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Micropropagation, *In Vitro* Regeneration and Molecular Diversity Study of *Moringa Stenopetala* (Bakf.) from Ethiopia

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Abstract

Micropropagation, *In vitro* Regeneration and Molecular diversity Study of *Moringa stenopetala* (Bakf.) from Ethiopia

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The tree species Moringa stenopetala *belongs to the family Moringaceae and represented only by a single genus* Moringa. M. stenopetala *is a native tree in Southern Ethiopia, North Kenya and Eastern Somalia. It plays a vital role for household food security, as source of income, medicine, purification of water, windbreak, fodder, fuel and shade tree all year round. Conventional propagation of* M. stenopetala *involves the use of seeds and cuttings . However, the application of fast and efficient biotechnological methods to propagate and improve different traits of interest is much needed. The objectives of this study were to develop micropropagation and* in vitro *regeneration protocols for* Moringa stenopetala *and to analyze its genetic diversity using ISSR molecular marker. Shoot tips obtained from* in vitro *germinated seedlings were used for culture initiation. All treatments with BAP in combination with NAA showed 100% shoot initiation beginning from the first culture. BAP at lower concentration was found to be the best for shoot multiplication. Of the treatments with BAP, KN, TDZ, IBA and NAA used for shoot multiplication, high quality and maximum mean number (11.16+0.14 and 10.86∧0.14) of shoot per shoot tip explant were attained on medium containing 0.5 mg/l BAP and 1.0 mg/l BAP with no significant difference. Similarly, application of 1.0 mg/l BAP resulted in the highest number of induced axillary shoots (13.66±0.20) per nodal explant. Half strength MS medium containing NAA combined with IBA was virtually better in* *promoting root induction than 1/2 MS medium containing IBA or NAA alone. The leaves of* in vitro *multiplied shoots were used to develop the* in vitro *regeneration protocol based on using TDZ, 2,4-D and NAA alone or in combination. The use ofTDZ alone at lower concentrations and application of 2,4-D, TDZ and/or NAA together led to very strong callus formation on MS medium. The maximum shoot regeneration percentage (63.66%) was achieved on a medium supplemented with BAP and KN each at 1.0 mg/l concentration. A genetic diversity study based on ISSR was also conducted on a total of 150 samples representing 14 populations from different parts of Ethiopia. The highest level ofpercent polymorphism (53.66%) was obtained from samples of Arba Minch Beto area and the least level of polymorphism (46.34%) was obtained from individuals in Arba Minch Merab Abaya area. The AMOVA results revealed that the genetic variation at population level accounted 21.47% of the total variation, while the within populations component accounted for 78.53% showing a higher within population variation than among populations. Jaccard's similarity coefficient for populations ranged from 0.601 to 0.749, with the highest similarity value (0.749) was shown between populations from Konso town and populations from outlying areas of Konso. Findings from this study could be used for mass propagation of disease free planting materials within relatively short period and contribute to further improvement of the plant. In addition, the results obtained here can contribute to food security of both human and cattle, solve the demand of plantlets both in quality and quantity and help to increase source of income for local residents as well as contribute to the genetic improvement of the species.*

Key words: In vitro *regeneration, inter simple sequence repeat (ISSR), micropropagation, Moringa, node, rooting*

Declaration by student

I, Belachew Seraw, hereby certify that I had personaly carried out the work depicited in the thesis entitled **"Micropropagation,** *In Vitro* **Regeneration and Molecular Diversity Study of** *Moringa Stenopetala* **(Bakf.) from Ethiopia".** No part of the thesis has been submitted for the award of any other degree or diploma prior to this date.

Signature____________________Date________________________

Declaration by supervisor

This is to certify that Belachew Seraw in the department of Microbial, Cellular and Molecular Biology has fulfilled the requirements prescribed for the PhD degree. The thesis entitled **"Micropropagation, *In Vitro* Regeneration and Molecular Diversity Study of *Moriitga Stenopetala* (Bakf.) from Ethiopia"** was carried out under my direct supervision. No part of the thesis was submitted for the award of any degree or diploma prior to this date.

Name of the supervisor: Dr. Tileye Feyissa Signature________ Date______________

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Dedicated to my mother and my late father :

Wube Worku and Seraw Taye

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1. Introduction

Moringa {*Moringa stenopetala,* Bakf.) belongs to the family *Moringaceae* which is represented only by a single genus *Moringa.* It is a softwood multipurpose tree and one of the native plants in the north-east tropical Africa (Yisehak Kechero *et al*., 2011). It is the second most important domesticated *Moringa* species after *M.oleifera* (Steinmuller *et al*., 2002).

As indicated by Price (2007), compared to *M. oleifera*, *M. stenopetala* has larger edible leaves, more drought resistance, and larger seeds (important for those using to purify water). Furthermore, *M. stenopetala* has vigorous growing green foliage (Fig 1) and grow during long dry season. But in spite of the initial rapid growth of *M. oleifera* in drier years the species does not do well without watering.

In Ethiopia *Moringa stenopetala* is grown as a backyard crop in the southern parts of the rift valley and adjoining lowlands for its edible leaves, flowers and tender pods. The tree is grown for daily use in more than six million households of Southern Ethiopia (Haile-Gebriel Endeshaw, 2003). Yisehak Kechero *et al*. (2011) indicated that *Moringa* has attracted enormous attention of plant genetic resource conservationists due to its widespread use in agriculture and medicine. Anbes Tenaye *et al*. (2009) pointed out that cultivation of the plant is on the increase because of its multiple uses and ability to thrive under harsh environments. The leaves are particularly important as human food because they appear towards the end of the dry season when few other sources of green vegetables are available.

Jahn (1991) indicated that the successful domestication of a little known *Moringa stenopetala* which is indigenous to East Africa was made by the African tribes. The ongoing dissemination to highlands and lowlands is due to the achievements of remote African tribes who developed exceptional skills and dedication to farming in harsh environment. For example, in parts of Southern Ethiopia, *Moringa stenopetala* has become a striking feature in the surrounding areas of native villages, small towns, on terraces and in fields.

Moringa stenopetala is known by different vernacular names such as Shiferaw (Amharic), Aleko, Aluko, Halako (Gamo/Wolita), Haleko, Shelchada (Konso), Haleko (Burji), Haleko (Dherashe) and Cabbage Tree (Eng) (Edwards *el al.*, 2000; Dechasa Jiru, 1995; Demeulenaere, 2001).

Moringa stenopetala is highly valued especially in the southern part of Ethiopia. It is fast growing, drought tolerant and easily adapted to poor soil and arid conditions (Yisehak Kechero *el al.*, 2011). However, it has not received significant research attention to select and develop potential ecotypes that might be valuable both as horticultural and medicinal crops.

Nowadays, *M. stenopetala* is mostly known from cultivation in southern Ethiopia around home gardens, home compounds, farmlands, abandoned farmlands and abandoned settlement areas. Its wild habitat has shrunk which may eventually lead to drastic genetic erosion. This situation entails the assessment of diversity of *M. stenopetala* to select and develop elite genotypes. Efforts should also be made to restore and conserve the genetic resource of this highly valuable species.

Conventional propagation of *M. stenopetala* involves the use of seeds and cuttings to get seedlings. Trees grown from cuttings are known to have much shorter root. In addition, trees obtained from seeds vary in genotypes and hence in their phenotypes leading to variation in fruit (pod) production and nutritional values. In this regard, clonal multiplication through tissue culture is needed. The use of seeds for various other purposes such as water purification (ICRAF, 2006), medication (ICRAF, 2006; Jahn, 1991) and oil extraction (Lalas *et al.*, 2003) also limits the use of seeds for large scale propagation. In addition, seed harvesting requires growing seedlings to achieve tree size and flower.

Assessing diversity in germplasm resources can help to identify elite genotypes for crop improvement programs. Crop genetic resource improvement requires a periodic assessment of diversity to select elite genotypes. Rapid clonal multiplication is also effective in large scale multiplication systems of selected genotypes. Tissue culture is one of the most commercially exploited components of biotechnology that has been used for the rapid clonal multiplication of selected genotypes of diverse groups of plant species (Rani and Raina, 2000).

1.2 Literature Review

1.2.1 Taxonomy, Botanical description and Distribution of *Moringa stenopetala*

Moringa stenopetala belongs to family *Moringaceae* that is represented only by a single genus *Moringa*. The genus is represented by about 14 species to which *M. stenopetala* belongs. Northeast tropical Africa is a center of endemism plus diversity to the genus (Mark, 1998). The taxonomic position of the family is not clear. It has some features similar to those of Brassicaceae and Capparidaceae but the seed structure is not similar to either of the above families. Pollen studies have not provided any other information but recent molecular studies have pointed to a relationship with the Carricaceae. These indicate that the taxonomic position of the family is not yet fully settled and is open for further studies (Edwards *et al.*, 2000).

Mark (1998) pointed out that the genus follows the distribution pathway from Rajasthan (India) to south West Africa (Africa, Madagascar and parts of Asia, including Arabia and India). It is indicated that the genus *Moringa* is indigenous to several countries (http://www.mobot.org/gradstudents/olson/moringahome.html). These countries include Madagascar, Namibia, Angola, Kenya, Ethiopia, India, Pakistan, Bangladesh and Afghanistan. List of countries and indigenous species of the genus *Moringa* also include: (i) Kenya: *M. arborea, M. borziana, M. longituba, M. rivae, M. stenopetala;* (ii) Somalia: *M. borziana, M. longituba, M. pygmaea*; (iii) Ethiopia: *M. longituba, M. rivae, M. ruspoliana, M. stenopetala* (iv) Madagascar: *M. drouhardii, M. hildebrandtii.* (v) Namibia: *M. ovalifolia.* (vi) Angola: *M. ovalifolia.* (vii) India: *M. concanensis, M. oleifera.* (Viii) Egypt: *M. peregrina.*

Moringa stenopetala is 6-10 m tall tree; trunk: more or less 60 cm in diameter at breast height; crown: strongly branched sometimes with several branches; thick at base; bark: white to pale gray or silvery, smooth; wood: soft; Leaves: up to 55 cm long; inflorescence: pubescent, dense many flowered panicles ca. 60 cm long (Edwards *et al.*, 2000) (Fig 1.1).

Moringa stenopetala is a native tree in Southern Ethiopia, North Kenya and Eastern Somalia. That is why it is often named as the African Moringa (ECHO, 1999). In Ethiopia, *M. stenopetala* is found in many arid areas of the Southern regions most extensively between Arbaminch and Konso at altitude of up to 1800 m a. s. l. (Stenz and Mayer, 1990). It is distributed in the lowland ecology of the southern part of the country (Anbes Tenaye *et al.*, 2009).

The habitat where the genus occurs in Ethiopia as summarized from the herbarium vouchers of the national herbarium includes: rocky areas along rivers, dry scrub land, Acacia-Commiphora woodland, water courses with some evergreens, open Acacia-Commiphora bush land on gray alluvial soil and in cultivation around village. The species is cultivated in terraced fields, gardens and small towns (Edwards *et al.*, 2000).

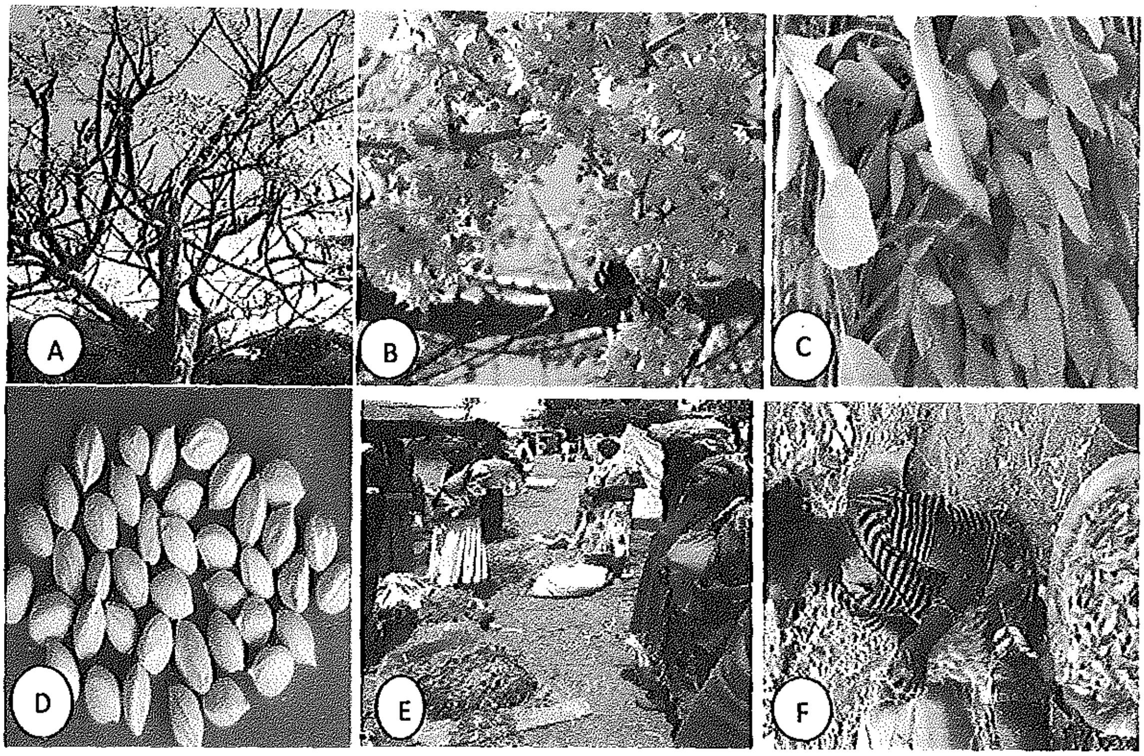

Figure 1.1: *Moringa stenopetala.* (A) M. *stenopetala* tree, (B) M. *stenopetala* inflorescence, (C) M. *stenopetala* leaves, (D) *M. stenopetala* seed kernel, (E) *M. stenopetala* leaves ready to be sold in a local market and (F) A girl stripping *M. stenopetala* leaves to cook and feed her family.

(Photos: Belachew seraw, 2016)

Yalemthehay Mekonnen (1999) also pointed out that the species is found to grow in Keffa, Gamo Gofa, Bale, Sidamo, Borana and Debub Omo zones, and in Konso and Dherashe especial woredas in Ethiopia. It is a strategic plant in being a unique food tree in drought prone areas and has been recently distributed to Wello, Shoa, Harargie and Sidamo for demonstration purpose by the Soil and Water Conservation of the Ministry of Agriculture.The Forestry Research Center also tried an alley cropping in Fontenina (Wello), Dhera (Arsi) and a windbreak trial at Ziwai, Hawassa College of Agriculture and International Livestock Center for Africa (ILCA) planted the tree in Ziwai (Dechasa Jiru, 1995). *Moringa stenopetala* has a wide range of adaptation from arid to humid climates with a prospect to be grown in a wide range of land use classes. The species grow in the area that has the rainfall range of 300-1400 mm per year (Mayer, 1990).

There are no reports on the exact pattern of mating of *M. stenopetala*. But Muluvi *et al.* (2004) estimated the pattern of mating by using AFLP markers in the closely related species, *M. oleifera*, to be 26% selfing and 74% out crossing rates. In addition, Bhattacharya and Mandal (2004) reported that *M. oleifera* shows delayed stigma receptivity to promote cross pollination.

1.2.2 Uses of Moringa stenopetala

Educational Concerns foe Hunger Organization (1999) mentioned that all parts of the tree except the wood are edible, providing a highly nutritious food for both humans and animals. The flowers are a good nectar source for honey and the seeds are a rich oil source for cooking and lubricant uses. Many parts of the plant have been used in medicinal preparations. Whole plants have been used as living hedges, fences, and windbreaks. The tree is also resistant to most pests.

Moringa stenopetala trees can be used to combat malnutrition, especially among infants and nursing mothers. Leaves can be eaten fresh, cooked, or stored as dried powder. It is especially promising as a food source because the tree is in full leaf at the end of the dry season when other foods are typically scarce.

Farmers in the southern regions of Ethiopia use *M. stenopetala* during both dry and wet seasons in their diet. It plays a vital role for household food security, as source of income, medicine, windbreak, fodder, fuel and shade tree all year round (Anbes Tenaye *et al.*, 2009). The foliage and fruit pods are rich in protein (including good amounts of the sulfur-containing aminoacids, methionine and cystine) and good sources of vitamins A, B and C (when raw), calcium and iron (Ram, 1994). Many parts of the plant have been used in medicinal preparations traditionally in the region against malaria and internal parasites. The wood is very soft; useful for paper but makes low-grade firewood and poor quality charcoal.

Documentation on the use of *M. stenopetala* in the southern regions of Ethiopia has shown that the leaves of shiferaw are cooked and eaten. In the Arba Minch and Wolita areas, the local people cook the leaves of M. *stenopetala* tree and eat them with their traditional kurkufa (a cereal dish made of maize and sorghum).The people of Konso and the surrounding communities in southern Ethiopia also use the plant both as food and to treat various ailments (Yalemtsehay Mekonnen and Amare Gessesse, 1998; Jahn, 1991).

In Southern Ethiopia, the leaves could be harvested twice a month and have diverse uses among the local people. Leaves are used as a cabbage, to treat malaria, hypertension, stomach disorders, to expel retained placenta, to treat asthma and diabetes and has anti trypanosomal activity (Yalemtsehay Mekonnen *et al.*, 1999).

Moringa stenopetala leaves are rich in crude protein, minerals and essential amino acids (Melesse Abera *et al.* 2008) and low in tannin content (Tegene Negesse *et al.* 2009). 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 Abera *et al.* 2008). *M. stenopetala* leaves are available during most part of the year and could be a good source of feed mainly during the dry season as a protein and energy supplement for ruminant livestock (Melesse Abera *et al.* 2008; Tegene Negesse *et al.* 2009).

Gram for gram, leaves of *M. stenopetala* contain seven times more vitamin C than oranges, four times more calcium and two times more protein than milk, four times more vitamin A than carrots, three times the potassium of bananas, three times more iron than spinach and twice the protein of yogurt. Moringa also contains all eight of the essential amino acids and ten of the non-essential ones required for the human body, plus several antibiotic properties (Charles, 2010)

Attracting attention is also the use of the dried, crushed seeds as a coagulant in flood water purification (Jahn, 1984). Even very muddy water can be cleared when crushed seeds are added. Solid matter and some bacteria will coagulate and sink to the bottom of a container. Powder from crushed seed kernels worked as a natural flocculent, binding to the solids in water and causing them to sink to the bottom (CWS, 2000). Since bacteria in water are generally attached to solid particles, treatment with this powder can purify water with 90-99% of the bacteria removed. Additional treatment of the water by boiling or adding chlorine or bleach is needed to render it completely safe to drink.

1.2.3 Propagation of *Moringa stenopetala*

In many areas of Ethiopia, propagation of *Moringa stenopetala* is made traditionally from seeds and cuttings. As reported by Jahn (1991), propagation of better performing Moringa trees, for example in Konso, is a trial and error practice by local farmers. If a young tree with poor leaf development is observed, it will be transplanted to another site to develop better. For this purpose, the stem should be "as big as a man" and the tap root has to be preserved at about half of this length. Then all branches are removed and the termini of stem and root are chopped off before trans-plantation during the major rainy season in March/April. The first leaves appear after about one and a half months.

Other type of propagation consists of the transplantation of seedlings which have developed from seeds germinating spontaneously under a mother tree. In this case, great care is taken not to damage the tap root and transplantation is made at the start of the rains. Trees grown from seed need at least two and half years before they produce their first fruit (Jahn, 1991). Another type of propagation involves collecting and sowing seeds to get seedlings. Trees obtained from seeds vary in genotypes and hence in their phenotypes leading to variation in fruit (pod) production and nutritional values.

Price (2007) also explained conventional cultivation of *M. stenopetala* trees by regeneration of plantlets from cuttings. Cuttings of 45-100 cm long with stems 4-10 cm diameter should be taken from the woody parts of the branches. It should be wood from the previous year. Cuttings can be treated for three days in the shade and then planted in a nursery or in the field. Some sources say that trees grown from cuttings produce inferior fruit and have a shallow root development, thus making them more susceptible to drought. Longer roots are an advantage for stabilization or access to water. In addition, it was observed that Termites can be a problem when cuttings are planted (http://moringatrees.org/tmtcultivation.html;http://miracletrees.org/growing_moringa.html). So seedlings are clearly preferable. In this regard, rapid multiplication of *M. stenopetala* through tissue culture to produce large number of seedlings is required. Yisehak Kechero *et al.* (2011) also noted that *M. stenopetala* has not received significant research attention to select and develop potential ecotypes.

1.2.4 Plant tissue culture

Plant tissue culture broadly refers to the *in vitro* cultivation of plants, seeds and parts of the plants such as leaf, immature and mature embryos, flower buds, anthers, microspores, ovaries, tissues, single cells, protoplasts and others. *In vitro* cell and tissue culture methodology is envisaged as a means for germplasm conservation to ensure the survival of endangered plant species, rapid mass propagation for large scale revegetation, and for genetic manipulation studies.

Tissue culture techniques are part of a large group of strategies and technologies, ranging through molecular genetics, recombinant DNA studies, genome characterization, gene-transfer techniques and aseptic growth of cells, tissues, organs, and *in vitro* regeneration of plants in the field of Biotechnology (Brown and Thorpe, 1995).

Aulinger (2002) pointed out that biotechnological improvements of plants should focus on three main areas: *in vitro* culture, genetic transformation and molecular markers analysis. Plant tissue culture is an integral part of molecular approaches to plant improvement and acts as an intermediary whereby advances made by the molecular biologists in gene isolation and modification are transferred to plant cells.

