODS

4.1. Source of explants

Seed are collected from Mojo around Debrezeit, then multiplied in plant tissue culture laboratory in Addis Ababa University, Young leaves from *in vitro* multiplied shoots were used as explants source for *in vitro* regeneration.

4.2. Preparation of stock solution and culture media

4.2.1. Preparation of stock solution

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

4.2.2. Growth regulators stock solutions preparation

Different plant growth regulators, BAP, NAA and IBA were used in different concentrations. Each of these hormones was prepared by dissolving 50 mg of powdered hormone in 50 ml of distilled water to prepare 1.0 mg/ml stock solution. The dissolving process was started by adding a drop of 1M NaOH for auxin (NAA and IBA) and for cytokinin (BAP). Then the volume was adjusted by adding enough distilled water. Then the solutions were stored in refrigerator until used.

4.2.3. Culture media preparation

Murashig and Skoog (1962) MS basal medium was used throughout the experiments. MS stock solution was prepared by mixing 50ml/l macronutrient, 5ml/l micronutrient, 5ml/l vitamin and 5ml/l iron EDTA. Then 30g/l sucrose was added followed by addition of growth regulators. The pH was adjusted to 5.8 by using 1M NaOH or 1M HCl. Finally, 7g/l agar was added and dissolved by heating in microwave oven until agar was melted. Then 50 ml of the medium was dispensed in to culture vessel. The medium was sterilized by autoclaving at a temperature of 121^{o}C and a pressure of 105 Kpa for 15 minute. After autoclaving, the medium was transferred to laminar air flow cabinet for cooling. But in the case of callus induction, 25 ml of the medium was dispensed in to each Petri dish in laminar airflow cabinet immediately after autoclaving.

4.3. Culture Conditions

Except callus induction, 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 light intensity. But for callus induction the cultures were kept at a temperature of 25±2 ° C under dark condition.

4.4. Callus induction

Young leaves from in vitro multiplied shoots were used for callus induction. The leaves from multiplied shoots were excised and wounded perpendicular to midrib. The wounded leaves were cultured upside down on a Petri dish containing 25 ml MS medium supplemented with different concentrations of NAA (0.0, 2.0, 3.0, 4.0, 5.0, 6.0 mg/l) in combination with (0.0, 0.5, 1.0, 2.0, 3.0 mg/l) BAP . Six explants were cultured in each Petri dish with 5 replications for each treatment. The cultures were maintained at 25 ± 2°C under dark conditions until callus was induced.

4.5. Shoot regeneration

The induced calli were removed aseptically from the Petri dishes on a sterilized glass plate inside the laminar air flow cabinet and transferred to jars containing 50 ml MS medium with different concentration of NAA (0.0, 1, 1.5, 2, mg/l) in combination with BAP (0.0, 0.5, 1.5, 2, mg/l). The cultures were kept in dark condition at 25 ± 2°C until shoots of about 0.5 cm appear from the callus. Then the cultures with regenerated shoots were transferred to light intensity of 22 µmol $m^{-2}s^{-1}$ at 16 h photoperiod with the same temperature of the above. A total of 30 samples were used. Five explants per jar with 6 replications per treatment were used.

4.6. Shoot multiplication

Shoots regenerated from calli were excised aseptically in side laminar air flow cabinet and placed into culture jars containing 50 ml of MS medium supplemented with different concentrations of IBA (0.0, 0.5, 1.5, 2.5, 3.5 mg/l) in combination with BAP (0.5, 1.0, 1.5, 2.0 mg/l). Five explants were cultured in each jar with 6 replications. The cultures were maintained under the same condition used for shoot regeneration for 4 weeks.

4.7. Rooting

The multiplied shoots were excised aseptically inside laminar air flow cabinet and cultured in culture jar containing 50 ml half strength MS medium supplemented with different concentrations of NAA (0.5 1.5, 2.0, 2.5, mg/l or IBA (0.0, 0.5, 1.0, 2.0, 3.0 mg/l) and 15 g/l

sucrose was added.. Five explants were cultured in each jar with 6 replications. The cultures were maintained at 25 ± 2°C with 16 h photo period at light intensity of 22 μmol $m^{-2}s^{-1}$.

4.8. Acclimatization

Well rooted plantlets were taken out of the medium without damaging the roots and washed thoroughly in running tap water to remove all the residues of culture medium. Then the plantlet was planted in plastic pots containing a sterile a sterile garden soil and covered with light plastic covers. The pots were kept in the culture room for two weeks by giving adequate water. After two weeks, the plants were transferred to greenhouse. The plastic covers were gradually removed after 2 weeks. Finally, the plants were fully exposed to the normal growth conditions and the survival rate was evaluated after a month.

4.9. Data analysis

A completely randomized design (CRD) was used for all experiments. Callus induction and regeneration percentage was calculated. For multiplication and rooting, data were subjected to one-way analysis of variance (ANOVA) to detect if there are significant differences among treatments. To detect homogeneity of variance, the means of different treatments were analyzed by using Turkey's test using statistical data analysis software SPSS 20.0 version at 5% probability level.

5. RESULTS

5.1. Callus induction

The effect of NAA, BAP and the combination of the two for callus induction after 5 weeks of culture were compared. The results of the present study showed that using high concentration of NAA (6 mg/l) and low concentration of BAP (0.5mg/l) is better than the combination of BAP and NAA for callus induction. In all treatments, there were differences in the rate of callus induction. The percentage of callus induction ranged from 0.0% to 73.3% (Tables 1). Among all the treatments, 0.5 mg/l BAP resulted in the highest percentage of callus induction (73.3%) (Table1). The second (70%) and third (66.6%) highest percentage of callus induction were obtained on medium containing 6.0 mg/l and 5 mg/l NAA respectively (Table 1).

Table 1 : Effects NAA and BAP on callus induction

NAA(mg/l)	BAP(mg/l)	% of callus induction
0	0	23.3%
2	0	40%
3	0	16.16%
4	0	60%
5	0	66.6%
6	0	70%
0	0.5	73.3%
0	1	6.6%
0	2	43.3%
0	3	25%

0	0	23.3%
2	**0.5**	**16%**
2	**1**	**23.3%**
2	**2**	**10%**
2	**3**	**16%**
3	**0.5**	**23.3%**
3	**1**	**3.3%**
3	**2**	**30%**
3	**3**	**40%**
4	**0.5**	**10%**
4	**1**	**0%**
4	**2**	**0%**
4	**3**	**0%**
5	**0.5**	**30%**
5	**1**	**0%**
5	**2**	**6.6%**
5	**3**	**0%**

6	**0.5**	**20%**
6	**1**	**0%**
6	**2**	**0%**
6	**3**	**33.3%**

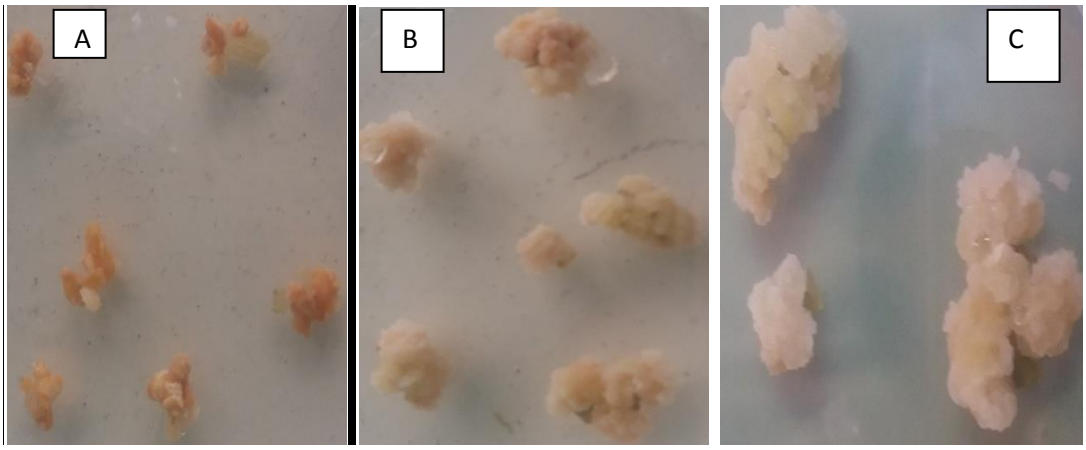

Figure 6: Callus induction from leaf explants of *Moringa oleifera* on MS medium supplemented with BAP and NAA. (A) 6.0 mg/l NAA; (B) 5.0 mg/l NAA and (C) 0.5 mg/l BAP