Some of the plant tissue culture techniques that are more approachable and have been found to be applied directly in plant propagation and genetic improvement of plants are (i) micropropagation, (ii) meristem culture, (iii) somatic embryogenesis, (iv) somaclonal variation, (v) embryo culture, (vi) *in vitro* regeneration (vii) anther culture, and (viii) protoplast culture (Smith and Drew, 1990). Most applied and well translated among these is the technique of micropropagation, which has revolutionized the modern agriculture industry (Singh and Shetty, 2011).

Many new complete plants can be obtained through direct or indirect morphogenesis and through somatic embryogenesis (Gamborg and Witter, 1975). *In vitro* regeneration and micropropagation are the practices of rapidly multiplying stock plant material to produce large numbers of progeny plants. Direct morphogenesis (direct micropropagation or simply micropropagation) is the production of shoots from explants without passing through callus (unorganized tissue) phase known as organ culture, which include meristem cultures, shoot cultures, embryo cultures and isolated root cultures. Whereas indirect morphogenesis (*in vitro* regeneration or indirect micro propagation) refers to induction of shoots through callus phase grouped as unorganized tissue cultures, which include callus cultures, suspension or cell cultures or anther cultures (Fig. 2). The integrated approaches of plant culture systems will provide the basis for the future development of novel, safe, effective, and high-quality products for consumers (Erica, 2000; Khanna, 2003).

The main advantage of tissue culture technology lies in the production of high quality and uniform planting material that can be multiplied on a year-round basis under disease-free conditions, and supplied anywhere irrespective of the season and weather (Singh and Shetty, 2011). For example, Singh and Shetty (2011) pointed out that banana is being cultivated in India in an area of about 500,000 hectares with an average productivity of about 15 kg of yield per plant. However, by replacing the conventional methods of use of suckers with tissue cultured plantlets, the productivity is enhanced to about 50 kg per plant from the same area.

Among the Asian countries that are active in commercial tissue culture plants production, it is in India that about 125 commercial units are functioning, while Indonesia and Japan have 33 units each, Korea has 20 and Thailand has 18 units. Other global countries involved in tissue culture plants production include European countries, USA, Canada, Australia, New Zealand, Israel, Middle East, South and Central America and Africa. All these countries together produced about 900 million plants in the year 2003 (Singh and Shetty, 2011).

1.2.5 Direct regeneration

Direct regeneration involves regenerations directly from explants without passing through callus phase. It is the application of tissue culture technique to the propagation of plants starting with very small parts grown aseptically in a test tube or other suitable containers (Hartmann *et al.*, 1993). It is the simplest and most commercially useful tissue culture method (Trigiano and Gray, 2005).

Use of tissue culture for micro propagation was initiated by Morel in 1960, who found this as the only commercially viable approach for orchid propagation (Razdan, 2003). Since then several crop species have been micropropagated. Micropropagation is a multibillion dollar industry with an estimated annual global market of more than 15 billion US dollar, and is practiced in thousands of nurseries and commercial biotechnology laboratories throughout the world (Rani and Raina, 2000).

Use of micropropagation has also many advantages over conventional methods of plant propagation of which the major ones are: (i) it is possible to generate pathogen-free plants, even from explants of infected mother plants; (ii) it is important in terms of multiplying plants throughout the year, with control over most facets of production; (iii) it is possible to produce haploid plants; (iv) it can be used for further studies in genetic transformation (v) it enables the production of a large number of plants in a short time from a selected number of genotypes, where the traditional methods of multiplication are either not available or are ineffective in large scale multiplication systems (Rani *et al.*, 1995).

Micropropagation can be used as one of the key tools of plant biotechnology to meet the growing demands for elite planting material. It can help us to supply clones of superior quality plants in ornamental, horticultural, floricultural and agro-forestry sectors. This need has been successfully tapped through micropropagation by the application of techniques of plant tissue culture thereby effectively translating the concept of technology for the commercial needs. As a result, several hundred plant tissue culture laboratories have come up worldwide (Singh and Shetty, 2011).

1.2.6 Indirect regeneration

Indirect regeneration involves regeneration from callus and cell suspension (Fig 2). Indirect regeneration is applicable to induce desirable, heritable changes in regenerated plants by subjecting a population of cells to a selection pressure. It is important to have a reproducible system for the regeneration of large numbers of plants from stressed cells as the selecting agent may lower the ability to regenerate plants. It is important that tolerance to the stress operates at both the cellular and whole plant levels so that there is a greater chance of recovering desirable plants (Tomes and Swanson, 1982).

Plant regeneration from cell cultures is also central to the application of gene transfer techniques. Not all plants are readily amenable to *in vitro* regeneration and there is thus a need to continuously develop regeneration protocols for the recalcitrant species if they are to benefit from genetic engineering technologies (FAO, 2005). *In vitro* regeneration usually results in high genetic and phenotypic variability in individuals derived from cultures, which is called somatic variation. Somatic variation can be beneficial in crop improvement especially on traits for which somaclonal mutants can be enriched during *in vitro* culture, including resistance to disease, pathotoxins, herbicides and tolerance to environmental or chemical stress, as well as for increased production of secondary metabolites.

1.2.7 Factors that affect response of explants in tissue culture

Several factors including the explants source, variations of the culture medium composition and environment such as CO_2 enrichment and light intensity associated with the sucrose content can affect the micropropagation performance (Mayak *et al.*, 1998).

The basic nutritional requirements of *in vitro* cultured plant cells are very similar to those utilized by plants in nature. However, the nutritional composition used *in vitro* varies depending on the type of protoplasts, cells, tissues, organs, and plant species. A nutrient medium is defined by its composition of mineral salts, carbon sources, vitamins, plant growth regulators, and other organic supplements. A particular medium is identified by its salt composition unless and otherwise identified. Other additives such as amino acids, vitamins, growth regulators, and other organic supplements can be added in any concentrations to a given salt concentrations to get the desired results (Murashige, 1974; Deen and Mohamoud, 1996).

Although all plants are composed of the basic unit of life, all cells are not considered totipotent. Only some cells are competent to divide and give rise to complete plant in tissue culture. Thus, not all plant species are equally amenable to tissue culture. In general, herbaceous species are more amenable to tissue culture techniques than woody perennials. Somaclonal variation can also be a problem with some micropropagation techniques. Field evaluation of plants is required to verify trueness-to-type and to check for genetic variants (Hussey, 1983 and Withers, 1989).

Although several media have been developed, the medium of Murashige and Skoog (1962) is widely used in different types of tissue culture systems. However, the most important factors that show recalcitrant of plant tissue culture depend on the species used, organic additives and the type and concentration of growth regulators (Erica, 2000) (Fig 1.2). In addition, other additives such as activated charcoal, ascorbic acid, acetic acid and polyamines and exogenous factors such as light, temperature, light quality and intensity are important in determining the response of some tissues in culture (Erica, 2000).

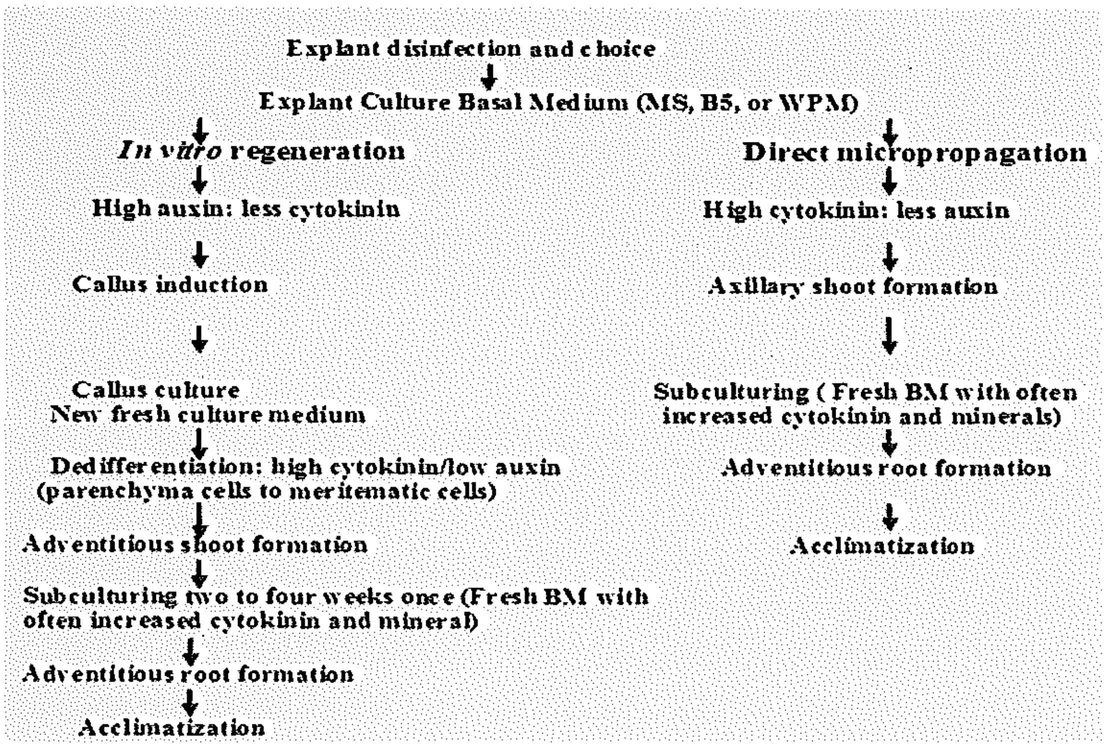

Figure 1.2: A general illustration of the stages of plant tissue culture

Source: Hartmann *et al.* (2002)

1.2.8 Plant Genetic diversity

The understanding of the amount, the extent and the distribution of genetic variation is vital to the development of effective conservation strategies and use plans. The amount of variation can be very different among species and populations of a species (Hodgkin, 1997). Understanding the genetic structure of natural populations is one of the central issues in population genetic studies (Epperson and Li, 1996). Knowledge of the genetic structure is a fundamental aspect in the understanding speciation, adaptation or genetic change in plant populations and species (Syamsuardi and Okada, 2002).

Traditionally, genetic variation of tree species has been understood through an assessment of survival and growth performance parameters (Jahn, 1991). However, such exercise can be time consuming, labor-intensive and costly. Molecular markers have been proved to be powerful tools in the assessment of genetic variation both within and among plant populations by analyzing large number of loci distributed throughout the genome (Powell *et al.*, 1995). The development and utilization of different marker systems have also paramount importance to assess the genetic diversity of a plant species at different levels. Molecular markers based on PCR amplification are efficient tools for plant breeding programs (Powell *et al.*, 1995; Gupta and Varshney, 2000).

Molecular marker systems contribute to a better understanding of the factors influencing genetic diversity and its patterns of distribution.

1.2.9 Molecular markers

Although the whole genome sequence is now available for a few plant species to identify specific genes located on a particular chromosome, most scientists use an indirect method called genetic markers for the tracing of a specific region of DNA (Semagn *et al.*, 2006).

Since the markers and the genes they mark are close together on the same chromosome, they tend to stay together as each generation of plants is produced. As scientists learn where markers occur on a chromosome, and how close they are to specific genes, they can create a genetic linkage map. Such genetic maps serve several purposes, including detailed analysis of associations between economically important traits and genes or quantitative trait loci (QTLs) and facilitate the introgression of desirable genes or QTLs through marker-assisted selection.

Genetic markers fall into one of the three broad classes: those based on visually assessable traits (morphological and agronomic traits), those based on gene product (biochemical markers), and those relying on a DNA assay (molecular markers).

As the full genome sequence will not be available for most species of interest in the near future, it is important to find strategies for developing and using molecular markers when sequence resources are limited. The presence of various types of molecular markers, and differences in their principles, methodologies, and applications require careful consideration in choosing one or more of such methods. No molecular markers are available yet that fulfill all requirements needed by researchers. According to the kind of study to be undertaken, one can choose among the variety of molecular techniques, each of which combines at least some desirable properties (Kumar *et al.*, 2009).

The various molecular markers can be classified into different groups based on: (i) Mode of transmission (biparental nuclear inheritance, maternal nuclear inheritance, maternal organelle inheritance, or paternal organelle inheritance), (ii) Mode of gene action (dominant or codominant markers) and (iii) Method of analysis (hybridization-based or PCR based markers) (Semagn, *et al.*, 2006).The focus of this section is on the later.

The various PCR-based techniques are of two types depending on the primers used for amplification: (i) Arbitrary or semi-arbitrary primed PCR techniques that are developed without prior sequence information (e.g., RAPD, AFLP and ISSR) and (ii) Site-targeted PCR techniques that are developed from known DNA sequences (e.g., EST, SSR and SCAR). ISSR was developed as PCR-based nuclear marker that could be applied to any species without the requirement for prior sequence information (Zietkiewicz *et al.*, 1994).

1.2.10 ISSR markers

ISSR marker system is a DNA markers which relies on one primer for PCR that anneals to an SSR region and amplifies region between inversely oriented adjacent SSRs. ISSR assay can be undertaken for any species that contains a sufficient number and distribution of SSR motifs and has the advantage that genomic sequence data is not required. This technique amplifies large numbers of DNA fragments per reaction, representing multiple loci from across the genome; it is an ideal method for fingerprinting varieties (Maheswaran, 2004).

ISSR requires low cost and labor but with high reliability. It is a technique with wide acceptance in plant improvement studies as plant breeding makes use of the fact that certain DNA markers are closely linked to important agronomic traits such as diseases tolerance and seed size (Zietkiewicz *et al.*, 1994). ISSR is a RAPD-like technique that shares the simplicity of RAPD markers but uses longer PCR primers. Hence, it is more reproducible than the original RAPD method. It has relatively low start up costs and easy to use. ISSR markers have the added advantage of permitting convenient development of SSR markers from amplified ISSRs (Van der Nest *et al.*, 2000).

The method involves PCR amplification of regions between two adjacent and inversely oriented microsatellites using a single, usually 16-25 base pair long (Reddy *et al.*, 2002) SSR-containing primer anchored at the 3' or 5' end by two to four nucleotides (Fig 1.3) . The primer can be based on any of the SSR motifs (di, tri, tetra, - or penta nucleotides) found at microsatellite loci. ISSRs can be visualized on agarose gels, on silver stained polyacrylamide gels or fluorescently labeled for detection with an automated DNA sequencer (Edwards and McCouch, 2007).

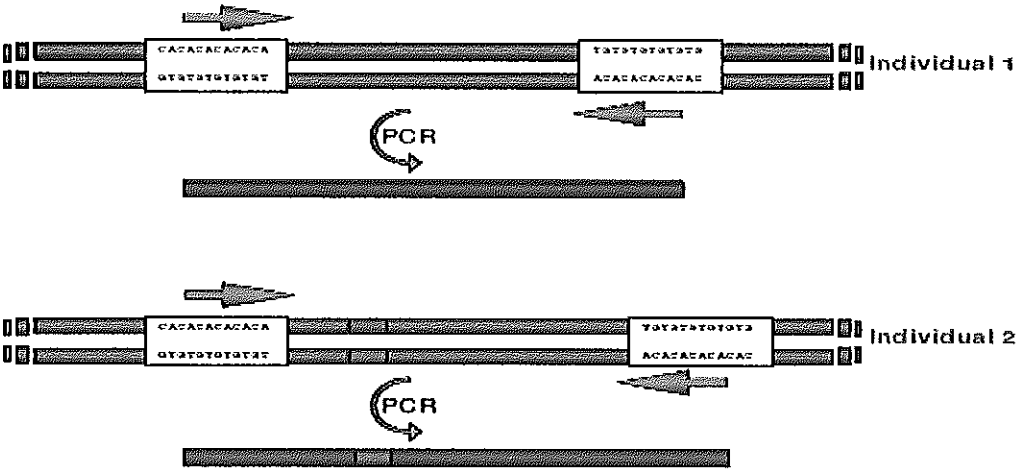

Figure 1.3: Principles of the amplification of DNA with a single oligonucleotide primer in ISSR marker system. DNA segments delimited by the inverted simple sequence repeats (SSR) (individual 1 and 2) are amplified with a single ISSR primer (green). ISSR variation may result from insertion or deletion (red mark) in different individuals produces PCR fragments of different sizes (blue) of the segment and mutation at PCR priming sites can also be the source of variation (Meyer *et al.*, 1993; Gupta *et al.*, 1994; Wu *et al.*, 1994; Zietkiewicz *et al.*, 1994).

1.3 Objectives

The aim of this thesis was to establish protocols for micropropagation and *in vitro* regeneration as well as to evaluate the molecular genetic diversity of *Moringa stenopetala* to provide a basis for its conservation and improvement.

1.3.1 General objective

To develop micropropagation and *in vitro* regeneration protocols for *Moringa stenopetala* and to analyze its genetic diversity using ISSR molecular marker.

1.3.2 Specific objectives

The specific objectives were:

- To develop an efficient micropropagation protocol of *Moringa stenopetala* from the plant shoot tip and node.
- To establish an efficient *in vitro* regeneration protocol using leaf explants.
- To evaluate molecular genetic diversity among and within populations collected from different parts of Ethiopia using ISSR DNA markers

2. Micropropagation of *Moringa stenopetala* from shoot tips

Moringa stenopetala plays a vital role for household food security, as source of income, medicine, windbreak, fodder, fuel and shade tree all year round in many areas of the southern regions of Ethiopia (Anbes Tenaye *et al.*, 2009 and personal observation). As indicated by Price (2007), when compared to *M. oleifera*, moringa native to Ethiopia (*M. stenopetala*) has larger edible leaves, more drought resistance, and larger seeds. Nowadays, *M. stenopetala* is mostly known from cultivation in southern Ethiopia around home gardens, home compounds, farmlands, abandoned farmlands and abandoned settlement areas but rarely so in the wild as in the past. Edwards *et al.* (2000) warned that in Ethiopia, *M. stenopetala* is nearly endangered in the wild. Yisehak Kechero *et al.* (2011) indicated that *M. stenopetala* has not received significant research attention to select and develop potential ecotypes. Therefore, steps should be taken for improvement, conservation and mass propagation of *M. stenopetala* through tissue culture techniques.

Shoot development directly from the meristem avoids callus formation, ensuring that genetic instability and somaclonal variation are minimized. Grout (1995) also summarized the major advantages of merrstem culture as that it provides: clonal propagation *in vitro* with maximal genetic stability; the potential for removal of viral, bacterial, and fungal pathogens from donor plants; the meristem tip as a practical propagule for cryopreservation and other techniques of culture storage; a technique for accurate mircro propagation of chimeric material; and cultures that are often acceptable for international transport with respect to quarantine regulations.

To our knowledge, this is one of the few reports on the *in vitro* propagation of *Moringa stenopetala.* The approach taken here in is to expand upon the earlier observations by Stephenson and Fahey (2004) and Mitiku Muanenda (2013). Stephenson and Fahey (2004) made the first attempt of tissue culture of *M. stenopetala.* But they were not successful and indicated that examination of a wider range of seed sizes and conditions are required. Mitiku Muanenda (2013) also studied the *in vitro* propagation of *M. stenopetala* from shoot explants using some cytokinins and auxins with few treatments and less number of combined treatments compared to the present study. The present study used a different approach in seed sterilization, seed culture and media preparation

2.1 Materials and methods

Source of explant and surface disinfection

Matured seeds of *M.stenopetala* were collected from Merab Abaya, near Arba Minch area, in the SNNPR during November and December of 2012. Fruits were cut open and the seeds were separated from pods and washed with detergent for 10 minutes and rinsed in running tap water for five minutes then thoroughly washed and rinsed again in sterilized distilled water for about 24 hours to speed up seed germination. Then the seeds were surface sterilized with 70% alcohol for one minute and with 10 % calcium hypochlorite $[Ca(ClO)_2]$ solution for 25 minutes followed by five washings with sterile distilled water. The surface sterilized seeds were cultured in culture jars containing 50 ml plant growth regulators-free MS (Murashige *et al.*, 1962) medium.

Shoot initiation

Shoot tips of size 0.5 to 1cm were cut from *in vitro* germinated seedlings and were cultured on MS medium supplemented with BAP (0.0,0.5,1.0,1.5,2.0 and 2.5 mg/l) in combination with NAA (0.1, 0.5 and 1.0 mg/l). The medium was supplemented with 30 g/l sucrose (w/v) and the pH was adjusted to 5.8 before addition of 8 g/l agar (w/v) and autoclaved at 121°C with a pressure of 105 Kpa for 15 min. The cultures were maintained in culture room under light intensity of 40 µmol m^{-2} s^{-1} and 16 hour photoperiod provided by cool-white fluorescent lamps at a temperature of 25±2 °C. Data were recorded as percentage of initiation, mean number of shoots per explant and mean shoot length per explant after 3 weeks.

Shoot multiplication

Shoot multiplication medium was enriched with different concentrations (0.0, 0.5, 1.0, 1.5, 2.0, 2.5, 3.0 and 4.0 mg/l) of BAP or KN or TDZ, BAP (0.0, 1.0, 2.0 and 3.0 mg/l) in combination with KN (0.0, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l), BAP (0.0, 0.5, 1.0, 1.5,2.0 and 2.5 mg/l) in combination with IBA (0.0, 0.01, 0.1 and 0.5 mg/l), BAP (0.0, 1.5, 2.0, 2.5, 3.0 and 4.0 mg/l) in combination with NAA (0.0,0.1,0.5 and 1.0 mg/l), and TDZ (0.0, 0.5, 1.0, 1.5, 2.0 and 2.5 mg/l) in combination with IBA (0.0, 0.01, 0.1 and 0.5 mg/l).

Shoot multiplication data were collected after three subsequent subculture following initiation. Five shoots per culture vessel and six replications per treatment were used.

Data were collected on number of shoots and leaves per explant and shoot length per explant at three weeks interval. Cultures were maintained in growth room with the same culture condition as indicated for shoot initiation.

Rooting

Shoots obtained from the best multiplication medium were used for root induction. Half strength MS medium supplemented with NAA or IBA (0.0, 0.5, 1.0, 1.5 and 2.0 mg/l), NAA (0.5, 1.0, 1.5 and 2.0 mg/l) combined with IBA (0.5, 1.0, 1.5 and 2.0 mg/l), 30 g/l sucrose (w/v) and 8 g/l agar (w/v) were used for root induction. pH of the medium was adjusted to 5.8 prior to autoclaving at 121°C with a pressure of 105 Kpa for 15 min. Thirty explants per treatment, five shoots per culture vessel with six replicates, were used. Cultures were maintained in growth room with the same culture condition as indicated for culture initiation and shoot multiplication experiments. The percentage of shoots producing roots, number and length of roots were recorded after four weeks.

Acclimatization

Roots of four-week-old rooted plants were thoroughly washed with running tap water to avoid all media residues, and then planted in plastic pots filled with autoclaved sand soil: Red soil: Compost in a ratio of 2:1:2 (v/v) respectively. One hundred twenty plantlets were used for acclimatization.The plantelets were covered with polyethylene bags and irrigated with sterile distilled water for two weeks in a greenhouse. After two weeks the plastic bags were removed and watering with tap water was done when required. The number of survived plantlets was recorded after 60 days of acclimatization.

Experimental design and data analysis

A completely randomized design (CRD) was used throughout the experiments and mean values of various treatments were subjected to one way ANOVA and the means were separated by Duncan Multiple Range Tests (DMRT) using SPSS statistical package (Version 20.0).

2.2 Results

Culture initiation

Shoot tips obtained from *in vitro* germinated seedlings (Fig 2.1) were used for culture initiation.

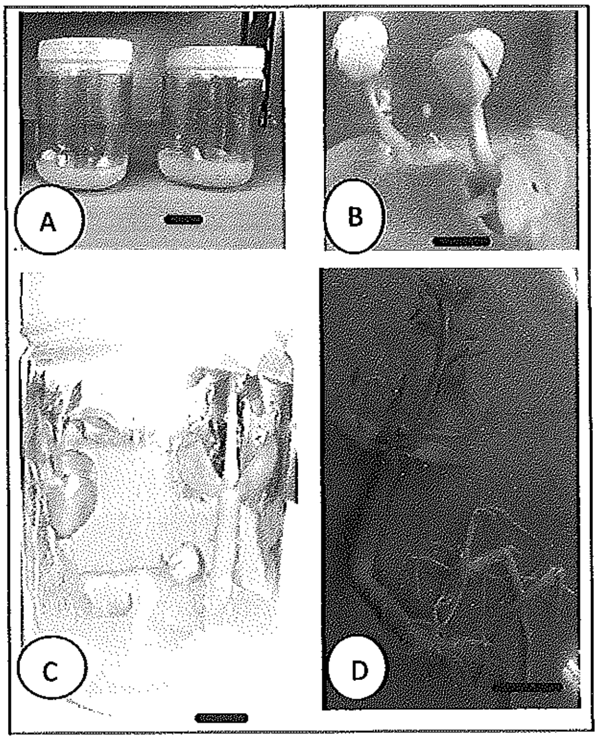

Figure 2.1: *In vitro* seed germination of *M. stenopetala.* (A) Seed culture, (B) Germinating seed, (C) Main shoots and (D) Axillary shoots. Bars= 1cm

Shoot initiations were clearly observed after four days from the beginning of culture (Fig 2.2). Cultures on MS medium supplemented with BAP alone and BAP in combination with NAA showed a significant difference at $P = 0.05$ (Table 2.1). All treatments with BAP in combination with NAA showed 100% shoot initiation beginning from the first culture and all treatments of BAP alone showed 76 to 85% shoot initiation in the first culture and 100% shoot initiation starting from the second culture.

Among the different treatments used for shoot initiation, the medium containing BAP (2.0 mg/l) combined with NAA (0.1 mg/l) and BAP (1.0 mg/l) alone produced maximum mean number of shoots (5.80±0.30 and 5.56±0.20) per explant respectively, with no significant difference. On the other hand, the lowest number of shoots (2.50±0.01 and 2.50±16) per explant was observed on the control and the medium containing 2.0 mg/l BAP combined with 1.0 mg/l NAA respectively, with no significant differences. The greatest shoot height (1.51±0.05cm) was achieved on the medium containing 1.0 mg/l BAP combined with 0.5 mg/l NAA.

In general, MS media supplemented with BAP in combination with NAA were found to be superior over BAP alone for shoot initiation. Treatments of BAP combined with NAA can also solve the problem of shoot initiation from shoot tip explants of *M. stenopetala* at the first culture.

Table 2.1: The effect of BAP alone or in combination with NAA in shoot initiation of *M. stenopetala* from shoot tip explants

PGR(mg/l)		Shoot initiation frequency (%)	Mean shoot no. per explant (cm)	Mean shoot length per explants
BAP				
0		0	2.50 ± 0.01^{j}	1.38 ± 0.01^{b}
0.5		85	5.40 ± 0.20^{ab}	1.40 ± 0.06^{b}
1.0		85	5.56 ± 0.20^{a}	1.26 ± 0.03^{c}
1.5		80	5.00 ± 0.01^{bc}	1.32 ± 0.03^{bc}
2.0		78	4.76 ± 0.15^{cd}	0.98 ± 0.01^{ef}
2.5		76	4.20 ± 0.13^{ef}	0.92 ± 0.01^{f}
BAP	NAA			
1.0	0.1	100	4.50 ± 0.09^{de}	1.38 ± 0.07^{b}
1.0	0.5	100	3.97 ± 0.08^{fg}	1.51 ± 0.05^{a}
1.0	1.0	100	3.50 ± 0.21^{h}	1.07 ± 0.04^{de}
1.5	0.1	100	4.00 ± 0.07^{fg}	1.14 ± 0.03^{d}
1.5	0.5	100	3.75 ± 0.07^{gh}	1.13 ± 0.02^{d}
1.5	1.0	100	3.70 ± 0.09^{gh}	0.88 ± 0.01^{f}
2.0	0.1	100	5.80 ± 0.30^{a}	1.10 ± 0.02^{d}
2.0	0.5	100	3.80 ± 0.13^{fgh}	0.94 ± 0.01^{f}
2.0	1.0	100	2.50 ± 0.16^{j}	0.65 ± 0.02^{g}
2.5	0.1	100	3.00 ± 0.13^{i}	1.06 ± 0.03^{de}
2.5	0.5	100	2.70 ± 0.01^{ij}	1.14 ± 0.06^{d}
2.5	1.0	100	2.60 ± 0.50^{ij}	0.74 ± 0.03^{g}

Means within columns having different letter (lower case) in superscript are significantly different at $p < 0.05$. The values represent mean ± S.E

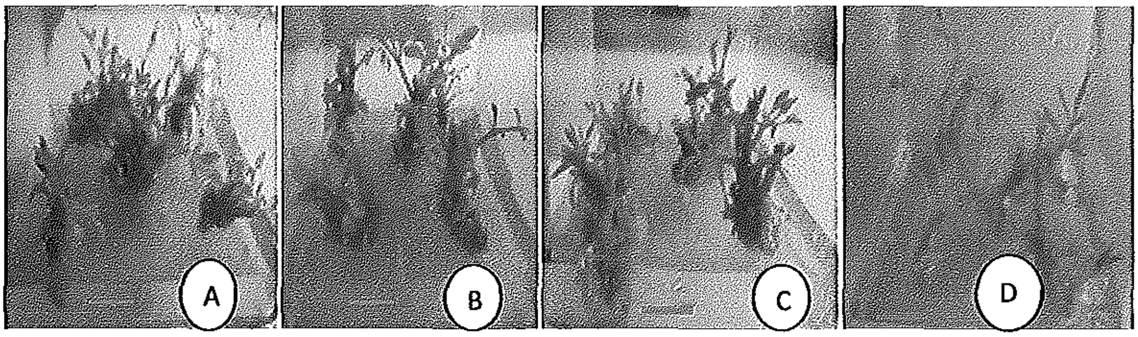

Figure 2.2: Shoot initiation from shoot tips of *in vitro* germinated seedlings on MS medium using BAP and NAA. (**A**) 0.5 mg/l BAP, (**B**) 1.0 mg/l BAP, (**C**) 1.0 mg/l BAP + 0.1 mg/l NAA and (**D**) Control. Bars =1 cm

Shoot multiplication

The Effect of BAP, KN and TDZ

A significant difference of *in vitro* shoot multiplication was detected at various concentrations of KN, BAP and TDZ after 3 weeks at $p < 0.05$ (Table 2.2). High quality and maximum mean number (11.16±0.14 and 10.86±0.14) of shoot per explant were attained on medium containing 0.5 mg/l BAP and 1.0 mg/l BAP with no significant difference. The next highest mean number (10.18±0.15 and 10.10±0.21) of shoot was obtained in the presence of 1.5 mg/l BAP and 2.0 mg/l BAP respectively. There was a general decline in shoot number and shoot elongation when the concentrations of BAP were more than 2.0 mg/l. Among the different treatments of KN, maximum shoot numbers (6.03±0.23 and 5.50±0.09) and leaf numbers (7.03±0.23 and 6.26±0.15) per explant were achieved on MS medium supplemented with 1.5 mg/l KN and 2.0 mg/l KN respectively. Among the three cytokinins used, 1.0 mg/l BAP showed the highest (7.50±0.09) leaf number per explant. The treatments with TDZ showed the lowest proliferation of shoot, shoot elongation and leaf number. Plantlets on the culture also showed abnormal morphology and were very stunt (Fig 2.3). In case of similar concentrations of BAP, KN and TDZ, generally BAP alone showed the best performance in terms of shoot number, shoot length and number of leaves per explant.

Table 2.2: The Effect of BAP, KN or TDZ alone on Shoot multiplication

PGR(mg/l)	Mean no. of shoot per explants	Mean shoot length per explant (cm)	Mean no. of leaf per explant
BAP			
0.0	3.36 ± 0.08^{i}	1.38 ± 0.01^{ab}	4.43 ± 0.11^{g}
0.5	11.16 ± 0.14^{a}	1.46 ± 0.07^{a}	6.40 ± 0.33^{c}
1.0	10.86 ± 0.14^{a}	1.36 ± 0.04^{ab}	7.50 ± 0.09^{a}
1.5	10.18 ± 0.15^{b}	1.36 ± 0.04^{ab}	5.00 ± 0.01^{def}
2.0	10.10 ± 0.21^{b}	1.06 ± 0.05^{de}	5.26 ± 0.08^{d}
2.5	9.43 ± 0.78^{c}	1.04 ± 0.04^{de}	4.40 ± 0.09^{g}
3.0	5.65 ± 0.07^{e}	0.96 ± 0.01^{ef}	4.80 ± 0.13^{efg}
4.0	5.53 ± 0.06^{e}	0.94 ± 0.01^{efg}	5.40 ± 0.18^{d}
Kn			
0.5	3.46 ± 0.07^{hi}	1.25 ± 0.04^{bc}	4.48 ± 0.06^{g}
1.0	4.50 ± 0.09^{f}	1.00 ± 0.01^{def}	5.20 ± 0.20^{de}
1.5	6.03 ± 0.23^{d}	1.12 ± 0.02^{cd}	7.03 ± 0.23^{b}
2.0	5.50 ± 0.09^{e}	1.13 ± 0.03^{cd}	6.26 ± 0.15^{c}
2.5	4.03 ± 0.13^{g}	0.94 ± 0.03^{efg}	4.73 ± 0.08^{fg}
3.0	3.73 ± 0.08^{gh}	0.93 ± 0.03^{efg}	4.43 ± 0.20^{g}
4.0	2.70 ± 0.05^{jk}	1.20 ± 0.05^{c}	3.62 ± 0.05^{h}
TDZ			
0.5	3.00 ± 0.16^{j}	0.74 ± 0.04^{hi}	2.20 ± 1.34^{i}
1.0	2.80 ± 0.13^{j}	1.04 ± 0.07^{de}	2.30 ± 0.21^{i}
1.5	2.40 ± 0.09^{kl}	0.82 ± 0.04^{ghi}	2.20 ± 0.07^{i}
2.0	1.83 ± 0.13^{m}	0.80 ± 0.04^{ghi}	1.20 ± 0.13^{k}
2.5	2.20 ± 0.07^{l}	0.71 ± 0.04^{i}	2.00 ± 0.11^{ij}
3.0	1.69 ± 0.05^{m}	0.86 ± 0.44^{fgh}	1.86 ± 0.09^{ij}
4.0	0.99 ± 0.12^{n}	0.57 ± 0.52^{j}	1.56 ± 0.06^{jk}

Means within columns having different letter in superscript are significantly different at $p < 0.05$.

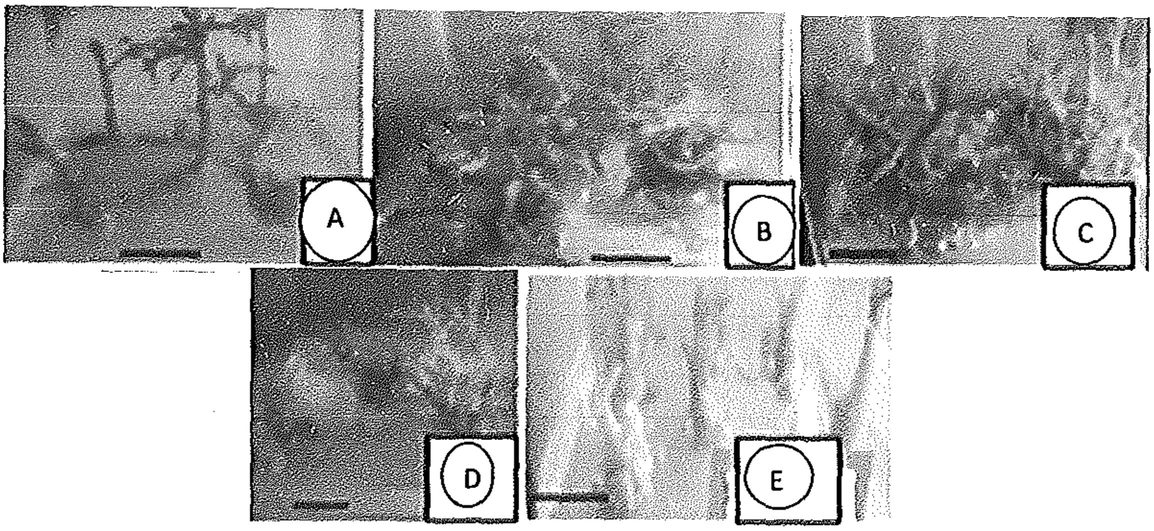

Figure 2.3: Shoot multiplication from shoot tips of *M. stenopetala* using BAP, KN and TDZ. **(A)** Control, **(B)** 0.5 mg/l BAP, **(C)** 1.0 mg/l BAP, **(D)** 1.5 mg/l KN and **(E)** 0.5 mg/l TDZ

Bars =1 cm

Synergetic effect of BAP and KN on shoot multiplication

Among the experiments laid to investigate the effect of various combinations of BAP and KN, the MS medium containing 1.0 mg/l BAP + 1.0 mg/l KN, 1.0 mg/l BAP + 2.0 mg/l KN, 2.0 mg/l BAP + 1.0 mg/l KN and 2.0 mg/l BAP + 1.5 mg/l KN produced the highest mean number of shoots (7.50±0.15, 8.03±0.21, 7.46±0.15, 7.66±0.15) per explant respectively with no significant differences (Table 2.3 and Fig 2.4). Better number of leaves (6.73±0.71 and 5.76±0.33) was also produced in the MS medium containing 1.0 mg/l BAP + 2.0 mg/l KN and 2.0 mg/l BAP + 1.5 mg/l KN respectively. Plant growth regulators-free MS medium showed the highest mean (1.38±0.01) shoot length.

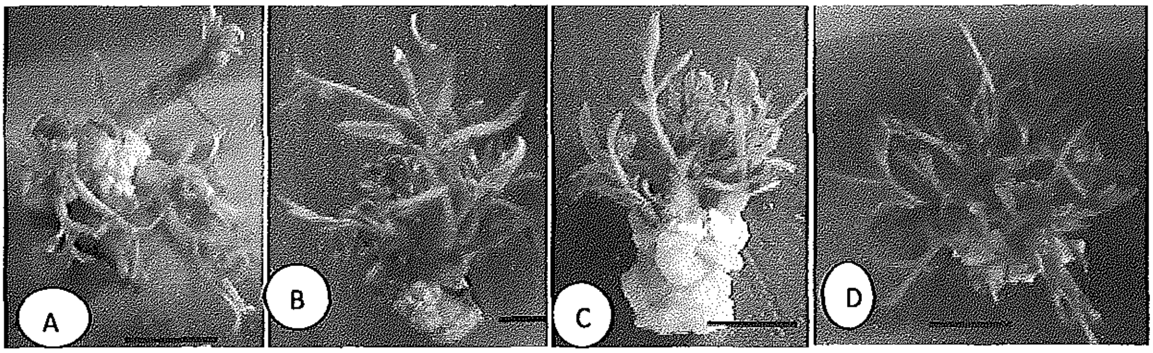

Figure 2.4: Individual shoots showing multiplication from shoot tips of *M. stenopetala* using BAP and KN. **(A)** BAP combined with KN each 1.0 mg/l, **(B)** 1.0 mg/l BAP +2.0 mg/l KN, **(C)** 2.0 mg/l BAP +1.0 mg/l KN and **(D)** 2.0 mg/l BAP+1.5 mg/l KN. Bars =1 cm

Table 2.3: The combined effect of BAP and KN on shoot multiplication

PGR(mg/l)		**Mean no. of shoot per explant**	**Mean shoot length per explant (cm)**	**Mean no. of leaves per explant**
BAP	**KN**			
0	0	3.36 ± 0.08^{f}	1.38 ± 0.01^{a}	4.43 ± 0.11^{de}
1.0	1.0	7.50 ± 0.15^{a}	1.00 ± 0.01^{def}	5.03 ± 0.13^{de}
1.0	1.5	6.70 ± 0.15^{bc}	0.92 ± 0.15^{efg}	5.26 ± 0.20^{d}
1.0	2.0	8.03 ± 0.21^{a}	1.00 ± 0.01^{def}	6.73 ± 0.71^{a}
1.0	2.5	6.50 ± 0.30^{bc}	0.81 ± 0.03^{h}	3.60 ± 0.30^{f}
1.0	3.0	5.66 ± 0.70^{de}	1.00 ± 0.01^{def}	4.50 ± 0.09^{de}
2.0	1.0	7.46 ± 0.15^{a}	0.94 ± 0.01^{efg}	4.43 ± 0.20^{de}
2.0	1.5	7.66 ± 0.15^{a}	0.89 ± 0.04^{fgh}	5.76 ± 0.33^{b}
2.0	2.0	6.80 ± 0.14^{b}	1.03 ± 0.05^{de}	5.20 ± 0.29^{d}
2.0	2.5	5.16 ± 0.14^{e}	0.88 ± 0.02^{gh}	4.26 ± 0.08^{ef}
2.0	3.0	5.70 ± 0.16^{de}	0.89 ± 0.03^{fgh}	5.00 ± 0.18^{bcde}
3.0	1.0	6.50 ± 0.21^{bc}	1.26 ± 0.08^{b}	5.43 ± 0.28^{bc}
3.0	1.5	5.66 ± 0.16^{de}	0.87 ± 0.02^{gh}	3.50 ± 0.09^{f}
3.0	2.0	5.76 ± 0.16^{d}	1.12 ± 0.02^{c}	4.70 ± 0.23^{de}
3.0	2.5	6.40 ± 0.32^{bc}	1.00 ± 0.01^{def}	5.70 ± 0.20^{b}
3.0	3.0	6.16 ± 0.25^{cd}	1.09 ± 0.05^{de}	4.73 ± 0.08^{de}

Means within columns having different letter in superscript are significantly different at $p < 0.05$.

The synergetic effect of BAP and IBA on shoot multiplication

Among the various concentrations of BAP combined with IBA, the medium enriched with 0.5 mg/l BAP + 0.1 mg/l IBA resulted in the largest mean number of shoot (5.40± 0.09), leaves (7.40±0.39) and shoot elongation (1.70±0.03). It was clearly observed that there is a general trend whereby each BAP level (except at 0.5 mg/l BAP) when combined with the lowest levels of IBA treatments resulted in better mean shoot number, shoot length and leaf number (Table 2.4). The plantlets were also green and healthy (Fig 2.5).