5.2. Shoot regeneration

MS medium supplemented with 0.5 mg/l NAA resulted in the highest percentage of regeneration (33.3 %) followed by medium supplemented with 0.5 mg/l BAP resulted in 30% regeneration (Table 2). Except combination of 0.5 NAA with 0.5BAP and 2 NAA with 0.5 BAP, all MS media supplemented with a combination of NAA and BAP did not give any response (no shoot formed).

Table 2: Effect of NAA and BAP on regeneration of shoot from callus

NAA	BAP	% regeneration
0	0	3.3%
0.5	0	33%
1.5	0	20%
2	0	3.3%
2.5	0	0%
3	0	0%
0	0.5	30%
0	1.5	26.6%
0	2	0%
0	2.5	0%
0.5	0.5	3.3%
0.5	1.5	00%
0.5	2	00%
1.5	0.5	00%
1.5	1.5	00%
1.5	2	00%
2	0.5	6.6%
2	1.5	00%
2	2	00%

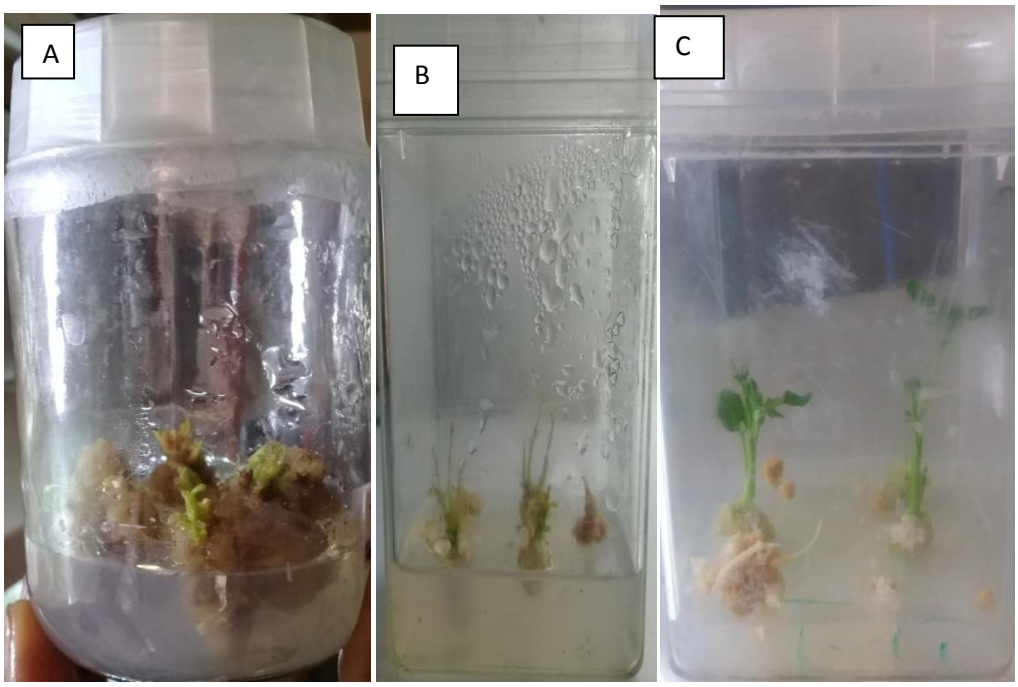

Figure 7: Regeneration of shoots from callus on MS medium supplemented with NAA and BAP alone (A) After 15 days of regeneration on 0.5 BAP (b) 0.5 BAP (C) 0.5 NAA

5.3. Shoot Multiplication

The response of explants cultured on MS medium supplemented with different concentrations of BAP in combination with IBA is presented in Table 3.