Table 2.4: The combined effect of BAP and IBA on shoot multiplication

PGR(mg/l)		Mean no. of shoot per explant	Mean shoot length per explant (cm)	Mean no. of leaves per explant
BAP	IBA			
0	0	3.36±0.08^{def}	1.38±0.00^{b}	4.43±0.11^{de}
0.5	0.01	4.20±0.07^{cde}	1.40±0.03^{b}	5.60±0.25^{c}
0.5	0.1	5.40± 0.09^{a}	1.70±0.03^{a}	7.40±0.39^{a}
0.5	0.5	4.80± 0.18^{b}	1.26±0.04^{c}	6.60±0.25^{b}
1.0	0.01	4.40±0.09^{c}	1.46±0.03^{b}	4.80±0.18^{d}
1.0	0.1	4.40±0.09^{c}	1.43±0.07^{b}	6.23±0.23^{b}
1.0	0.5	3.73±0.08^{fg}	1.13±0.04^{d}	4.26±0.08^{de}
1.5	0.01	4.36±0.08^{cd}	1.11±0.18^{d}	4.46±0.09^{de}
1.5	0.1	3.80±0.18^{f}	1.05±0.01^{d}	4.00±0.18^{ef}
1.5	0.5	3.36±0.08^{gh}	0.95±0.01^{ef}	3.86±0.06^{ef}
2.0	0.01	4.00±0.13^{def}	1.27±0.03^{c}	5.60±0.30^{c}
2.0	0.1	3.80±0.15^{f}	0.94±0.01^{ef}	4.00±0.13^{ef}
2.0	0.5	3.20±0.15^{h}	0.88±0.01^{f}	3.46±0.09^{f}
2.5	0.01	3.96±0.13^{ef}	0.94±0.01^{ef}	3.46±0.09^{f}
2.5	0.1	3.20±0.84^{h}	0.89±0.01^{f}	3.53±0.09^{f}
2.5	0.5	3.00±0.18^{h}	1.04±0.05^{de}	2.73±0.24^{g}

Means within columns having different letter in superscript are significantly different at $p < 0.05$.

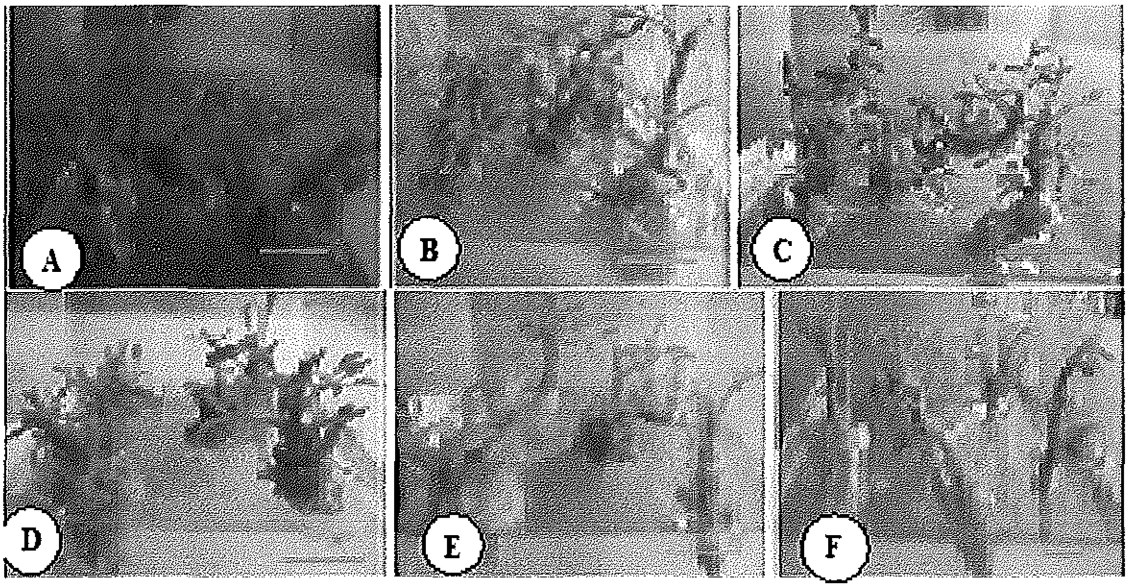

Figure 2. 5: Shoot multiplication from shoot tips of *M. stenopetala* in MS medium containing BAP and IBA. (A) 0.5 mg/l BAP + 0.1 mg/l IBA, (B) 1.0 mg/l BAP + 0.1 mg/l IBA, (C) 1.0 mg/l BAP + 0.5 mg/l IBA, (D) 2.0 mg/l BAP + 0.01 mg/l IBA, (E) 2.5 mg/l BAP + 0.01 mg/l IBA and (F) 2.5 mg/l BAP + 0.5 mg/l IBA Bars =1 cm.

The synergetic effect of BAP and NAA on shoot multiplication

To examine the combined effect of BAP and NAA, MS medium containing various concentrations of BAP in combination with low levels of NAA were examined. The highest three mean numbers of shoot (5.60±0.30, 4.60±0.32, 4.20±0.13) were obtained in the medium containing 2.0 mg/l BAP + 0.1 mg/l NAA, 3.0 mg/l BAP + 0.1 mg/l NAA and 2.0 mg/l BAP + 0.5 mg/l NAA with significant differences and the lowest mean number of shoot (2.00±0.16) was obtained in the medium containing 4.0 mg/l BAP plus 1.0 mg/l NAA (Table 2.5, Fig 2.6). The highest mean shoot length (1.5±0.05, 1.5±0.06) was obtained in the medium containing 1.0 mg/l BAP + 0.5 mg/l NAA and 1.0 mg/l BAP + 1.0 mg/l NAA respectively, without significant differences. The highest mean number of leaf (6.00± 0.26) per explant was also obtained on the medium containing 1.5 mg/l BAP + 0.5 mg/l NAA.

Table 2.5: The combined effect of BAP and NAA on shoot multiplication from shoot tip of *M. stenopetala*

PGR(mg/l)		Mean no. of shoot per explant	Mean shoot length per explant (cm)	Mean no. of leaves per explant
BAP	NAA			
0.0	0.0	3.36 ± 0.08^{def}	1.38 ± 0.01^{b}	4.43 ± 0.11^{de}
1.0	0.1	3.80 ± 0.21^{cd}	1.10 ± 0.03^{c}	4.40 ± 0.18^{e}
1.0	0.5	3.40 ± 0.09^{def}	1.5 ± 0.05^{a}	5.20 ± 0.27^{bc}
1.0	1.0	3.20 ± 0.07^{efg}	1.5 ± 0.06^{a}	4.43 ± 0.09^{de}
1.5	0.1	4.20 ± 0.07^{bc}	1.08 ± 0.02^{c}	5.00 ± 0.11^{cd}
1.5	0.5	3.80 ± 0.07^{cd}	1.10 ± 0.03^{c}	6.00 ± 0.26^{a}
1.5	1.0	3.60 ± 0.09^{de}	0.92 ± 0.18^{de}	3.80 ± 0.07^{f}
2.0	0.1	5.60 ± 0.30^{a}	1.00 ± 00^{cde}	5.60 ± 0.34^{ab}
2.0	0.5	4.20 ± 0.13^{bc}	0.96 ± 0.01^{de}	4.13 ± 0.13^{ef}
2.0	1.0	3.00 ± 0.16^{fgh}	0.64 ± 0.01^{h}	1.80 ± 0.29^{jk}
2.5	0.1	3.80 ± 0.76^{cd}	0.96 ± 0.01^{de}	4.20 ± 0.13^{ef}
2.5	0.5	3.00 ± 0.00^{fgh}	1.02 ± 0.05^{cd}	2.40 ± 0.14^{i}
2.5	1.0	2.60 ± 0.09^{hi}	0.76 ± 0.03^{g}	2.20 ± 0.13^{ij}
3.0	0.1	4.60 ± 0.32^{b}	1.08 ± 0.04^{c}	5.00 ± 0.26^{cd}
3.0	0.5	3.20 ± 0.13^{efg}	0.74 ± 0.04^{g}	2.60 ± 0.18^{hi}
3.0	1.0	2.80 ± 0.76^{ghi}	0.76 ± 0.03^{g}	2.20 ± 0.18^{ij}
4.0	0.1	2.80 ± 0.99^{ghi}	0.80 ± 0.03^{fg}	3.20 ± 0.21^{g}
4.0	0.5	2.40 ± 0.49^{ij}	0.90 ± 0.01^{ef}	3.00 ± 0.11^{gh}
4.0	1.0	2.00 ± 0.16^{j}	0.90 ± 0.11^{ef}	1.60 ± 0.14^{k}

Means within columns having different letter in superscript are significantly different at $p < 0.05$.

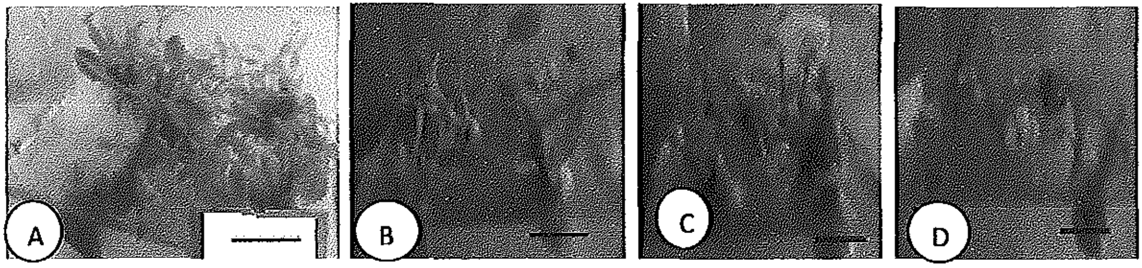

Figure 2.6: Multiplication from shoot tips of *M. stenopetala* using BAP and NAA. **(A)** 2.0 mg/l BAP + 0.1 mg/l NAA, **(B)** 2.0 mg/l BAP + 0.5 mg/l NAA, **(C)** 3.0 mg/l BAP + 0.1 mg/l NAA and **(D)** 4.0 mg/l BAP + 1.0 mg/l NAA. Bars =1 cm

Synergetic effect of TDZ and IBA on shoot multiplication

Among the combined effect of TDZ and IBA, the medium containing 0.5 mg/l TDZ + 0.01 mg/l IBA produced the highest mean number of shoots (4.26±0.22) per explant followed by 3.8±0.07 shoots per explant by 1.0 mg/l TDZ + 0.5 mg/l IBA. The highest mean shoot length (1.38±0.01cm) and the highest mean number of leaves per explant were produced by the control (Table 2.6). Generally, poor shoot elongation and leaf production were observed (Fig 2.7).

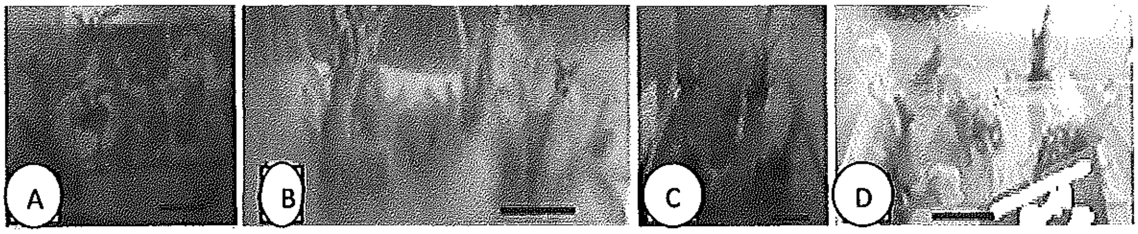

Figure 2.7: Multiplication from shoot tips of *M. stenopetala* using TDZ and IBA. **(A)** 0.5 mg/l TDZ + 0.01 mg/l IBA, **(B)** 1.5 mg/l TDZ + 0.1 mg/l IBA, **(C)** 1.5 mg/l TDZ + 0.5 mg/l IBA and **(D)** 2.0 mg/l TDZ + 0.1 mg/l IBA. Bars =1 cm

Table 2.6: The combined effect of TDZ and IBA on shoot multiplication

PGR(mg/l)		Mean no. of shoot per explant	Mean shoot length per explant (cm)	Mean no. of leaves per explant
TDZ	IBA			
0.0	0.0	3.36 ± 0.08^{bcd}	1.38 ± 0.01^{a}	4.43 ± 0.11^{a}
0.5	0.01	4.26 ± 0.22^{a}	1.25 ± 0.04^{bc}	3.80 ± 1.31^{b}
0.5	0.1	3.80 ± 0.17^{ab}	1.15 ± 0.05^{cde}	3.10 ± 1.22^{c}
0.5	0.5	2.80 ± 0.18^{efg}	1.04 ± 0.01^{ef}	2.40 ± 0.09^{e}
1.0	0.01	3.20 ± 0.18^{cde}	1.04 ± 0.01^{ef}	4.03 ± 0.12^{ab}
1.0	0.1	3.40 ± 0.14^{bcd}	1.04 ± 0.08^{ef}	3.80 ± 0.13^{b}
1.0	0.5	3.80 ± 0.07^{ab}	1.10 ± 0.03^{def}	2.80 ± 0.07^{cde}
1.5	0.01	3.60 ± 0.14^{bc}	1.30 ± 0.45^{ab}	3.20 ± 0.13^{c}
1.5	0.1	3.00 ± 0.26^{def}	1.20 ± 0.04^{bcd}	3.00 ± 0.20^{cd}
1.5	0.5	2.60 ± 0.22^{fgh}	0.90 ± 0.03^{gh}	2.60 ± 0.18^{de}
2.0	0.01	3.56 ± 0.18^{bc}	1.10 ± 0.03^{def}	3.20 ± 0.13^{c}
2.0	0.1	2.43 ± 0.50^{gh}	1.02 ± 0.04^{f}	3.20 ± 0.21^{c}
2.0	0.5	2.20 ± 0.18^{h}	0.80 ± 0.01^{h}	2.80 ± 0.07^{cde}
2.5	0.01	3.43 ± 0.09^{bcd}	1.30 ± 0.04^{ab}	3.23 ± 0.07^{c}
2.5	0.1	2.60 ± 0.49^{fgh}	0.86 ± 0.06^{h}	2.60 ± 0.49^{de}
2.5	0.5	2.60 ± 0.09^{fgh}	1.00 ± 0.05^{fg}	3.20 ± 0.07^{c}

Means within columns having different letter in superscript are significantly different at $p < 0.05$.

Root induction

Rooting was observed about two weeks after the transfer of multiplied shoots to rooting media (Fig 2.8). NAA alone was better and showed significant difference ($P < 0.05$) in promoting root induction than IBA alone. The combined effect of NAA and IBA also showed better rooting than media containing IBA alone (Table 2.7).

The best root number per shoot (8.33±0.92, 8.00±0.94, 8.00±0.80) were obtained on 1/2 strength MS medium supplemented with 0.5 mg/l NAA + 1.0 mg/l IBA, 2.0 mg/l NAA and 0.5mg/l NAA respectively, with no significant differences. Half strength MS medium fortified with 1.0 mg/l NAA + 0.5 mg /l IBA gave the best rooting response (86.41±0.23) followed by 80.62±0.11 and 75.82±1.00 percent rooting response in medium containing 1.0 mg/l IBA and 0.5 mg/l NAA respectively. The highest root length (2.96±0.22 cm) were also developed on 1/2 strength MS medium fortified with 1.0 mg/l IBA. The next highest mean root length (2.30±0.23 and 2.16±0.24 cm) were achieved on medium containing NAA + IBA both 1.0 mg/l, and 0.5 mg/l NAA + 1.0 mg/l IBA respectively.

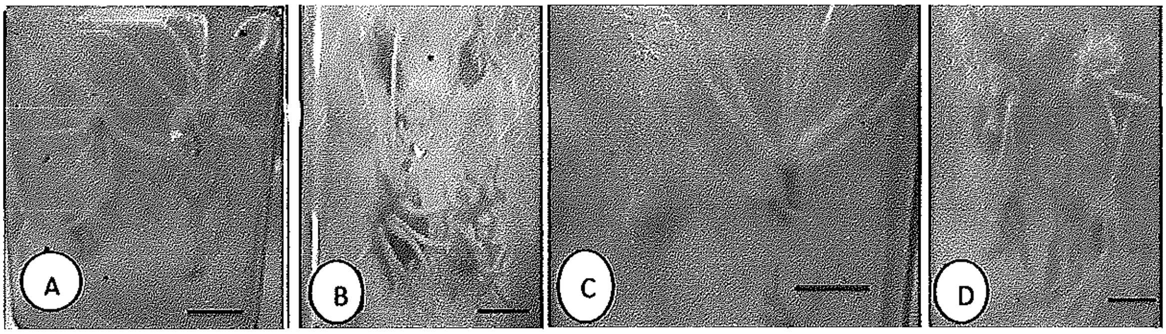

Figure 2.8: Root induction on 1/2 strength MS medium enriched with NAA and IBA. **(A)** 0.5 mg/l NAA + 1.0 mg/l IBA, **(B)** 0.5 mg/l NAA, **(C)** 1.0 mg/l IBA and **(D)** 1.5 mg/l NAA + 0.5 mg/l IBA. Bars = 1 cm

Table 2.7: The effect of NAA and IBA on rooting of *in vitro* propagated shoot from shoot tip of *M. stenopetala*

PGR(mg/l)		Mean no. of root per explant	Mean root length per explant (cm)	Mean rooting response of each treatment in percent
NAA	**IBA**			
-	0	4.80 ± 0.30^{def}	1.63 ± 0.16^{efgh}	54.05 ± 0.13^{h}
-	0.5	$2.86 \pm0.46g^{hi}$	1.39 ± 0.12^{fghi}	25.30 ± 0.24^{t}
-	1.0	6.66 ± 0.60^{bc}	2.96 ± 0.22^{a}	80.62 ± 0.11^{b}
-	1.5	1.20 ± 0.07^{j}	0.46 ± 0.02^{l}	6.81 ± 0.05^{w}
-	2.0	1.33 ± 0.08^{j}	1.96 ± 0.15^{bcde}	27.10 ± 0.09^{q}
0.5	-	8.00 ± 0.80^{a}	2.05 ± 0.19^{bcd}	75.82 ± 1.00^{c}
1.0	-	3.73 ± 0.15^{fg}	1.01 ± 0.06^{jk}	47.51 ± 0.16^{m}
1.5	-	3.50 ± 0.46^{fgh}	0.74 ± 0.06^{kl}	26.01 ± 0.14^{s}
2.0	-	8.00 ± 0.94^{a}	1.00 ± 0.07^{jk}	51.80 ± 0.29^{l}
0.5	0.5	2.23 ± 0.12^{hij}	1.31 ± 0.06^{ghij}	26.57 ± 0.22^{rs}
0.5	1.0	8.33 ± 0.92^{a}	2.16 ± 0.24^{b}	52.33 ± 0.20^{kl}
0.5	1.5	2.66 ± 0.08^{ghi}	2.05 ± 0.01^{bcd}	55.88 ± 0.25^{g}
0.5	2.0	4.20 ± 0.52^{ef}	1.73 ± 0.08^{def}	52.97 ± 0.06^{jk}
1.0	0.5	7.26 ± 0.38^{ab}	1.76 ± 0.14^{cdef}	86.41 ± 0.23^{a}
1.0	1.0	5.66 ± 0.23^{cd}	2.30 ± 0.23^{b}	54.04 ± 0.12^{h}
1.0	1.5	2.00 ± 0.01^{ij}	1.18 ± 0.05^{ij}	53.31 ± 0.02^{i}
1.0	2.0	7.36 ± 0.08^{ab}	2.11 ± 0.11^{bc}	45.74 ± 0.12^{n}
1.5	0.5	3.46 ± 0.34^{fgh}	1.66 ± 0.04^{efg}	70.64 ± 0.11^{d}
1.5	1.0	3.86 ± 0.30^{fg}	0.66 ± 0.04^{kl}	45.78 ± 0.03^{n}
1.5	1.5	2.13 ± 0.18^{ij}	0.52 ± 0.01^{l}	18.83 ± 0.02^{u}
1.5	2.0	5.40 ± 0.47^{de}	1.16 ± 0.11^{ij}	61.13 ± 0.19^{e}
2.0	0.5	4.80 ± 0.16^{def}	0.96 ± 0.01^{jk}	61.25 ± 0.23^{e}
2.0	1.0	3.50 ± 0.09^{fgh}	0.53 ± 0.01^{l}	31.54 ± 0.07^{p}
2.0	1.5	4.36 ± 0.13^{ef}	1.26 ± 0.04^{hij}	37.70 ± 0.04^{o}
2.0	2.0	4.66 ± 0.08^{def}	1.63 ± 0.02^{efgh}	59.09 ± 0.14^{f}

Means within columns having different letter in superscript are significantly different at $p < 0.05$.

Acclimatization

Acclimatization of *in vitro* rooted plantlets was successful with 85% plants survived and established as healthy plants (Fig 2.9).

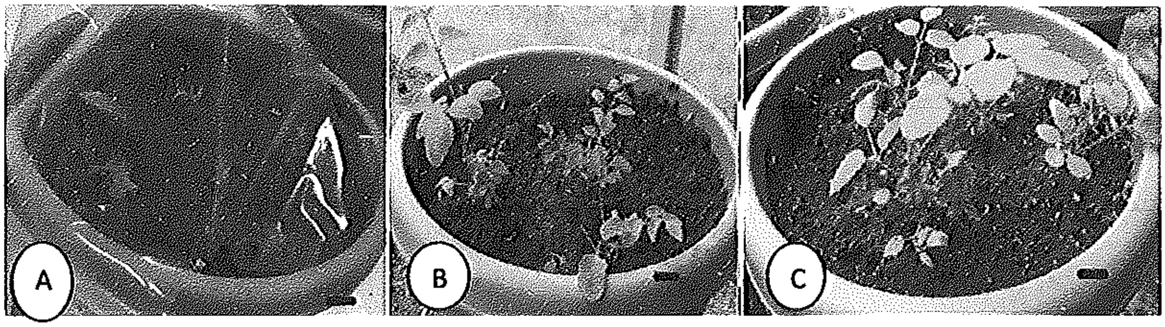

Figure 2.9: Acclimatization of *in vitro* rooted shoots of *M. stenopetala* in greenhouse.

(A) Day one, (B) After 1 month and (**C**) After 2 months Bars = 1cm

2.3 Discussion

In the present study, shoot initiation was made using BAP alone and BAP combined with NAA. All treatments with BAP along NAA showed 100% shoot initiation beginning from the first culture and all treatments of BAP alone showed 76-85% shoot initiation in the first culture and 100% shoot initiation starting from the second culture. Mitiku Muanenda (2013) indicated the problem of shoot initiation at the first culture from shoot tip explants of *in vito* grown seedlings and suggested that laying the explants on surface of the media can solve the problem. Our results did not prove this action to solve the problem but rather treatments of BAP combined with NAA could solve the problem of shoot initiation from shoot tip explants of *M. stenopetala* at the first culture. In general, MS media supplemented with BAP along NAA were found to be superior over MS media supplemented with BAP alone for shoot initiation.

In shoot multiplication study, the effect of the three cytokinins, BAP, KN and TDZ alone were evaluated. In addition, the synergetic effect of BAP and KN, BA and IBA, BAP and NAA, and TDZ combined with IBA were examined for shoot multiplication. It was found that the type, level and combination of the cytokinins and auxins used have profound effect on shoot multiplication. Of all these treatments, the highest number of shoots (11.16±0.14 and 10.86±0.14), were achieved at 0.5 mg/l BAP and 1.0 mg/l BAP respectively, without significant differences. This could indicate that lower concentrations (0.5mg/l and 1.0 mg/l) of BAP alone are the optimal concentrations for shoot multiplication of *M. stenopetala*. Mitiku Muanenda (2013) also reported that 1.0 mg/l BAP was optimal in producing the maximum mean number of shoots (17.40±0.74) per explant in *M. stenopetala*. The highest number of axillary shoots per explant was also obtained at 1.0 mg/l BAP in *Moringa oleifera* (Islam *et al.*, 2005; Saini *et al.*, 2012; Stephenson and Fahey, 2004) but more number of shoots per explant was obtained in the present study compared to these reports in *M. oleifera*. Variations in genotype, the genotype, type and age of the explant, culture medium composition and environmental factors such as CO_2 enrichment and light intensity can be the possible causes for the difference in shoot multiplication performance among these studies.

In all the three ctokinins (BAP, KN and TDZ) studied, more than 2.0 mg/l concentrations were less effective in shoot multiplication and also showed lesser shoot length and leaf numbers per shoot. The highest number of shoots per explant induced by KN was at 1.5 mg/l (6.03±0.23 shoots per explant) and for that of TDZ was at 0.5 mg/l (3.0±0.16 shoots per explant). This indicates that the use of TDZ for shoot multiplication has inhibitory effect. The plantlets also showed signs of senescence and subsequent growth was comprised of only very small, narrow leaves and shorten internodes. Magyar-Ta'bori *et al.* (2010) indicated that although TDZ is a highly active synthetic cytokinin as compared to other naturally occurring amino purine cytokinins, it can cause undesirable side effects such as inhibited shoot elongation and rooting. Coste *et al.* (2011) also reported that media containing TDZ induced hyperhydric malformation, shortening of internodes, and differentiation of smaller leaves in shoot cultures of *Hypericum hirsutum* and *H. maculatum*. The undesirable effects might be attributed to the fact that application of TDZ can affect the concentration of endogenous plant growth regulators. Many studies also reported that TDZ is known to affect pathways of purines and cytokinin metabolisms (Capelle *et al.*, 1983; Mok *et al.*, 1982; Laloue and Fox, 1989). For example, Laloue and

Fox (1989) reported that TDZ can prevent the breakdown of purines by inhibiting cytokinin oxidase. It was also reported that TDZ application causes alterations in nitrate reductase, ATP, ribulose diphosphate carboxylase oxidase and pentose phosphate enzymes (Kulaeva *et al.*, 1982; Mok *et al.*, 1987). Guol *et al.* (2011) also pointed out that morphological changes appeared in TDZ-induced tissues and organs might be the consequence of alterations in enzyme kinetics.

The synergetic effect of BAP and KN is more effective as compared to the other three combinations (BAP + IBA, BAP + NAA, TDZ + IBA). The synergetic effect of BAP and KN is most effective at 1.0 mg/l BAP + 1.0 mg/l KN (7.50±0.15 shoots per explant), 1.0 mg/l BAP + 2.0 mg/l KN (8.03±0.21 shoots per explant), 2.0 mg/l BAP + 1.5 mg/l KN (7.66±0.15 shoots per explant), 2.0 mg/l BAP + 1.0 mg/l KN (7.46±0.15shoots per explant), with no significant differences. Unlike the present study, Mitiku Muanenda (2013) reported that the synergetic effect of BAP + NAA was more effective than BAP + KN in shoot multiplication of *M. stenopetala.* This variation might be attributed to a difference in the endogenous cytokinin level of the explants used in the two studies due to genotype differences.

In the present study, the synergetic effect of the two combinations (BAP + IBA, BAP + NAA) was comparable and the application of 0.5 mg/l BAP along with IBA at 0.1 mg/l resulted in the highest number of shoots (5.40±0.09 shoots per explant) among the BAP and IBA combined treatments. Similarly, the highest number of shoots achieved at 2.0 mg/l BAP along with 0.1 mg/l NAA was 5.60±0.30 shoots per explant and it was the most effective among the BAP + NAA combined treatments. The application of TDZ and

IBA resulted in the least number of shoots per explant among the 4 combined treatments (BAP + KN, BAP + IBA, BAP + NAA, TDZ + IBA) and the highest shoot number (4.26±0.22) per explant was achieved at 0.5 mg/l TDZ + 0.01mg/l IBA. The addition of TDZ here also became inhibitory. The shoots developed very small and narrow leaves and short internodes.

The effects of different concentrations of NAA and IBA alone and NAA combined with IBA were also compared on root induction, root elongation and rooting response of micro shoots on 1/2 MS medium. Root formation, root length and rooting response were mainly related to growth regulator type and concentration as well as combination of growth regulators. Half MS media containing NAA + IBA and NAA alone were comparable in promoting root induction and the effect of IBA alone was less effective. Application of 0.5 mg/l NAA, 2.0 mg/l NAA and 0.5 mg/l NAA + 1.0 mg/l IBA resulted in the highest number of induced roots (8.0±0.8, 8.00±0.94, 8.33±0.92 per shoot respectively) after two weeks with no significant differences. The highest rooting response was also achieved at 1.0 mg/l NAA + 0.5 mg/l IBA. Among the treatments of IBA alone, the highest number of roots (6.66±0.60) per shoot was achieved at 1.0 mg/l IBA and the longest roots (2.96 cm) were also achieved at 1.0 mg/l IBA. Studies on *in vitro* induction of roots from microshoots of *M. stenopetala* were rare but Mitiku Muanenda (2013) reported that the highest number of roots (10.20±0.15) was achieved at 1.0 mg/l NAA indicating that NAA alone was more effective than IBA + NAA and IBA alone. There were more reports on the *in vitro* induction of roots from microshoots in the related species, *M. oleifera*. Marfori (2010) reported that the use of 0.05mg/l NAA alone is more effective (6.8 roots per shoot) in inducing roots. On the other hand, Saini *et al.* (2012) also indicated the application of two auxins, 0.5 mg/l IAA along with 1.0 mg/l IBA, resulted in the highest number of roots (15±1.3 per shoot) in *M. oleifera*. These reports are virtually similar with the present study. However, Islam *et al.* (2005) also reported that growth regulators-free MS medium gave more number of roots than the medium fortified with auxins. Shahzad *et al.* (2014) also reported that more roots were produced on growth regulators-free half and full salt strength MS media than the media containing auxins.These variable responses may be attributed to differences in the plant source and the type of explants used to establish tissue cultures. Eighty five percent of plants survived after acclimatization, similar with the results obtained by Shahzad *et al.* (2014) for *M. oleifera*.

3. Micropropagation of *Moringa stenopetala* using nodes as explants

Use of nodes as explants for clonal propagation has advantage by producing true to type plants from a single individual and has a considerable importance for large scale propagation in a short period of time (Ugandhar *et al.*, 2012).

Each *in vitro* produced shoot consist multiple nodes that potentially can be used as an explant to produce another shoot. But it is very important to develop a rational combination among growth regulators, level of growth regulators and explant types for the regeneration of multiple shoots. Regeneration of shoots from explants was found highly dependent on level and the type of plant growth regulator (Shahzad *et al.*, 2014). Induction of multiple shoots in *Moringa* was observed to be more successful in nodes as compared to other explants. Islam *et al.* (2005) also indicated that large scale propagation by tissue culture was feasible and several plantlets can be regenerated from one nodal explant in Moringa.

3.1 Materials and methods

Source of explant

Nodal explants were prepared from microshoots obtained from *in vitro* multiplication of shoot tips of *M. stenopetala* (chapter 2) and sub cultured in MS basal medium supplemented with 0.5 mg/l and 1.0 mg/l BAP.

Shoot multiplication

Nodal explants were transferred to shoot multiplication medium consisting of MS salts containing different concentrations of BAP (0.0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l) or Kinetine (0.0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l), BAP (0.0, 1.0, 2.0 and 3.0 mg/l) in combination with KN (0.0, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l), BAP (0.0, 0.5, 1.0, 1.5, 2.0 and 2.5 mg/l) in combination with IBA (0.0, 0.01 and 0.5 mg/l), BAP (0.0, 1.5, 2.0, 2.5, 3.0 and 4.0 mg/l) in combination with NAA (0.0, 0.1 and 0.5 mg/l) to determine their effect on multiple axillary shoot formation.The medium was supplemented with 30 g/l sucrose (w/v) and the pH was adjusted to 5.8 before addition of 8 g/l agar (w/v). Five shoots per culture vessel and six replications per treatment were used. The cultures were maintained in culture room under light intensity of 40 µmol m^{-2} s^{-1} and 16 hour photoperiod provided by cool-white fluorescent lamps at a temperature of 25±2 °C. Number of shoots and leaves per explant and shoot length were recorded 21 days after transfer to shoot multiplication medium.

Root induction

Multiple shoots obtained from nodal explants were used for root induction. Half strength MS medium supplemented with NAA or IBA (0.0, 0.5, 1.0, 1.5 and 2.0 mg/l), NAA (0.5 and 1.0 mg/l) combined with IBA (0.5, 1.0, 1.5 and 2.0 mg/l), 30 g/l sucrose (w/v) and 8 g/l agar (w/v) were used for root induction. pH of the medium was adjusted to 5.8 prior to autoclaving at 121°C with a pressure of 105 Kpa for 15 min. Thirty explants per treatment and five shoots per culture vessel with six replicates were used. Cultures were maintained in growth room with the same culture condition as indicated for shoot multiplication experiments. The percentage of shoots producing roots, number and length of roots were recorded after four weeks.

Acclimatization

The roots of four-week-old plantlets were thoroughly washed with running tap water to avoid all media residues, and then planted in plastic pots filled with autoclaved sand soil, red soil and compost in a ratio of 2:1:2 (v/v) respectively. The plantlets were covered with polyethylene bags and irrigated with sterile distilled water for two weeks in a greenhouse. Then the plastic bags were removed after two weeks and watering with tap water was done. One hundred twenty plantlets were used for acclimatization.The survived plantlets were recorded after 60 days of acclimatization.

Experimental design and data analysis

A completely randomized design (CRD) was used throughout the experiments. Data were subjected to analysis of variance (ANOVA) using statistical data analysis software SPSS version 20.0 at 5% probability level.

3.2 Results

Shoot Multiplication

The Effect of BAP and KN alone

The number of axillary shoots per nodal explant induced by BAP and KN alone at various concentrations after 21 days is shown in Table 3.1 and some multiplied shoots are shown in Figure 3.1. Application of 1.0 mg/l BAP resulted in the highest number of induced axillary shoots (13.66±0.20) and leaves (12.03±0.35) per nodal explant. The next highest mean number of shoot (6.96±0.49) and leaves (8.00±0.59) were obtained in the presence of 2.0 mg/l KN. At lower concentrations, KN was less effective than BAP in inducing axillary shoots per explant and vice versa. The lowest shoot proliferation was observed in the control medium. Both BAP and KN at higher and lower concentrations resulted in a reduction of shoot length and leaf number of shoots.

Table 3.1: The effect of BAP and KN alone on nodal micropropagation of *M.*

PGR(mg/l)	Mean no. of shoot per explants	Mean shoot length per explant (cm)	Mean no. of leaf per explant
BAP			
0.0	3.45 ± 0.01^{h}	1.21 ± 0.01^{b}	3.96 ± 0.15^{f}
0.5	5.40 ± 0.20^{ef}	1.50 ± 0.09^{a}	6.36 ± 0.32^{cd}
1.0	13.66 ± 0.20^{a}	1.25 ± 0.04^{b}	12.03 ± 0.35^{a}
1.5	5.13 ± 0.07^{ef}	1.41 ± 0.04^{a}	5.03 ± 0.07^{ef}
2.0	5.83 ± 0.40^{de}	$0.94\pm0.01d^{e}$	7.20 ± 0.59^{bc}
2.5	4.20 ± 0.15^{g}	0.98 ± 0.03^{d}	4.46 ± 0.10^{ef}
3.0	4.06 ± 0.22^{gh}	0.85 ± 0.01^{ef}	4.43 ± 0.37^{ef}
Kn			
0.5	4.21 ± 0.12^{g}	0.99 ± 0.4^{d}	4.45 ± 0.23^{ef}
1.0	4.73 ± 0.08^{fg}	0.95 ± 0.01^{de}	5.50 ± 0.09^{de}
1.5	5.23 ± 0.15^{ef}	1.02 ± 0.02^{ed}	6.20 ± 0.29^{cd}
2.0	6.96 ± 0.49^{b}	1.10 ± 0.01^{c}	8.00 ± 0.59^{b}
2.5	6.53 ± 0.16^{bc}	1.00 ± 0.01^{cd}	6.96 ± 0.33^{bc}
3.0	6.20 ± 0.29^{cd}	1.02 ± 0.02^{cd}	7.70 ± 0.48^{b}

Means within columns having different letter in superscript are significantly different at $p < 0.05$.

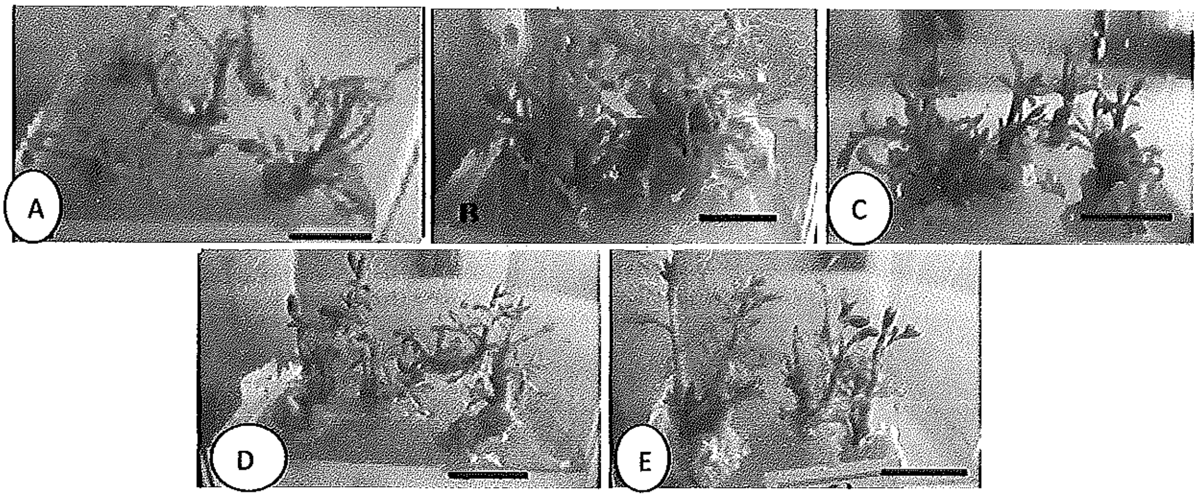

Figure 3.1: Multiple shoots obtained from nodal explants of *M. stenopetala* on MS medium supplemented with BAP and KN. **(A)** Control, **(B)** 1.0 mg/l BAP, **(C)** 2.0 mg/l BAP, **(D)** 1.0 mg/l KN and **(E)** 2.0 mg/l KN. Bars =1 cm

Synergetic effect of BAP and KN

The effects of different concentrations of BAP (1.0, 2.0, and 3.0 mg/l) with five different concentration of KN (1.0, 1.5, 2.0, 2.5, 3.0 mg/l) was compared on shoot induction from node culture (Table 3.2). Growth regulator free MS basal medium was also used as a control. Among the various level of BAP combined with KN, the medium containing 2.0 mg/l BAP + 1.0 mg/l KN produced the highest mean number of shoot (9.80±0.27) and leaves (10.86±0.54) followed by 8.26±0.08 shoot and 9.26±0.36 leaves per explant in the medium containing 1.0 mg/l BAP + 1.5 mg/l KN. The highest shoot length (1.21±0.01cm) was recorded in the control. Shoots on all culture media had healthy appearance (Fig 3.2).

Table 3.2: The effect of BAP and KN on shoot multiplication of nodal explants of *M. stenopetala*

PGR(mg/l)		Mean no. of shoot per explant	Mean shoot length per explant (cm)	Mean no. of leaves per explant
BAP	Kn			
0.0	0.0	3.45 ± 0.01^{g}	1.21 ± 0.01^{a}	3.96 ± 0.15^{hi}
1.0	1.0	6.26 ± 0.34^{d}	0.92 ± 0.01^{efg}	7.80 ± 0.27^{c}
1.0	1.5	8.26 ± 0.08^{b}	0.90 ± 0.01^{efg}	9.26 ± 0.36^{b}
1.0	2.0	6.33 ± 0.41^{d}	0.84 ± 0.02^{fg}	7.16 ± 0.51^{cd}
1.0	2.5	2.76 ± 0.26^{h}	0.81 ± 0.03^{g}	3.63 ± 0.30^{i}
1.0	3.0	3.80 ± 0.08^{fg}	1.06 ± 0.04^{bcd}	4.53 ± 0.09^{ghi}
2.0	1.0	9.80 ± 0.27^{a}	1.05 ± 0.01^{bcd}	10.86 ± 0.54^{a}
2.0	1.5	7.00 ± 0.37^{c}	0.95 ± 0.01^{def}	7.53 ± 0.38^{c}
2.0	2.0	5.73 ± 0.32^{d}	0.90 ± 0.01^{efg}	6.56 ± 0.43^{de}
2.0	2.5	3.90 ± 0.10^{fg}	1.01 ± 0.08^{bcde}	4.40 ± 0.10^{ghi}
2.0	3.0	3.86 ± 0.16^{fg}	1.06 ± 0.08^{bcd}	5.10 ± 0.18^{fg}
3.0	1.0	5.00 ± 0.13^{e}	0.80 ± 0.01^{g}	5.26 ± 0.15^{fg}
3.0	1.5	3.33 ± 0.22^{gh}	0.87 ± 0.02^{fg}	3.63 ± 0.1^{i}
3.0	2.0	3.86 ± 0.15^{fg}	1.12 ± 0.02^{ab}	4.83 ± 0.24^{gh}
3.0	2.5	4.60 ± 0.10^{e}	1.00 ± 0.01^{cde}	5.83 ± 0.20^{ef}
3.0	3.0	4.33 ± 0.17^{ef}	1.09 ± 0.05^{bc}	4.80 ± 0.08^{gh}

Means within columns having different letter (lower case) in superscript are significantly different at $p < 0.05$. The values represent mean ± S.E

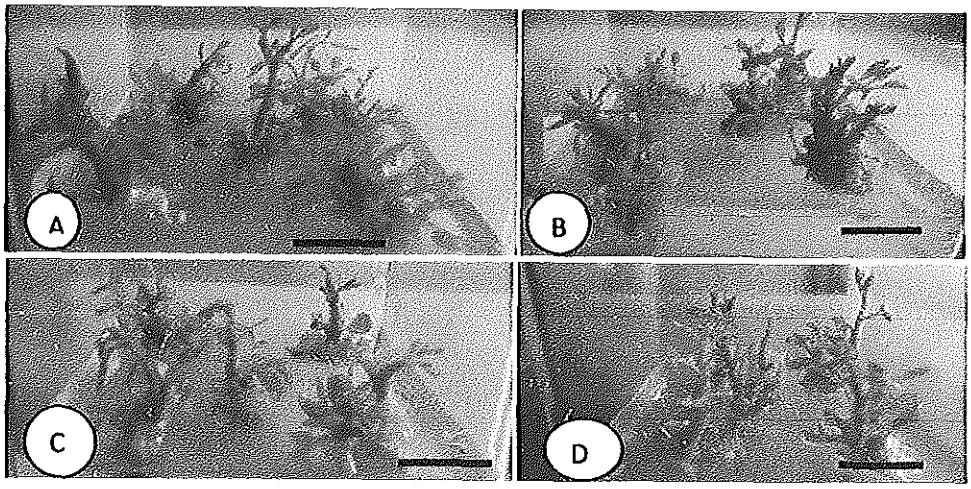

Figure 3.2: Multiple shoots obtained from nodal explants of *M. stenopetala* on MS medium supplemented with BAP and KN. (**A**) 2.0 mg/l BAP + 1.0 mg/lKN, (**B**) 1.0 mg/l BAP + 1.5 mg/l KN, (**C**) 1.0 mg/l BAP + 1.0 mg/l KN and (**D**) 3.0 mg/l BAP + 1.0 mg/l KN. Bars = 1 cm

Synergetic effect of BAP and IBA

The effect of different concentrations of BAP (0.5, 1.0, 1.5, 2.0, 2.5 mg/l) combined with 0.01 and 0.5 mg/l IBA on shoot induction from nodal explants culture was compared (Table 3.3). The medium containing 0.5 mg/l BAP + 0.01 mg/l IBA produced the best number of shoot (12.76±0.15) and the best number of leaves (14.73±0.08) per nodal explant. The medium containing 0.5 mg/l of both BAP and IBA produced the second highest number of shoots (9.86±0.46) and leaves (9.26±0.15). The highest shoot length (1.21±0.01) was recorded in the control. Shoots on all culture media had grown vigorously and had healthy appearance (Fig 3.3).