The results of the present study showed that using BAP alone resulted in better shoot multiplication as compared to IBA in combination with BAP. The MS medium containing various concentrations of lower BAP and combination of low BAP with low level of IBA produced shoots that are not significantly different at P = 0.05 after 4 weeks (Table 3). The mean number of shoots per explants ranged from 1.12±0.55 to 3.13 ±0.73 (Tables 3). Among all the treatments, medium containing 1.0 mg/l BAP resulted in the highest number (3.13) of shoots per explants. Shoots cultured on MS medium supplemented with 1.5 mg/l BAP in combination with 2.5 mg/l IBA and 1.5 mg/l BAP in combination with 3.5 mg/l IBA produced the lowest mean number of shoots per explants, 1.15 ±0.65 and 1.12 ± 0.55 respectively.

Shoot explants culture on 0.5 mg/l BAP result the highest shoot length (4.18 ± 0.86 cm) per explants and minimum shoot length (1.32±0.95) resulted in the MS medium supplemented with a combination of 1.5mg/l BAP with 2.5mg/l IBA

Table .3. Effects of BAP and IBA on multiplication of shoot

BAP	IBA	No of shoots /explants	Shoot length
00	**00**	1.77 ± 0.60 ^{c}	2.58 ±0.75 ^{bc}
0.5	**00**	2.76 ±0.91 ^{a}	4.18 ±0.86 ^{a}
1	**00**	3.13 ±0.73 ^{a}	3.26 ±0.79 ^{ab}
1.5	**00**	3.01 ±0 .52 ^{a}	2.57 ±0.74 ^{bc}
2	**00**	2.23±0.73 ^{b}	2.51 ±0.85 ^{bc}
0.5	**0.5**	2.74 ± 0.67 ^{a}	3.39±0.72 ^{b}
0.5	**1.5**	2.54 ± 0.81 ^{ab}	3.68±0.39 ^{a}
0.5	**2.5**	1.43 ±0.68 ^{cd}	2.86±0.67 ^{bc}
0.5	**3.5**	1.94 ± 0.72 ^{c}	1.81±0.55 ^{e}
1	**0.5**	1.71 ± 0.41 ^{c}	1.41±0.65 ^{fg}
1	**1.5**	1.69 ± 0.59 ^{cd}	1.53±0.59 ^{f}
1	**2.5**	1.29 ±0.50 ^{de}	1.72±0.28 ^{e}
1	**3.5**	1.30 ± 0.36 ^{de}	1.40±0.91 ^{fg}
1.5	**0.5**	1.15 ± 0.57 ^{ef}	1.51±0.75 ^{f}
1.5	**1.5**	1.19 ± 0.47 ^{ef}	1.50±0.69 ^{f}
1.5	**2.5**	1.15 ±0.65 ^{f}	1.32±0.95 ^{g}
1.5	**3.5**	1.12 ± 0.55 ^{f}	1.35±0.39 ^{g}

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

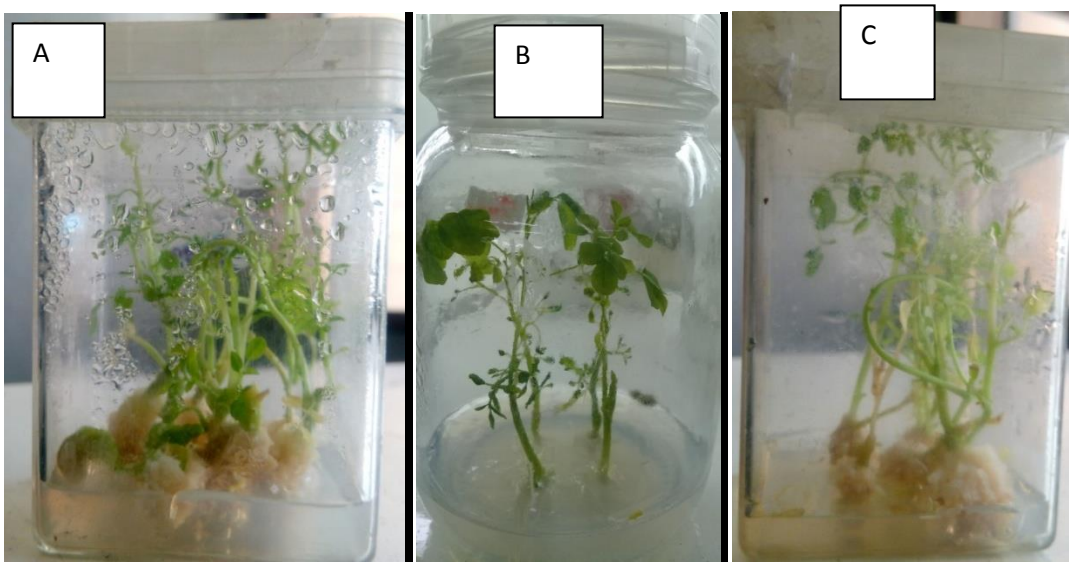

Figure 8: Shoot multiplication from shoot tip explants of *Moringa oleifera* on MS medium containing BAP alone and in combination with IBA (A) 1.0 mg/l BAP ; (B) 0.5 mg/l BAP + 0.5 mg/l IBA; (C) 1.5 mg/l BAP

5.4. Rooting and acclimatization

The shoots cultured on half strength MS basal media supplemented with different concentrations of IBA and NAA resulted in different rooting responses. Among all the treatments, 1/2 strength MS medium supplemented with 0.5 mg/l IBA resulted in the highest number of roots per explant (9.60±0.86) (Table 4) followed by the control, which was growth regulators free ½ strength MS medium with 2.84±0.48 mean root number and 1.0 mg/l IBA that produced 2.65±0.91 mean root number (Table 4).

Shoot explants cultured on 1/2 strength MS medium supplemented with 2.5 mg/l NAA and 3.0 mg/l IBA produced the lowest mean root number per explants (0.74±0.77) and (0.98±0.83). The highest mean length of roots per explants (2.27±0.06 cm) was obtained on growth regulators free half strength MS medium which was used as control, 2.12±0.08 and 2.06±0.09 were obtained from half strength MS medium supplemented with 0.5 mg/l NAA and 1.0 mg/l IBA respectively. After one month of acclimatization, from the total of 30 plantlet, 27(90 %) plantlet was survived in the green house.

Table 4: Effects of different concentration of NAA and IBA on rooting

IBA(mg/l)	NAA (mg/ l)	**No. of roots/ explants Mean±SE**	**Root length (cm) Mean±SE**
00	**00**	2.84±0.48^{b}	2.27±0.06^{a}
0.5	**00**	9.60±0.86 ^{a}	1.41±0.09 ^{b}
1	**00**	2.65±0.91 ^{b}	2.06±0.09^{a}
2	**00**	1.24±0.52^{cd}	1.00±0.08 ^{c}
3	**00**	0.98±0.83 ^{cd}	1.06±0.09 ^{c}
0	**0.5**	2.58±0.81^{b}	2.12±0.08^{a}
0	**1.5**	2.09±0.76^{bc}	1.36±0.07^{b}
0	**2**	1.25±0.72^{cd}	0.76±0.04^{d}
0	**2.5**	0.74±0.77	0.53±0.05^{e}