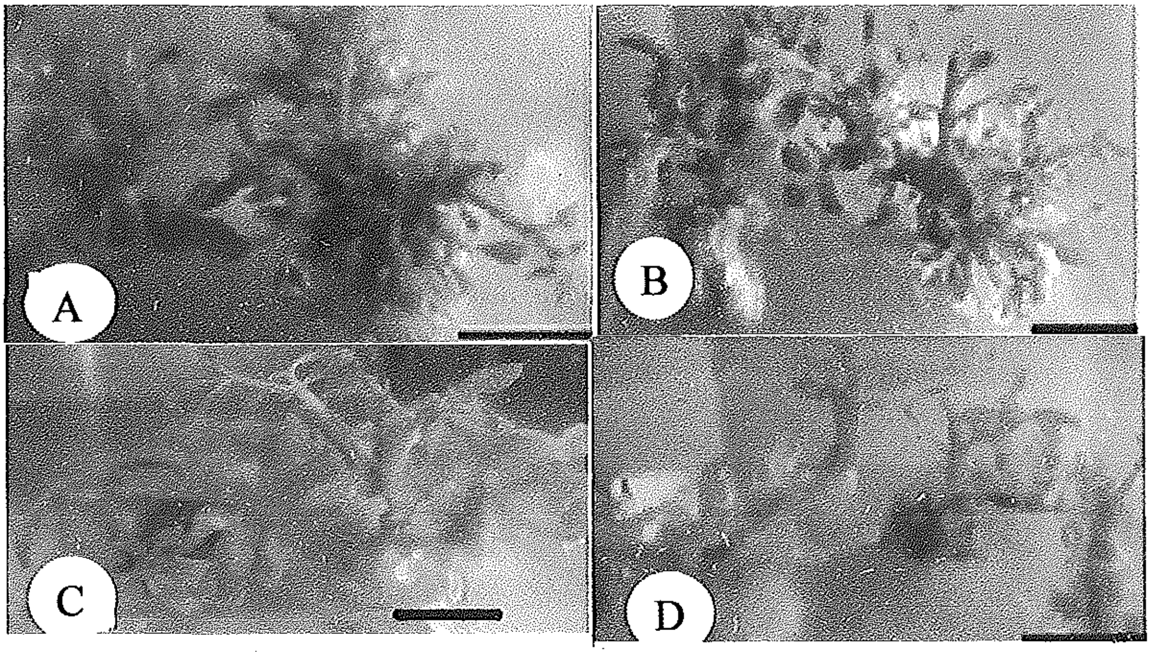

Figure 3.3: Multiple shoots obtained from nodal explants of *M. stenopetala* on MS medium supplemented with BAP and IBA. (**A**) 0.5 mg/l BAP + 0.01mg/l IBA, (**B**) 0.5 mg/l BAP + 0.5 mg/l IBA, (**C**) 1.0 mg/l BAP + 0.01 mg/l IBA, (**D**) 2.5 mg/l BAP + 0.01 mg/l IBA.

Bars = 1 cm

Table 3.3: The combined effect of BAP and IBA on shoot multiplication from nodal explants of *M. stenopetala*

PGR(mg/l)		Mean no. of shoot per explants	Mean shoot length per explant (cm)	Mean no. of leaves per explant
BAP	IBA			
0.0	.00	3.45±0.01^{f}	1.21± 0.01^{a}	3.96 ±0.15^{f}
0.5	0.01	12.76 ±0.15^{a}	1.06 ±0.06^{b}	14.73± 0.08^{a}
0.5	0.5	9.86 ±0.46^{b}	0.97 ±0.01^{c}	9.26 ±0.15^{b}
1.0	0.01	7.66 ±0.33^{c}	0.90± 0.01^{d}	8.06± 0.26^{cd}
1.0	0.5	5.73± 0.34^{de}	0.83 ±0.03^{d}	7.63 ±0.42^{d}
1.5	0.01	7.26 ±0.15^{c}	0.85 ±0.01^{d}	8.53± 0.30^{c}
1.5	0.5	6.00± 0.18^{d}	0.75± 0.02^{e}	7.43 ±0.35^{d}
2.0	0.01	5.03± 0.23^{e}	0.75 ±0.01^{e}	6.36± 0.42^{e}
2.0	0.5	6.10± 0.44^{d}	0.85± 0.01^{d}	6.56 ±0.27^{e}
2.5	0.01	3.50± 0.09^{f}	0.75± 0.01^{e}	3.20± 0.15^{g}
2.5	0.5	3.50± 0.09^{f}	0.65± 0.01^{f}	4.03± 0.13^{f}

Means within columns having different letter in superscript are significantly different at $p < 0.05$.

Synergetic effect of BAP and NAA

The effect of different concentrations of BAP (1.0, 1.5, 2.0, 2.5, 3.0 and 4.0 mg/l) in combination with 0.1 and 0.5 mg/l NAA was examined for shoot proliferation, elongation and number of leaves of microshoots of nodal explants (Table 3.4 and Fig 3.4). The maximum number of shoots (6.90±0.47) and leaves (8.00±0.84) per explant was observed in the medium containing 1.0 mg/l BAP + 0.1 mg/l NAA. The medium containing 2.0 mg/l BAP + 0.1 mg/l NAA produced the second highest number of shoot (4.93±0.27) per explant. The highest shoot length (1.21±0.01cm) was recorded in the control experiment. In general, the different concentrations of BAP combined with the lower level (0.1 mg/l) of NAA showed better shoot proliferation, elongation and number of leaves per explant than BAP combined with the higher concentration (0.5 mg/l) of NAA.

Table 3.4: The combined effect of BAP and NAA on shoot multiplication from nodal explants of *M. stenopetala*

PGR(mg/l)		Mean no. of shoot per explants	Mean shoot length per explant (cm)	Mean no. of leaves per explant
BAP	NAA			
0	0	3.45±0.01^{de}	1.21±0.01^{a}	3.96±0.15^{d}
1	0.1	6.90±0.47^{a}	1.04±0.24^{ab}	8.00±0.84^{a}
1	0.5	4.26±0.08^{bc}	0.87±0.01^{bc}	5.26±0.08^{c}
1.5	0.1	4.76±0.15^{bc}	0.92±0.01^{bc}	6.36±0.52^{b}
1.5	0.5	3.26±0.15^{def}	0.55±0.01e	2.96 ±0.13^{d}
2	0.1	4.93±0.27^{b}	0.57±0.01e	3.73±0.08^{d}
2	0.5	3.73±0.82^{cd}	0.75±0.01^{cde}	3.73±0.08^{d}
2.5	0.1	3.50±0.21^{d}	0.75±0.03^{cde}	5.06±0.32^{c}
2.5	0.5	2.76±0.15^{fg}	0.75±0.01^{cde}	3.30±0.20^{d}
3.0	0.1	2.50±0.09^{g}	0.65±0.01^{de}	3.30±0.08^{d}
3.0	0.5	2.73±0.08^{fg}	0.65±0.01^{de}	1.96±0.13^{e}
4.0	0.1	2.90±0.18^{efg}	0.83±0.05^{bcd}	3.30±0.21^{d}
4.0	0.5	2.50±0.11^{g}	0.90±0.09^{bc}	3.13±0.14^{d}

Means within columns having different letter in superscript are significantly different at $p < 0.05$.

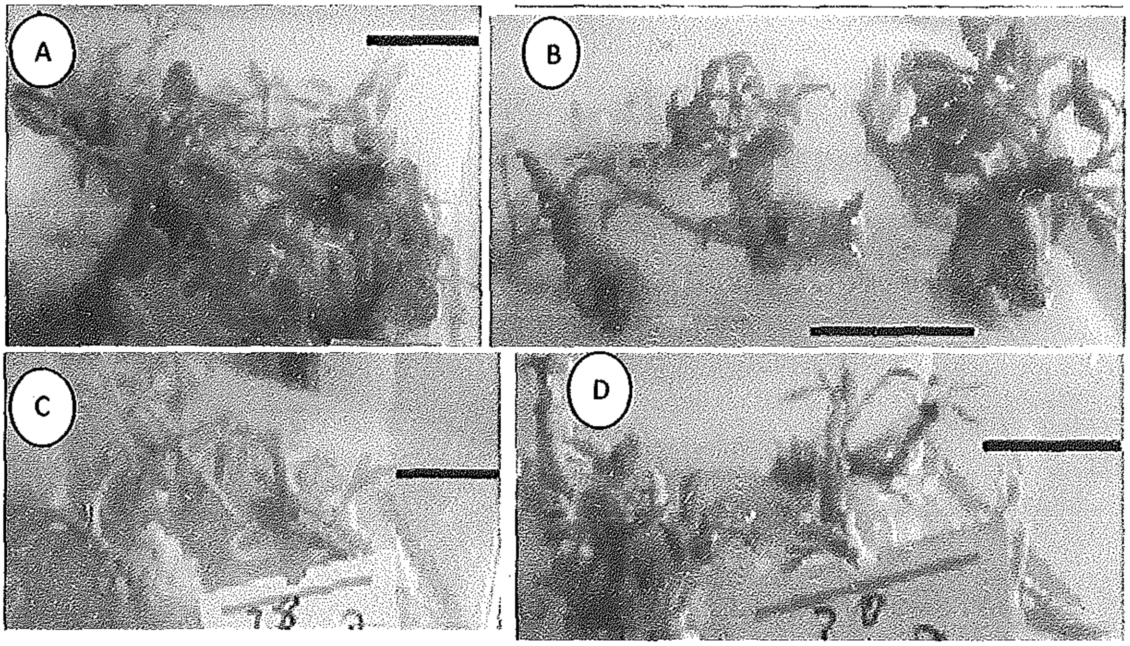

Figure 3.4: Microshoots obtained from nodal explants of *M. stenopetala* on MS medium supplemented with BAP and NAA. **(A)** 1.0 mg/l BAP + 0.1 mg/l NAA, **(B)** 2.0 mg/l BAP + 0.1 mg/l NAA, **(C)** 1.5mg/l BAP + 0.1 mg/l NAA, **(D)** 4.0 mg/ l BAP + 0.5 mg/l NAA.

Bars = 1 cm

Root induction

Rooting was observed after two weeks of transfer of microshoots to rooting media (Fig 3.5). Half strength MS medium containing NAA combined with IBA was virtually better in promoting root induction than 1/2 MS medium containing IBA or NAA alone(Table 3.5). In addition, 1/2 MS medium containing NAA alone showed better rooting than 1/2 MS medium containing IBA alone.

The highest root numbers per shoot (6.00±0.49) were obtained on half strength MS medium supplemented with 0.5 mg/l NAA + 1.0 mg/l IBA. Half strength MS medium fortified with 1.0 mg/l NAA alone and NAA + IBA (both 0.5 mg/l) produced the second highest number (4.63±0.08 and 4.60±0.30) of roots per explant respectively, with no significant differences. The highest rooting response (55.50±5.53 percent) was obtained on a medium containing 1.0 mg/l NAA + 1.5 mg /l IBA. The highest root length (3.33±0.08cm) was also obtained on 1/2 strength MS medium containing 0.5 mg/l NAA + 1.5 mg/l IBA.

Table 3.5: The effect of NAA and IBA on rooting of multiplied shoots from nodes of *M. stenopetala*

PGR(mg/l)		Mean no. of root per explants	Mean root length per explant (cm)	Mean rooting response of each treatment in percent
NAA	**IBA**			
-	0	3.88±0.01^{cd}	1.73±0.10^{e}	47.00±0.17^{b}
-	0.5	1.50±0.09^{gh}	1.25±0.04^{g}	24.20±0.18^{e}
-	1.0	1.43± 0.09^{gh}	2.23±0.14^{d}	24.30±0.13^{e}
-	1.5	1.00±0.01^{hi}	0.70±0.07^{h}	9.30±0.13^{f}
-	2.0	1.66±0.08^{fg}	2.66±0.08^{b}	49.86±0.17^{ab}
0.5	-	1.00± 0.00^{hi}	0.38±0.01^{i}	6.34±0.32^{f}
1.0	-	4.63±0.08^{b}	0.62±0.02^{hi}	24.50±0.09^{e}
1.5	-	0.93±0.01^{hi}	0.45±0.01^{hi}	8.53±0.21^{f}
2.0	-	4.20±0.14^{bcd}	0.55±0.01^{hi}	25.86±0.17^{de}
0.5	0.5	4.60±0.30^{b}	2.30±0.13^{cd}	48.00±0.41^{b}
0.5	1.0	6.00±0.49^{a}	2.51±0.07^{bc}	50.83±3.69^{ab}
0.5	1.5	1.96±0.14^{fg}	3.33±0.08^{a}	34.60±3.75^{c}
0.5	2.0	3.03±0.13^{e}	1.56±0.09^{ef}	31.50±2.81^{cd}
1.0	0.5	2.15±0.04^{f}	1.25±0.12^{g}	34.40±2.20^{c}
1.0	1.0	0.48±0.01^{i}	0.41±0.01^{i}	6.46±0.25^{f}
1.0	1.5	4.26 ±0.38^{bc}	1.63± 0.08^{ef}	55.50±5.53^{a}
1.0	2.0	3.60 ±0.41^{de}	1.40 ±0.09^{fg}	25.00±0.01^{e}

Means having same letter (s) in a column are not significantly different by Duncan's comparison test at $P < 0.05$ level.

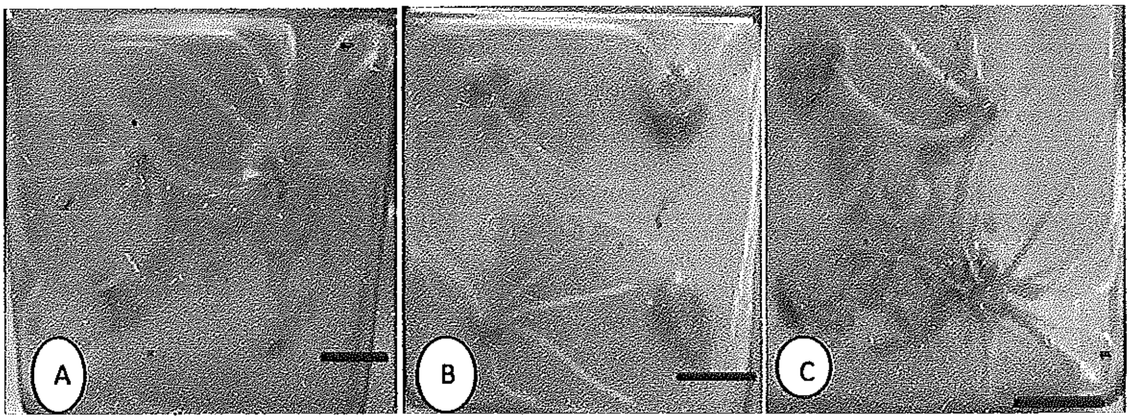

Figure 3.5: Root induction on 1/2 strength MS medium enriched with IBA and NAA. **(A)** 0.5 mg/l NAA + 1.0 mg/l IBA, **(B)** 0.5 mg/l NAA + 0.5mg/l IBA, **(C)** 1.0 mg/l NAA

Bars = 1 cm

Acclimatization

Acclimatization of plantlets was successful with 80% plants survived and healthy (Fig 3.6).

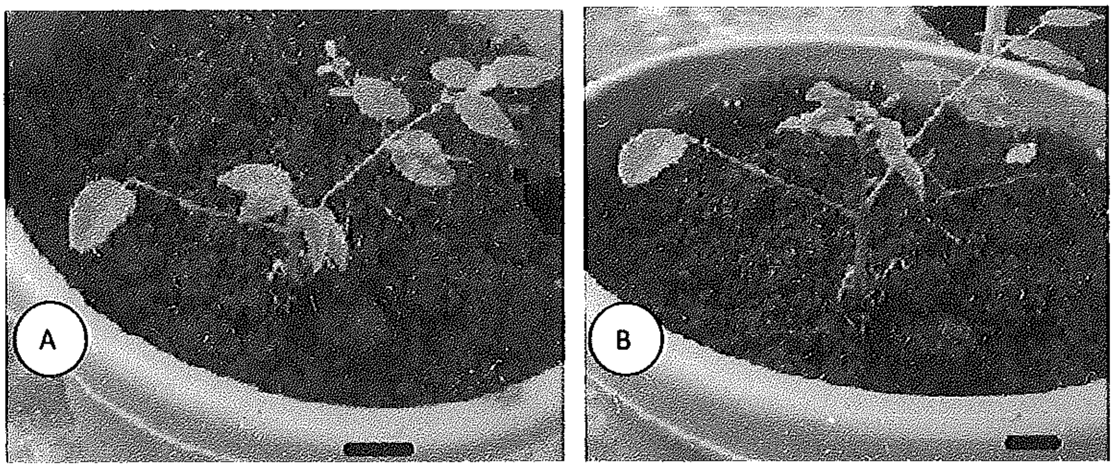

Figure 3.6: Acclimatization of *in vitro* rooted microshoots from nodal explants of *M. stenopetala* in greenhouse. **(A)** After 30 days and **(B)** After 60 days. Bars represent 1 cm

3.3 Discussion

In the present study, the effect of BAP and KN alone was evaluated for shoot induction and proliferation. The type and concentration of cytokinin used have profound effect on shoot multiplication. BAP was most effective at 1.0 mg/l inducing an average of 13.66±0.20 axillary shoots per explant whereas the highest number of axillary shoots per explant (6.96±0.49) induced by KN was at a concentration of 2.0 mg/l. In previous studies (Islam *et al.*, 2005; Saini *et al.*, 2012), the highest number of axillary shoots per explant (4.0±0.20 and 9.0±1.0) was also obtained at 1.0 mg/l BAP respectively in *Moringa oleifera* but less number of shoots per explant was obtained in these studies. The highest number of axillary shoots per explant (≈ 4.5) was also obtained at 0.5 mg/l BAP in some other studies (Marfori, 2010; Förster *et al.*, 2013). However, more than 5 axillary shoots per explant was obtained at 0.5 to 2.0 mg/l BAP or at 1.5 to 3.0 mg/l KN alone in the present study. These all variable responses may be attributed to differences in the plant source, the type of explants used to establish tissue cultures, medium composition and species type. For example, Saini *et al.* (2012) used nodal sections of young, aseptically grown seedlings. On the other hand, Islam *et al.* (2005) used stem explants obtained from field-grown plants. It was also noted that BAP at lower concentrations (0.5 - 2.0 mg/l) and KN at higher concentrations (2.0 - 3.0 mg/l) are found to be more effective in inducing axillary shoots. Unlike the present study, low concentrations of KN resulted in more number of shoots in *Moringa oleifera* (Riyathong, 2010; Abdellatef and Khalafallah, 2010).

The present study also examined the synergetic effect of different PGRs (BAP and KN, BAP and IBA, BAP and NAA). The BAP + KN and BAP + IBA combined treatments showed better synergistic effect than the BAP + NAA treatments on multiple shoot proliferation. Earlier, no reports were available on the use of such combination of PGRs to induce multiple shoots from nodal explants of *M. stenopetala*. Among the three different combinations (BAP and KN, BAP and IBA, BAP and NAA), the synergetic effect of BAP and IBA produced the highest (12.76±0.15) number of axillary shoots per explant at 0.5 mg/l BAP + 0.01 mg/l IBA. The second highest mean number of axillary shoots (≈ 9.8) per nodal explant was achieved at both 2.0 mg/l BAP + 1.0 mg/l KN and 0.5 mg/l BAP + 0.5mg/l IBA. The combined effect of BAP and IBA virtually induced more axillary shoots per explant than the combined effect of BAP and KN. The synergetic effect of BAP and NAA induced callus which later hindered shoot-bud production and it produced the least number of axillary shoots per explant among these three different combinations. The highest number of axillary shoots per each nodal explant, in BAP and NAA combinations, was 6.90±0.47 at 1.0 mg/l BAP + 0.1 mg/l NAA. Sivanesan and Jeong (2009) also reported that MS medium containing BAP with IBA enhanced the number of shoots per explant as compared to BAP with NAA in other plant species.

The effects of different concentrations of NAA and IBA alone and NAA (0.5 mg/l, 1.0 mg/l) combined with IBA (0.5, 1.0, 1.5, 2.0 mg/l) were compared on root induction, root elongation and rooting response of microshoots arose from nodal explants culture. Root formation, root length and rooting response were mainly correlated with growth regulator type and level as well as combination of growth regulators. Half strength MS medium containing NAA combined with IBA was virtually better in promoting root induction than individual performance of these growth regulators. Application of 0.5 mg/l NAA along with IBA at 1.0 mg/l on half strength MS medium resulted in the highest number of induced roots (6.00±0.49 per shoot) after two weeks. The highest rooting response (55.50±5.53 percent) was achieved at 1.0 mg/l NAA + 1.5 mg /l IBA. The highest root length (3.33±0.08cm) was obtained on half strength MS medium fortified with 0.5 mg/l NAA + 1.5 mg/l IBA. In addition, 1/2 MS medium with NAA alone showed better rooting than 1/2 MS medium containing IBA alone. The optimum concentrations of NAA (1.0 mg/l) and IBA (2.0 mg/l) alone produced 4.63±0.08 and 1.66±0.08 mean roots per shoot respectively.