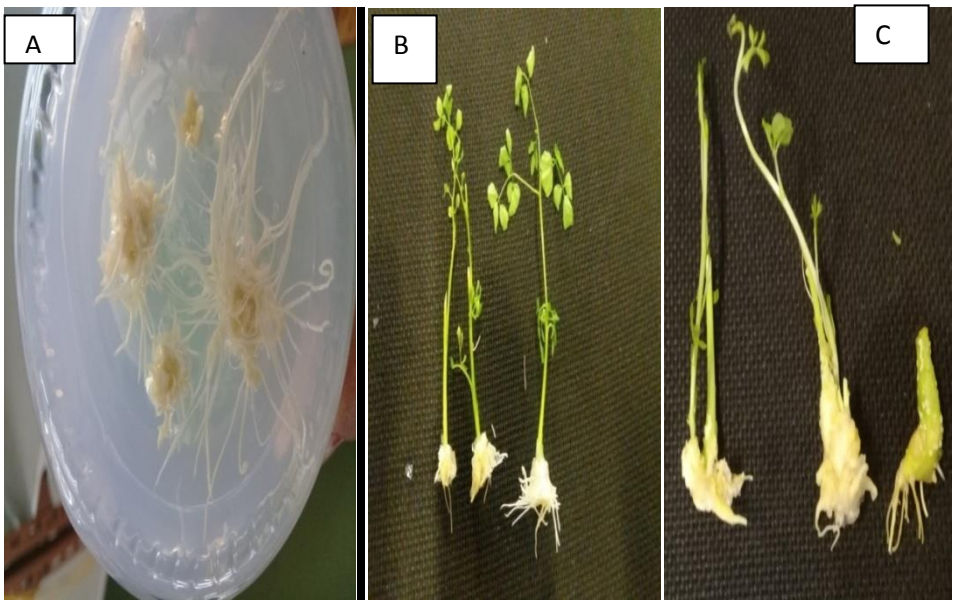

Figure 9: *In vitro* rooting of *Moringa oleifera* shoots on half strength MS medium containing IBA alone and MS media without growth regulators. A and B: 0 .5 mg/l IBA and (C) Growth regulators free

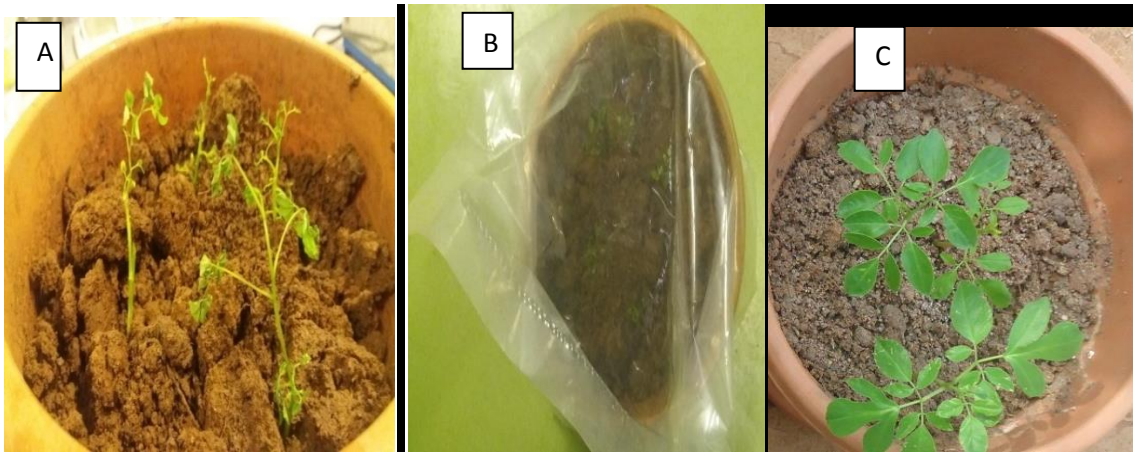

Figure 10: Acclimatization of *in vitro* rooted shoots of *Moringa oleifera* in greenhouse. (A) Plants transferred from the medium to the pots; (B) Plants covered with plastic bags and (C) after 4 weeks of acclimatization

6. DISCUSSION

6.1. Callus induction

In callus induction experiment, BAP, NAA and the combination of both were compared. Application of BAP or NAA alone resulted in higher callus induction rate as compared to the combination of both.

Cytokinins affected callogenesis by resulting in decrease of the cell wall lignification, facilitating callus initiation and the appropriate concentration of BA promoted callus induction and differentiation in many plant species. In this study, low concentration of BAP gave better callus induction rate. The findings of Riyathong *et al.* (2010) showed that the MS medium supplemented with 0.5 mg/l BAP result 100% callus induction from the shoot of *moringa oleifera.* The finding of these authors is similar to the present one. In the present study, the maximum callus induction rate (73.3) was recorded on MS medium supplemented with 0.5 mg/l BAP. The result reported by Riyathong *et al.* (2010) was higher than the present study. This difference may occur due to the genotype difference and type and source of explants.

Among leaf explants cultured on MS medium supplemented with different concentration NAA, an explants culture on higher concentration of NAA (6mg/l) gives a result of 70 % callus induction rate. In contrast Devendra *et al.* (2012) reported that the frequency of callus formation increased when the concentration of NAA increased 0.2mg/l - 2mg/l in *Moringa oleifera.* But further increasing of NAA concentration will be decrease the rate of callus induction.

Minimum callus induction rate was recorded in a combination of NAA with BAP. Among all leaf explants cultured on MS medium supplemented with different concentration NAA in combination with BAP, a combination of 3.0 mg/l NAA with 3.0 mg/l BAP, 6.0 mg/l NAA with 3.0 mg/l BAP and 5.0 mg/l NAA with 0.5 mg/l BAP result better callus induction rate (40%, 30% and 33.3% respectively). No callus was induced from leaf explants cultured on MS medium supplemented with 4 mg/l NAA in combination with 1 mg/l BAP, 4 mg/l NAA in combination with 2 mg/l BAP, 4 mg/l NAA in combination with 3 mg/l BAP, 5 mg/l NAA in combination with 1 mg/l BAP, 5mg/l NAA in combination with 3 mg/l BAP, 6 mg/l NAA in combination with 1 mg/l BAP, 6mg/l NAA in combination with 2 mg/l BAP.

The MS medium supplemented with 0.5 mg/l of 2, 4-D was the most effective medium for callus induction of *Moringa oleifera* with 100% of callus induction from week-3 after culturing of shoot (Shank, 2013).

Generally the present study was showed that the explants responded positively with callus induction when low BAP or higher NAA alone were used.

6.2. Regeneration of shoots from callus

The response of regeneration from callus was tested by using NAA in combination with BAP. The percentages of regeneration ranged from 0 to 33.3%. The highest percentage (33.3%) was resulted from the MS medium supplemented with 0.5 NAA. Similarly, Riyathong *et al.* (2010) reported the maximum (80%) regeneration rate of callus was obtained from MS medium supplemented with the same NAA concentration in *Moringa oleifera* and the difference is only on the regeneration rate.

Among all callus explants cultured on MS media supplemented with 0.5 mg/l BAP gave higher (30%) regeneration rate. In line with this result, Islam *et al.* (2005) used BAP for *in vitro* shoot regeneration of *Moringa oleifera* and found that BAP at 1.0 and 1.5 mg/L concentrations produced 100% regeneration response.

As the result showed in the present study, the regeneration rate for shoot from callus is very low more than half of the treatment did not give any response. Especially except 0.5 NAA with 0.5BAP and 2 NAA with 0.5 BAP, all MS media supplemented with a combination of NAA and BAP did not give any response (no shoot formed).

5.3. Shoot multiplication

In the present study, full strength MS medium containing different types and concentrations of PGRs have been used to multiply the already regenerated shoots of *Moringa oleifera.* Among all the treatments, the maximum numbers of shoots per explants were recorded on medium BAP alone and combination of low BAP and IBA concentration. Proliferation of multiple shoots in different tree plants had been previously characterized with different growth regulators. Islam *et al.* (2005) obtained (4±0.29) shoot number per explants from MS medium supplemented with 1 mg/l BAP in *Moringa oleifera*. The finding of these authors is similar to the present one. In the present study, the maximum mean number of shoots per explants (3.13 ±0.73) was recorded on MS medium supplemented with 1mg/l BAP. The findings of Islam *et al.* (2005) were higher than the present study. This difference may occur due to the genotype difference.

In the present study Lower (0.5 mg/l) BAP alone recorded the maximum shoot length (.4.18 ±0.86). In contrast, with the findings of Shahzad *et al.* (2014) who reported, the lowest shoot

length (2.18±0.31cm) was attained when these hypocotyls were cultured on the MS medium supplemented with BAP in *moringa oleifera.*

In this investigation, higher concentrations of BAP (2mg/l) relatively reduced the shoot number as well as shoot length. This is due to inhibitory effect of higher concentrations of BAP on proliferation (Bhojwani, 1996). In contrast, Saini *et al.* (2013) reported MS medium containing 2.0 mg/l BAP produced the highest mean number (10.8) of shoots per explants followed by those treated with 1.0.

In the present study, using different concentrations of BAP alone resulted in formation of callus at the cut ends of the shoots. Similarly, Islam *et al*. (2005) reported that higher concentration of the cytokinin increased the callus initiation at the cut ends of the shoots. Most of the shoots were observed with the callus development at the base and this phenomenon was most prominent when the concentration of the growth regulator was increased.