Although auxins are known to promote rooting response, their type and level in the nutrient medium were found to vary from tissue to tissue and species to species (D'silva and D'souza, 1992). There were different reports on the optimum *in vitro* root inducing treatments in microshoots derived from nodal explants to be used in Moringa. Marfori (2010) reported that the use of NAA (0.05 mg/l) alone is more effective (6.8 roots per shoot) in inducing roots. On the other hand, Förster *et al.* (2013) reported the highest root development (60%) to be in MS medium without growth regulators. Saini *et al.* (2012) also indicated the application of two auxins, 0.5 mg/l IAA along with IBA at 1.0 mg/l, resulted in the highest number of induced roots (15±1.3 per shoot) after 7 days. Shahzad *et al.* (2014) also reported that the mean roots produced (3.83±0.40 mean roots per shoot) on growth regulator free half and full MS media was more as compared to the mean number of roots produced on the media augmented with auxins. Islam *et al.* (2005) also concluded that growth regulator free MS media resulted in more number of roots (4 roots per shoot) than the media fortified with auxins. In the present study, PGRs-free half strength MS medium also induced more roots (3.88±0.01 roots per shoot) than the medium fortified with IBA alone where the optimum roots per shoot induced was 1.66±0.08 at 2.0 mg/l IBA. Similar with the results obtained by Marfori (2010) and Saini *et al.* (2012) in *M. oleifera*, eighty percent of the rooted plants survived after being transplanted in the soil in the present study.

The tissue culture protocol reported in this study is an alternative means of propagation of *M. stenopetala* plantlets with uniform genotypes for breeding selection and field experiments.

surface in direct contact with the medium. MS medium containing 30 g/l sucrose and different concentrations of TDZ (0.01, 0.02, 0.2, 1.0 and 2.0 mg/l) or NAA (0.002, 0.02, 0.1, 0.2, 0.5 and 1.0 mg/l) or 2,4-D (0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, and 4.0 mg/l) or three levels (0.0, 0.01 and 0.02 mg/l) of TDZ combined with 0.2 mg/l of both NAA and 2,4-D were used for callus induction. Growth regulator-free MS medium was used as control.

The pH of the medium was adjusted to 5.8 with 1N NaOH or HCl before addition of 8 g/l agar. Then the medium was autoclaved at 121°C for 15 min at 105 Kpa. Twenty five milli liter of the medium was dispensed into each Petri dish (90 mm diameter) after autoclaving. A total of 10 leaves per Petridish with four replications were used. The culture was maintained in full darkness for six weeks at room temprature. After two subcultures carried out every three weeks on the original callus induction medium, the number of leaves that induced callus (percentage of response) was recorded.

Shoot Regeneration

After six weeks, the calli produced were transferred to shoot regeneration medium containing various concentrations of BAP (0.5, 1.0, 1.5 and 2.0 mg/l) or KN (1.0, 1.5,2.0 and 2.5 mg/l) or 1.0 mg/l BAP in combination with KN (1.0, 1.5, 2.0 and 2.5 mg/l) or 0.5 mg/l BAP in combinations with NAA (0.01, 0.1, 0.5 and 1.0 mg/l) or 1.0 mg/l KN in combination with NAA (0.01, 0.1, 0.5 and 1.0 mg/l). Then the cultures were transferred to a 16-h photoperiod and covered with loose transparent soft papers for fifteen days. The cultures were then uncovered and maintained under dim light conditions at 25°C ± 2°C for another 15 days after which they were transferred to full light of 40 $\mu molm^{-2} \cdot s^{-1}$ and

16-h photoperiod. After 3 months, the number of calli that regenerated shoot, shoot length and number of regenerated shoots per explant were recorded.

Shoot Multiplication

Regenerated microshoots were cultured on shoot multiplication medium containing different concentrations of BAP (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l) or KN (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 mg/l) or BAP (1.0 and 2.0 mg/l) in combination with KN (1.0, 1.5, 2.0, 2.5 and 3.0 mg/L). Fifty milli liter medium was dispensed into each Magenta GA-7 culture vessels. A total of 30 shoots per treatment with five shoots per culture vessel in 6 replications were used. The cultures were maintained in growth room under light intensity of 40 μmolm^{-2} s^{-1} and 16-h photoperiod provided by cool-white fluorescent lamp at 25°C ± 2°C.The number of shoots and shoot length per explant was recorded after three weeks.

Rooting

Well developed microshoots were cultured on half strength MS medium containing 30 g/l sucrose and supplemented with different concentrations of IBA (0.5, 1.0, 1.5 and 2.0 mg/l) or NAA (0.5, 1.0, 1.5 and 2.0 mg/l) or NAA (0.5, 1.0, 1.5 and 2.0 mg/l) in combination with IBA (0.5, 1.0, 1.5 and 2.0 mg/l). A total of 30 shoots per treatment with five shoots per culture vessel in 6 replications were used. Then the cultures were placed in growth room in the same condition as indicated for shoot multiplication. The number and length of roots were recorded after four weeks of culture.

Acclimatization

Plantlets were carefully removed from culture vessels and the roots were carefully washed under running tap water followed by transfer to plastic pots containing sand, red soil, and compost in 1:3:2 ratio respectively and kept in greenhouse. The potted plantlets were covered with polyethylene bags for the first two weeks and then removed. One hundred twenty plantlets were used for acclimatization. The number of survived plants was recorded after two months.

Experimental Design and Data Analysis

In all experiments, Completely Randomized Design (CRD) was used. Data were subjected to one way ANOVA and least significant difference (LSD) test using statistical data analysis software SPSS version 20.0 at 5% probability level.

4.2 Results

Callus Induction

Significant differences for callus induction were observed among different concentrations and combinations of growth regulators (Table 4.1). The use of TDZ alone at lower concentrations and application of 2,4-D, TDZ and/or NAA together led to vigrous callus formation on MS medium. One hundred percent of callus formation was observed in the medium supplemented with TDZ (0.01 and 0.02 mg/l) alone, NAA + 2,4-D (both 0.2mg/l) + TDZ (0.0, 0.01, 0.02 mg/l), and 2,4-D (0.2 mg/l) + TDZ (0.0, 0.01, 0,02 mg/l). Among the different treatments of NAA and 2,4-D alone, 1.0 mg/l NAA and 3.0 mg/l 2,4-D showed the highest response (70.40±0.55 and 70.33±0.28) in callus induction respectively, with no significant differences. Callus formation was observed after 15 days of culture and numerous white, friable and compact calli were seen. Callus induction was not observed in the presence of 0.002 and 0.5 mg/l NAA and on the control medium.

Table 4.1: Effect of NAA, TDZ and 2, 4-D on callus induction from leaves of *M. Stenopetala*

Growth regulators (mg/l)			Callus formation (%)
NAA	TDZ	2,4-D	
0.0	0.0	0.0	0.0^{l}
.....	0.01	.....	100 ± 0.0^{a}
.....	0.02	.....	100 ± 0.0^{a}
....	0.2	.....	83.83 ± 0.27^{c}
.....	1.0	.....	86.50 ± 0.27^{b}
.....	2.0	.....	84.80 ± 0.30^{c}
0.002	.....	.....	0.0 ± 0.0^{l}
0.02	.....	.....	39.83 ± 0.81^{g}
0.1	.....	.....	50.66 ± 0.78^{f}
0.2	.....	.....	26.83 ± 0.44^{j}
0.5	.....	.....	0.0 ± 0.00^{l}
1.0	.....	.....	70.40 ± 0.55^{d}
.....	.....	0.5	20.16 ± 0.77^{k}
.....	.....	1.0	30.20 ± 0.18^{i}
.....	.....	1.5	38.26 ± 0.26^{h}
.....	.....	2.0	50.33 ± 0.63^{f}
.....	.....	2.5	65.33 ± 0.71^{e}
.....	.....	3.0	70.33 ± 0.28^{d}
.....	.....	3.5	27.20 ± 0.55^{j}
.....	.....	4.0	20.50 ± 0.80^{k}
0.2	0.0	0.2	100 ± 0.0^{a}
0.2	0.01	0.2	100 ± 0.0^{a}
0.2	0.02	0.2	100 ± 0.0^{a}
0.0	0.0	0.2	100 ± 0.0^{a}
0.0	0.01	0.2	100 ± 0.0^{a}
0.0	0.02	0.2	100 ± 0.0^{a}

Means within columns having different letter (lower case) in superscript are significantly different at $p < 0.05$. The values represent mean ± S.E

Shoot Regeneration

The maximum shoot regeneration percentage (63.66%), mean shoot number (5.13±0.25) and mean shoot length (1.24±0.01cm) per callus was achieved on a medium supplemented with BAP and KN each at 1.0 mg/l concentration. The medium fortified with 1.0 mg/l BAP + 1.5 mg/l KN showed the next highest shoot regeneration percentage (45.00±0.18), mean shoot number (3.96±0.13) and mean shoot length (1.16 ± 0.01cm). Callus grown on MS medium without growth regulator (control), callus grown on MS medium supplemented with BAP at 0.5 and 1.0 mg/l and callus grown on MS medium supplemented with 0.5 mg/l BAP + 1.0 mg/l NAA and the medium with KN + NAA each at 1.0 mg/l concentrations did not result in the regeneration of shoots from the callus cultures (Table 4.2).

Table 4.2: Effect of BAP, KN and NAA on shoot regeneration from leaf derived callus of *M. stenopetala*

PGRs			Regeneration percentage	Mean shoot no. per callus	Mean shoot length (cm)
BAP	KN	NAA			
0.0	0.0	0.0	.....	....	.....
0.5	.....	.....	.....	.....	.....
1.0	.....	.....	.....	.....	.....
1.5	.....	.....	5.00 ± 0.30^{ij}	1.02 ± 0.10^{f}	0.94 ± 0.01^{e}
2.0	.....	.....	9.90 ± 0.46^{g}	1.70 ± 0.03^{e}	0.89 ± 0.01^{f}
.....	1.0	.....	16.36 ± 0.48^{d}	2.20 ± 0.03^{d}	0.90 ± 0.01^{f}
.....	1.5	.....	17.40 ± 0.37^{d}	2.54 ± 0.07^{c}	1.09 ± 0.03^{d}
.....	2.0	.....	11.46 ± 0.44^{f}	2.36 ± 0.07^{cd}	1.12 ± 0.01^{cd}
.....	2.5	.....	5.73 ± 0.30^{i}	0.86 ± 0.02^{f}	0.77 ± 0.01^{h}
1.0	1.0	.....	63.66 ± 1.12^{a}	5.13 ± 0.25^{a}	1.24 ± 0.01^{a}
1.0	1.5	.....	45.00 ± 0.18^{b}	3.96 ± 0.13^{b}	1.16 ± 0.01^{b}
1.0	2.0	.....	19.70 ± 0.49^{c}	2.19 ± 0.03^{d}	0.85 ± 0.02^{g}
1.0	2.5	.....	10.30 ± 0.20^{g}	1.58 ± 0.07^{e}	1.08 ± 0.02^{d}
0.5	.....	0.01	10.30 ± 0.31^{g}	1.71 ± 0.03^{e}	1.13 ± 0.01^{c}
0.5	.....	0.1	8.33 ± 0.33^{h}	0.98 ± 0.04^{f}	0.78 ± 0.01^{h}
0.5	.....	0.5	2.93 ± 0.22^{k}	0.45 ± 0.01^{g}	0.71 ± 0.01^{i}
0.5	.....	1.0	.....	.....	.....
.....	1.0	0.01	13.07 ± 0.14^{e}	2.23 ± 0.03^{d}	1.11 ± 0.01^{cd}
.....	1.0	0.1	5.50 ± 0.33^{i}	0.86 ± 0.01^{f}	0.76 ± 0.02^{h}
.....	1.0	0.5	4.01 ± 0.11^{j}	0.56 ± 0.52^{g}	0.67 ± 0.11^{j}
.....	1.0	1.0	.....	.....	.....

Means having same letter (s) in a column are not significantly different by Duncan's comparison test at $P < 0.05$ level.

Shoot Multiplication

Multiplication of shoots showed significant differences among the treatments (Table 4.3). BAP at 1.0 mg/l and BAP (2.0 mg/l) + 1.5 mg/l KN showed the highest number of shoots (7.83 ±0.20 and 7.80±0.53) per explant respectively, with no significant differences. Besides, the next highest shoot number (7.56±0.34) per explant was obtained from the MS medium fortified with 2.0 mg/l BAP. The highest shoot length (1.77±0.11cm) per explant was also recorded in the MS medium without PGRs (control) followed by 1.58±0.01 cm shoot length per explant at 1.5 mg/l BAP.

Table 4.3: The effect of BAP and KN on multiplication of *in vitro* regenerated shoots from calli of *M. stenopetala* derived from leaf explants

PGRs (mg/l)		Mean no. of shoot per explant	Mean shoot length per explant (cm)
BAP	Kn		
0.0	0.0	2.70 ±0.14^{j}	1.77 ±0.11^{a}
0.5	.....	5.60± 0.17^{ef}	1.46 ±0.07^{bcd}
1.0	....	7.83± 0.20^{a}	1.53± 0.01^{bc}
1.5	.....	6.86± 0.24^{bcd}	1.58± 0.01^{b}
2.0	.....	7.56± 0.34^{ab}	1.33 ±0.05^{def}
2.5	.....	6.23± 0.36^{de}	1.36 ±0.05^{cde}
3.0	.....	5.63± 0.19^{ef}	1.06± 0.05^{g}
.....	0.5	4.13 ±0.18^{hi}	1.24± 0.06^{efg}
.....	1.0	7.03± 0.14^{bc}	1.21± 0.04^{efg}
.....	1.5	6.16 0.22^{de}	1.33± 0.01^{def}
.....	2.0	6.16 ±0.12^{de}	1.13± 0.03^{g}
.....	2.5	4.50 ±0.09^{gh}	1.23 ±0.05^{efg}
.....	3.0	4.33 ±0.08^{h}	1.20 ±0.04^{efg}
1.0	1.0	5.53± 0.19^{ef}	1.36± 0.05^{cde}
1.0	1.5	5.06 ±0.23^{fg}	1.18 ±0.05^{efg}
1.0	2.0	7.03 ±0.39^{bc}	1.34± 0.05^{def}
1.0	2.5	3.56 ±0.19^{i}	1.11± 0.05^{g}
1.0	3.0	3.96 ±0.03^{hi}	1.21± 0.03^{efg}
2.0	1.0	6.16 ±0.12^{de}	1.18± 0.05^{efg}
2.0	1.5	7.80± 0.53^{a}	1.33± 0.06 ^{def}
2.0	2.0	6.60± 0.33^{cd}	1.16± 0.04fg
2.0	2.5	4.50± 0.09^{gh}	1.22±0.07^{efg}
2.0	3.0	3.96 ±0.13^{hi}	1.11± 0.07^{g}

Means having same letter (s) in a column are not significantly different by Duncan's comparison test at $P < 0.05$ level.

Rooting

Among the root induction treatments, the 1/2 MS medium supplemented with 1.0 mg/l NAA + 0.5 mg/l IBA showed the highest root number (9.24 ± 0.03) per explant. IBA at 1.0 mg/l, NAA at 0.5 mg/l and 1.0 mg/l NAA + 2.0 mg/l IBA induced the next highest root number (8.47±0.01, 8.50±0.09 and 8.23±0.11) per explant respectively, with no significant differences (Table 4.4).

Table 4.4: Effect of NAA and IBA on root induction of shoots obtained from regenerated calli

PGRs		Mean root no. per explant	Mean root length per explants (cm)
NAA	IBA		
0.0	0.0	2.13 ± 0.06^{o}	1.13 ± 0.01^{m}
…..	0.5	2.45 ± 0.02^{n}	1.33 ± 0.02^{k}
…..	1.0	8.47 ± 0.01^{b}	2.45 ± 0.05^{c}
…..	1.5	1.94 ± 0.0^{p}	1.55 ± 0.01^{gh}
…..	2.0	1.07 ± 0.02^{p}	1.54 ± 0.02^{ghi}
0.5	…..	8.50 ± 0.09^{b}	2.82 ± 0.01^{a}
1.0	…..	5.13 ± 0.18^{g}	2.15 ± 0.13^{e}
1.5	…..	3.90 ± 0.01^{kl}	2.46 ± 0.01^{c}
2.0	…..	4.79 ± 0.02^{h}	1.50 ± 0.01^{hij}
0.5	0.5	4.26 ± 0.17^{ij}	1.10 ± 0.10^{m}
0.5	1.0	7.20 ± 0.18^{c}	1.80 ± 0.10^{f}
0.5	1.5	3.66 ± 0.08^{lmn}	1.59 ± 0.01^{gh}
0.5	2.0	3.81 ± 0.01^{klm}	1.45 ± 0.02^{ij}
1.0	0.5	9.24 ± 0.03^{a}	2.57 ± 0.03^{b}
1.0	1.0	6.66 ± 0.35^{d}	2.28 ± 0.01^{d}
1.0	1.5	4.06 ± 0.14^{jk}	1.61 ± 0.01^{g}
1.0	2.0	8.23 ± 0.11^{b}	2.32 ± 0.01^{d}
1.5	0.5	3.56 ± 0.09^{lmn}	1.57 ± 0.04^{gh}
1.5	1.0	4.47 ± 0.01^{i}	1.53 ± 0.04^{ghi}
1.5	1.5	2.03 ± 0.02^{p}	1.11 ± 0.01^{m}
1.5	2.0	6.26 ± 0.08^{e}	1.80 ± 0.10^{f}
2.0	0.5	5.84 ± 0.02^{f}	2.63 ± 0.04^{b}
2.0	1.0	4.24 ± 0.02^{ij}	1.42 ± 0.04^{j}
2.0	1.5	3.94 ± 0.03^{jkl}	1.14 ± 0.05^{l}
2.0	2.0	3.48 ± 0.01^{mn}	0.96 ± 0.04^{m}

Acclimatization

Seventy five percent of plants survived after acclimatization in glasshouse. The plantlets were grown well and no morphological abnormality was observed (Fig 4.1).

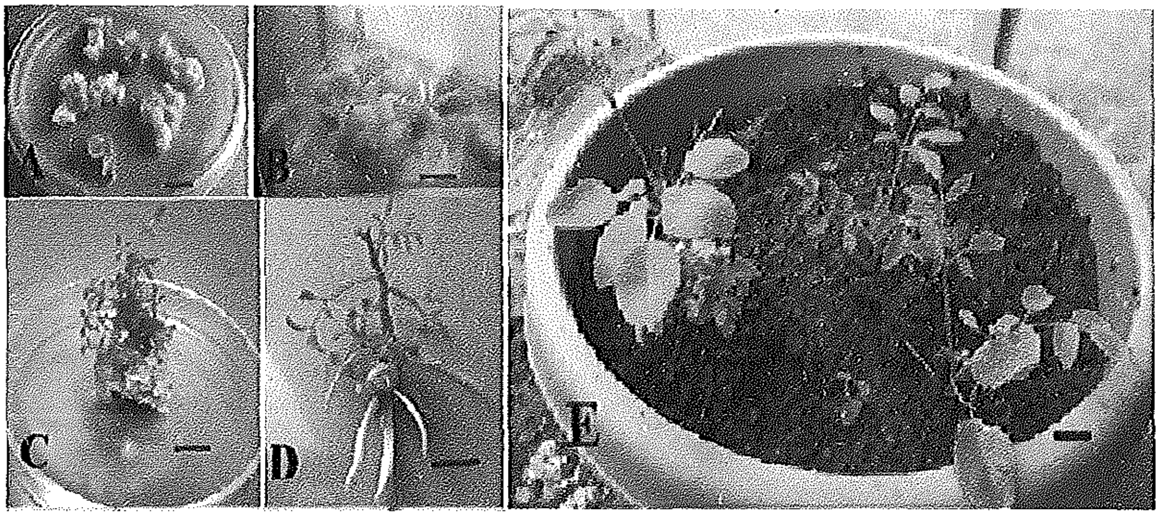

Figure 4.1: *In vitro* regeneration of *M. stenopetela.* (A) Callus induction, (B) Shoot formation, (C) Shoot proliferation, (D) Rooting and (E) Acclimatized plantlets after 2 months. Bars= 1cm

4.3 Discussion

Callus Induction

In the present study, the use of TDZ alone at lower concentrations and synergistic effects of 2,4-D, TDZ and/or NAA together led to vigrous callus formation on MS medium. Low concentration of TDZ (0.01 or 0.02 mg/l) alone or combined with NAA and/or 2,4-D (0.2 mg/l) showed 100% efficiency in callus formation on MS medium from leaf explants of *M. stenopetala*. Nieves and Aspuria (2011) also indicated that the BAP and TDZ are the commonly used ctyokinins for callus induction of woody plants which show synergistic effects with auxin at lower levels. In addition, Huetteman and Preece (1993) reported that callus induction and plant regeneration can be achieved using TDZ. Tileye Feyissa *et al.* (2005) also reported that high concentrations of TDZ promoted callus induction and shoot regeneration but inhibited shoot elongation from leaf explants of tree species: *Haygenia abyssinica*. The induction of strong callus at low concentration of TDZ alone or in combination with 2,4-D and NAA might be attributed to the fact that the high exogenous application of TDZ can affect the concentration of endogenous plant growth regulators especially in dicots (Capelle *et al.*, 1983; Mok *et al.*, 1982; Laloue and Fox, 1989).

Förster *et al.* (2013) also observed that the use of two phytohormones (IAA and TDZ) led to very strong callus formation of adaxial and abaxial oriented leaves of *M. oleifera* on MS medium. They also achieved 32-fold callus volume increase under dark conditions while the callus volume under light conditions was significantly lower. In addition, Nieves and Aspuria (2011) reported that BAP in combination with 2,4-D and TDZ significantly increased degree of callusing. They also noted the addition of TDZ to BAP and 2,4-D containing media further increases callus weight.