Among explants cultured on MS medium supplemented with different concentration BAP in combination with IBA show significance difference. Highest shoots per explants (2.74 ± 0.67) recorded on 0.5 mg/l BAP with 0.5mg/l IBA followed by 2.54 ± 0.81 recorded on 0.5 mg/l BAP with 1.5 mg/l IBA.

6.4. Rooting and acclimatization

The analysis of variance revealed that root number and root length varied significantly with half strength MS medium supplemented with NAA and IBA. Application of IBA alone exhibited the maximum mean root number per shoot as compared to NAA alone. The highest number of mean roots per shoot (9.60±0.86) and mean root length (2.27±0.06 cm) were obtained from MS medium supplemented with 0.5 mg/L IBA and growth regulators free MS medium respectively. In contrast, application of NAA alone exhibited the maximum mean root number per shoot as compared to IBA alone in *moringa oleifera* was reported by Saini *et al* (2012). Continuous increasing of the concentration of IBA from 0.5 to 3 resulted in reduction in mean root number from 9.60±0.86 to 0.98±0.83 and the continuous increasing of the concentration of NAA decreased the number of roots from 2.58±0.81 to 0.74±0.77. This is in agreement with the results of Aloufa *et al*. (2010). The number of roots produced per shoot increased when concentrations of IBA increased from 0.1 mg/l to 0.50 mg/l was applied in *Ximenia americana* L. However, further increase in the concentration of IBA to 2.0 mg/l showed a reduction in the mean root number per shoot .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 and ethylene retards 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. Moreover, the optimum concentration is may be between 0.1 and 0.5 mg/l as the present result indicated. In the present study comparison was made between the rooting hormones and IBA was found to be more effective in increasing root number and length than NAA.

Rooted plantlets were transferred to polyethylene bags containing autoclaved garden soil and placed inside the growth chamber for two weeks. After 2 weeks, the covers were removed. Then, they were taken into the greenhouse where 90 % plants survived and established as healthy. This result was better than previous report of Saini *et al.*, (2012) where about 80% of the plants were survived as health.

7. CONCLUSIONS

From the obtained result it could be concluded that for callus induction low concentration of BAP and high concentration of NAA is preferable. Among different combination of BAP and NAA tested for regeneration, BAP alone and NAA alone resulted in better shoot regeneration than different concentrations of BAP in combination with NAA. The highest percentage (33.3%) was resulted from the MS medium supplemented with 0.5 NAA. BAP is the most important cytokinin for shoot multiplication of *Moringa oleifera* The highest mean number of shoots per explants (3.13±0. 73) was obtained on full strength MS medium supplemented with 1.0 mg/l BAP alone for shoot multiplication and half strength MS medium supplemented with 0.5 mg/l IBA resulted in highest mean number of roots per explants (9.60±0.86).

8. RECOMMENDATIONS

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

- ✓ The effect of different concentrations of auxins and cytokinins on callus induction and regeneration of shoots and roots should be further studied
- ✓ The effect of different concentration of growth hormone for multiplication should be further studied
- ✓ Different rooting hormone and rooting response of multiplied shoots at each sub-culturing stage should be investigated.
- ✓ The effect of different concentrations of cytokinins and auxins on direct regeneration of shoots and roots should be further studied

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10. APPENDIX

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

Components	Concentration (g/L)	m/L during media preparation
Micronutrients		
ZnSO4.7H2O	1.72	
H3BO3	1.124	
* MnSO2.4H2O	3.38	
* MnSO4.H2O	0.05	5 ml/l
KI	0.166	
NaMoO4.2H2O	0.05	
CoCl2.6h2O	0.05	
Na2EDTA	7.472	
FeSO4.7H2O	5.56	
Macronutrients		
NH4NO3	33	
KNO3	38	
Cacl2.2H2O	8.8	50ml/l
MgSO4.7H2O	7.4	
KH2PO4	3.4	
Vitamins		
Myo-inositol	20	
Glycin (glycocoll)	0.4	
Nicotinic acid (NaOH)	0.1	5ml/l
Pyridoxin (B6)	0.1	
Thiamin (B1)	0.02	

***** Alternatives

 Na2EDTA and FeSO4.7H2O prepared alone