In the present study, among the different treatments of NAA and 2,4-D alone, 1.0 mg/l NAA and 3.0 mg/l 2,4-D showed the highest callus induction percentage (70.40±0.55 and 70.33±0.28) respectively, with no significant differences. Devendra *et al.* (2012) also reported 2,4-D (3.5 mg/l) and NAA(1.5 mg/l) showed 68.7% and 48.3% efficiency of callusing on MS medium from leaf explants of *M. oleifera*, respectively. In addition, Riyathong (2010) used MS medium supplemented with 0.5 mg/l 2,4-D for callus production from shoot explants for *in vitro* cultures of *M. oleifera*. Generally, the expanded range of concentrations observed here for callus induction might be attributed to differences in the species type, the possible effect of different genotypes, explant source and concentrations of the edogenous hormones.

Shoot Regeneration

Plant growth regulators play key role in the process of dedifferentiation and redifferentiation. These regenerative processes in cell and tissue cultures may be provoked by plant growth regulators. In the present study, the combined effect of BAP and KN had significant effect in the percentage of shoot regeneration, mean shoot number and mean shoot length per callus. The synergetic effect of BAP and KN (each 1.0 mg/l) on MS medium gave the maximum shoot regeneration percentage (63.66±1.12), mean shoot number (5.13±0.25) and mean shoot length (1.24±0.01cm) per callus derived from leaf explants of *M. stenopetala*. A promotive effect of BAP and KN in regeneration of shoots was also observed in other studies. Devendra *et al.*(2012) compared MS medium fortified with 3.0 mg/l BAP and KN (0.5, 1.0 and 1.5 mg/l concentrations) and it was observed that 3.0 mg/l BAP combined with 0.5 mg/l KN resulted in the highest percentage (61.3%) of shoot regeneration. However, they reported that the highest percentage of shoot regeneration (90.8%) was obtained in MS medium fortified with 3.0 mg/l BAP alone. These results might hold the fact that treatment of cytokinins result in shoot induction and formation (Dobranszki and Silva, 2010). On the other hand, Riyathong *et al.* (2010) reported that MS medium containing 0.5 mg/l NAA produced 80% shoot and 60% root formation with the highest average number of 3.13 shoots per explants in *M. oleifera*. The difference in shoot regeneration ability of different treatments observed in the present and previous studies might also be attributed to difference in species type, genotype and level of endogenous hormones.

Shoot Multiplication

This study revealed the efficiency of BAP for shoot multiplication in *M. stenopetala*. The promoting effect of BAP on shoot multiplication is in agreement with results from other related plant species such as in *M. oleifera* (Riyathong *et al*., 2010 and Förster *et al*., 2013). It was also observed that the application of BAP alone and the synergetic effect of BAP and KN had comparable effect on shoot multiplication of regenerated shoots from leaf callus of *M. stenopetala*.

Rooting

Different concentrations of IBA and NAA alone and in combination were used for root induction from microshoots in 1/2 MS medium. Virtually NAA was found to be superior over IBA for root induction in *M. stenopetala*. Similar results were also observed in other related species, *M. oleifera* (Devendra *et al*., 2012 and Nieves *et al*., 2011). In other woody species like *Mallotus repandus* (Prathanturarug *et al*., 2007), NAA was also effective in inducing root under *in vitro* conditions. However, the promotive effect of IBA on rooting has been also reported in *Pterocarpus marsupium* (Chand and Singh, 2004) and *Cinnamomum camphora* (Sharma and Vashistha, 2010). Further, the low salt medium (1/2 MS medium) was effective in root formation in other tree plants: *Aegle marmelos* (Nayak *et al*., 2007) and *Emblica officinalis* (Nayak *et al*., 2010). In the present study, the 1/2 MS medium supplied with 1.0 mg/l NAA combined with 0.5 mg/l IBA showed the highest root number (9.24 ± 0.03) per explant. Seventy five percent of plants survived after acclimatization in a greenhouse.

The protocol found in this study leads to regeneration of *M. stenopetala* plantlets from callus and makes the mass propagation of the species possible. In addition, secondary metabolites production and isolation of pharmaceuticals of interest from *M. stenopetala* callus is potentially possible.

5. Molecular diversity of *Moringa stenopetala* as revealed by ISSR marker

A prerequisite of any genetic improvement programs is a focus in genetic variability in the local germplasm so that breeding strategies can be mapped out. Studying the variability allows to identify duplicates, to estimate genetic linkage among individuals, to quantify genetic variability in the collection, to propose collections based on observed genetic diversity and to identify suitable breeding genotypes to improvement. The right genotype is especially important where the breeding or deployment strategy uses clonal propagation.

The development and use of molecular markers for the detection and exploitation of DNA polymorphism is one of the most significant developments in the field of molecular genetics. ISSRs are multi-locus systems making use of primers that simultaneously bind to many different parts of a genome resulting in the amplification of many loci at the same time. Edwards and McCouch (2007) suggested that when financial resources are restricted, ISSRs can provide large numbers of markers with a limited investment. ISSRs can provide high analysis using an automated sequencer and can be run on agarose gels with minimal investment in equipment.

ISSRs markers are quick and easy to handle. They have the reproducibility of SSR markers because of the longer length of their primers. Amplification in this technique does not require genome sequence information. ISSR is a dominant marker like RAPD (scored using presence or absence of bands). These features make ISSR better than other readily available marker systems in investigating the genetic variation among very closely related individuals and in crop cultivar classification (Fang *et al.,* 1997; Hormaza *et al.*1998). Little to no work to date has examined the application of the ISSR technique in genetic diversity study of *M. stenopetala* in Ethiopia and elsewhere.

5.1 Material and Methods

Plant materials

Leaf samples for DNA extraction were collected from Arba Minch, Wolaita, Sidama, Debub Omo zones, and Konso and Dherashe especial woredas in the SNNPR and from Shewa Robit in the northern part of Ethiopia (Fig 5.1; Table 5.1).

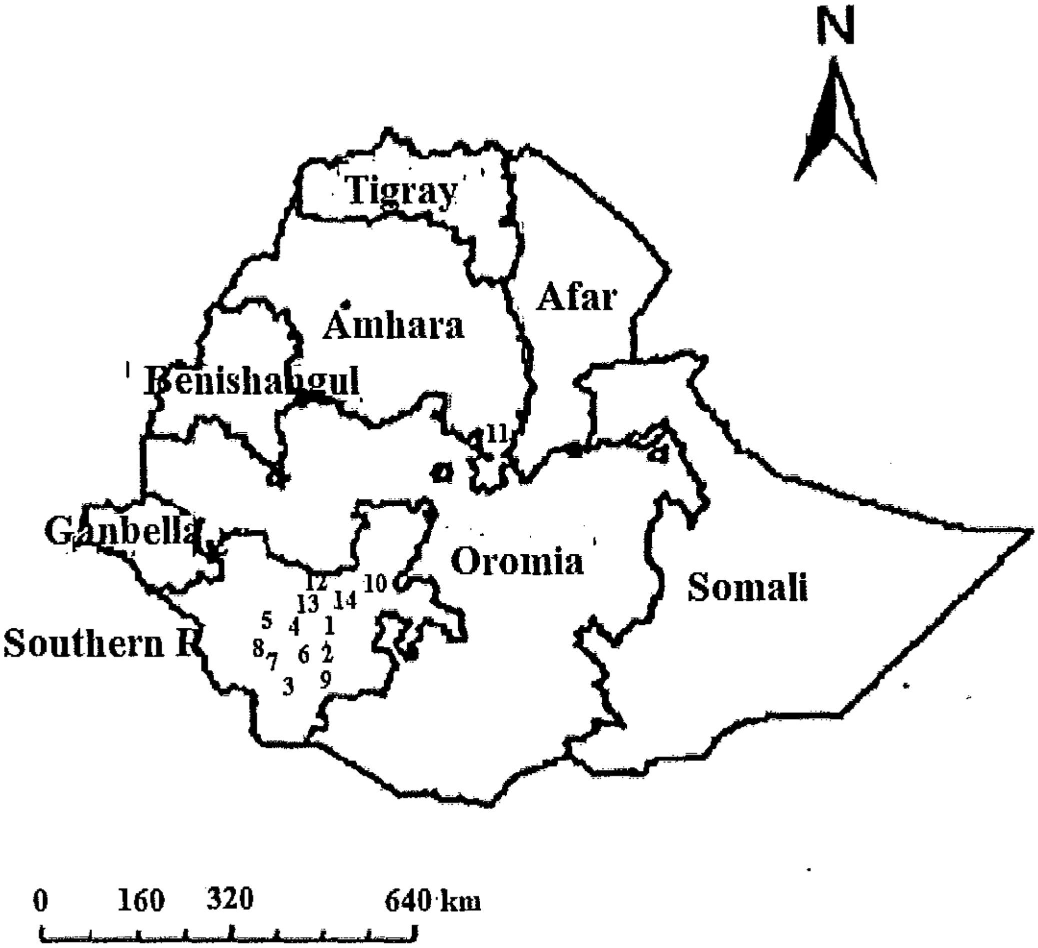

Figure 5.1: Map of Ethiopia showing the sampled populations of *Moringa stenopetala*. 1= Merab Abaya, 2 = Arba Minch town, 3 = Beto, 4 = Sawla town, 5 = Derashe, 6 = Jinka town, 7 = Male, 8 = Konso town, 9 = Konso-Degatu, 10 = Hawassa town, 11 = Shewa Robit town, 12 = Wolaita-Bele, 13 = Wolaita-Humbo and 14 = Wolaita-Humbo Tebela

Table 5.1: Sampling details of *M. stenopetala* populations in the present study

Population	Locality	Longitude (E)	Latitude (N)	Altitude (M)	No. of Individuals
Pop 1	AM-I (Arba minch zone, Merab abaya wereda, Merab abaya)	37°58.594’	06°11.228’	1219	11
Pop 2	AM-II (Arba minch zone, Arba minch town)	037°32.964’	06°02.666’	1309	10
Pop 3	AM-III (Arba minch zone , Beto Wereda, Beto kebele)	37°02.664’	06°45.121’	1067	11
Pop 4	AM-IV (Arba minch zone, Gofa wereda, Sawla town)	036°53.111’	06°18.012’	1346	10
Pop 5	DER (Derashe,Holte kebele)	037°26.535’	05°37.627’	1259	11
Pop 6	D.omo-I (Debub omo zone, Jinka town)	036°34.085’	05°47.117’	1411	11
Pop 7	D.omo-II (Debub omo zone, Male wereda, Baneta kebele)	036°45.915’	05°43.222’	1351	12
Pop 8	KON-I (Konso town)	037°26.323’	05°20.354’	1481	11
Pop 9	KON-II (Around Konso town-Degatu)	037°25.396’	05°19.952’	1616	10
Pop 10	SID (Sidama zone, Hawassa town)	38°28.895’	07°03.941’	1696	11
Pop 11	SR (Seimen shewa zone shewa robit town)	39°53.761’	9°59.758’	1239	11
Pop 12	WS-I (Wolita zone ,Kindo kesha wereda, Bele)	37°31.993’	6°55.126’	1251	11
Pop 13	WS-II (Wolita zone , Humbo Wereda, Humbo)	37°46.174’	6°42.254’	1608	10
Pop 14	WS-III (Wolita zone, Humbo wereda, Abela kebele)	37°45.101’	6°41.855’	1402	10

Sampling technique

Young leaves of 150 individuals (10 to 12 individuals from each population were sampled depending on the size of the population) of *Moringa stenopetala* representing 14 populations were collected in zip locked plastic bag containing silica gel as used by Muluvi *et al.* (1999) for *M. oleifera* and Olson (2000) for *Moringa* species (Table 5.1). The fourteen populations of *M. stenopetala* were subjected to genetic diversity analysis to evaluate the level of polymorphisms within populations and divergence among populations.

DNA extraction from leaf samples

Four to five leaves were ground to a fine powder using a grinding mortar with a pestle that was placed in liquid nitrogen and then ground to a fine powder manually. Nuclear genomic DNA was extracted for analysis from silica gel dried leaf tissues following the CTAB protocol procedure (Wang *et al.*, 1996) with slight modification.

Genomic DNA was extracted in 1.5 ml Eppendorf tubes using about 50 mg powder leaves. Then well crushed leave samples were mixed with 700 µl CTAB extraction buffer (2% CTAB, 5M NaCl, 2% PVP, 0.5M EDTA at pH 8.0, 1M Tris-HCl at pH 8.0 and 98% mercaptoethanol) pre-warmed in water at 65°C for 30min. The mixture was incubated for up to 30 min by thoroughly mixing at about 10 min interval in electrical constant temperature water box. Then centrifugation at 13,000 rpm for 7 min was followed and the same is repeated to attain quality DNA in the second extract. The supernatant of crude DNA extraction (the second) was transferred to new 1.5 ml Eppendorf tubes using cut blue tips. This crude DNA in the second extract was purified using 600 µl chloroform and followed by centrifugation at 13,000 rpm for 7 min. The supernatant of the second extraction was treated with isopropanol (2/3 of the solution volume) and incubated for more than 2h at 20°C. Then, it was centrifuged at 13,000 rpm for 12min, followed by aspiration and washed with 200 µl 70% ethanol. For further purification a pellet was dissolved with 100 µl 1xTE buffer and 50 µl of 7.5M NH_4Ac was added first, then 3M NaAc in the second round. Each salt treatment was followed by 100% ethanol (50µl) treatment. Then each mixture was incubated for more than 2h at - 20°C, centrifuged at 13,000 rpm for 35 min, followed by aspiration the fluid, washed pellet with 200 µl ethanol (70%) and aspirated using yellow tips. Finally DNA was resuspended in 100 µl 1×TE buffer (1M Tris-Hcl and 0.5 M EDTA pH 8.0) and stored at 4°C.

DNA quantification and quality test

The DNA concentration and its purity were determined by using nanodrop (Appendix 2). The quality and quantity of DNA was checked by running agarose gel electrophoresis and staining with ethidium bromide (Fig 5.2). The genomic DNA was tested using 1% agarose gel by applying 2µl genomic DNA loaded after mixing with 1µl 6× loading dye and 3µl deionized water. Agarose (0.5 g) and 50ml 1×TBE buffer (1M Tris-HCl, 0.5 M EDTA and 0.88M boric acid pH 8.0) were boiled using microwave oven for about 1min. It was casted on casting stand (Gel tray) after mixing with 2µl ethidium bromide for solidification. Subsequently, the DNA in agarose gel was subjected to electrophoresis in BIORAD min-sub® cell GT at 80V (from Biometra® standard power pack P25) for 40 min and visualized under UV using gel documentation system. The gel was photographed using Canon camera attached to gel documentation system.

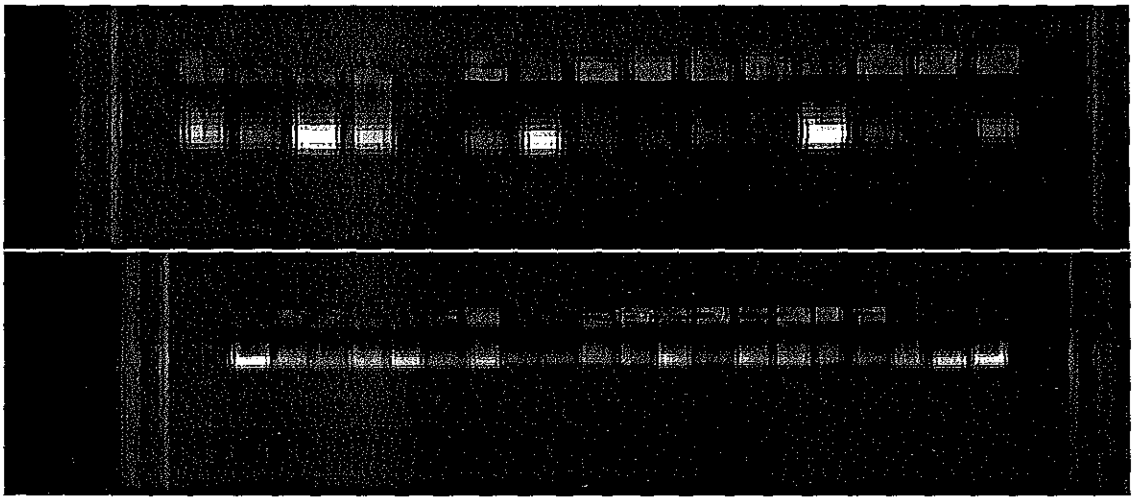

Figure 5.2: Test gel of genomic DNA of selected extracts among 150 Individual samples of M. stenopetala

ISSR Primer screening

A total of 12 primers were used for the initial testing of primer variability and reproducibility. Of these, four primers produced polymorphic and reproducible bands and were selected for the final analysis. ISSR primers that were used in the genus *Moringa* for assessment of genetic fidelity by Khateeb *et al.* (2013) and other primers were initially used to screen primers. Production of polymorphism and reproducible fragment patterns were used for screening of the best primers. Out of the 12 screened primers, four best performing ISSR primers (with the code 807, 835, 848, and 854) were used for further experimetns of detecting genetic diversity (Table 5.2).

Table 5.2: List of primers used for optimization, primer sequence, repeat motifs, annealing temperature and amplification patterns

ISSR primer	Primer Sequence (5' to 3')	Primer motif	T° (°C)	Amplification pattern	Remark
807	AGAGAGAGAGAGAGAGT	$(AG)_8T$	48	Polymorphic and reproducible	Accepted
818	CACACACACACACACAG	$(CA)_8G$	48	Not reproducible	Rejected
824	TCTCTCTCTCTCTCTCG	$(TC)_8G$	48	No amplification	Rejected
825	ACACACACACACACACT	$(AC)_8T$	48	Not reproducible	Rejected
827	ACACACACACACACACG	$(AC)_8G$	48	Not reproducible	Rejected
828	TGTGTGTGTGTGTGTGA	$(TG)_8A$	52	No amplification	Rejected
835	AGAGAGAGAGAGAGAGYC	$(AG)_8YC$	48	Polymorphic and reproducible	Accepted
848	CACACACACACACACARG	$(CA)_8RG$	48	Polymorphic and reproducible	Accepted
854	TCTCTCTCTCTCTCTCRG	$(TC)_8RG$	48	Polymorphic and reproducible	Accepted
857	ACACACACACACACACYG	$(AC)_8YG$	48	Not reproducible	Rejected
873	GAGAGAGAGAGAGACA	$(GA)_7CA$	50	No amplification	Rejected
880	GGAGAGGAGAGGAGA	$(GGAGA)_3$	45	No amplification	Rejected

Single-letter abbreviations for mixed base positions: R = (A, G), Y = (C, T); T° = Annealing temperature

PCR and gel electrophoresis

PCR amplification was carried out in a 25 µl reaction mixture containing 1 µl template DNA (25 ng) , 14.0 µl ddH_2O, 5.0 µl of dNTP mix (20mM), 2.5 µl Taq buffer (10x Thermopol reaction buffer), 2.0 µl $MgCl_2$ (2mM), 0.3 µl primer (20 pmol/µl) and 0.2 µl Taq Polymerase (5 u/µl).

The amplification program was set as 4 minutes preheating and initial denaturation at 94°C, followed by 40 cycles of 15 seconds at 94°C, 1 minute primer annealing at 45°C/ 48°C/50 °C /52 °C based on primers used, 1 minute and 30 second extension at 72°C and the final extension for 7 minutes at 72°C. The PCR products were stored at 4°C until loading on gel for electrophoresis. The lid temperature was held at 105°C.

The amplification products were separated by electrophoresis using an agarose gel (1.67% agarose with 100 ml 1xTBE) and 8µl amplification product of each sample with 2µl loading dye (6 times concentrated) was loaded on gel. DNA marker (100 bp molecular ladder) was used to estimate size of the fragments. Electrophoresis was done for 2 hours at constant voltage of 100V. The DNA was stained for 30 minutes with 10mg/ml ethidum bromide (EtBr) which was mixed and distained with 450 ml distilled water.

PCR product analysis

The PCR amplification products were electrophoresed on 1.2 % (w/v) agarose gel which consists of 0.5 µl/ml of 0.5 mg/ml ethidium bromide. TAE buffer (40 mM Tris acetate pH 8 and 1mM EDTA) was used as running buffer and also in the gel preparation. The PCR products were loaded into wells after 5µl of loading buffer (0.041% bromophenol blue, 0.041% xylene cynol and 6% glycerol) was mixed with them. The electrophoresis was done at 90 volts for about 2 and half hour. The gel DNA bands were scored through UV light illuminator and photographed by SensiCam digital camera.

Each ISSR band was considered as independent character or locus and assigned numbers in order of decreasing molecular weight. The position of each gel phenotypes was estimated against the DNA molecular ladder to avoid error while scoring.

Molecular data analysis

Clearly resolved, unambiguous bands were scored visually for their presence or absence for each primer and sample. The bands were recorded as discrete characters, presence '1' or absence '0' and '?' for missing data.The scored fragment data were assembled in data matrix for analysis. Each band was considered as a single and unique locus. Individuals with more than 5% missing data were removed from analysis. Finally, data were compiled as a binomial matrix. The total number of bands, distribution of bands across individuals, number of polymorphic bands in a set of individuals, and average number of bands per primer were calculated. Similarity matrix was generated based on the simple-matching coefficient, using the presence/absence data for individual PCR fragments.

Based on recorded bands different software was used for analysis. POPGENE version1.32 software (Yeh *et al.*, 1999) was used to calculate genetic diversity for each population as number of polymorphic loci and percent polymorphism. Analysis of molecular variance (AMOVA) was used to calculate variation among and within population using Areliquin version 3.01 (Excoffier *et al.*, 2006). NTSYS- pc version 2.02 (Rohlf, 2000) and Free Tree 0.9.1.50 (Pavlicek *et al.*, 1999) software were used to calculate Jaccard's similarity coefficient.

The Unweighted Pair Group Method with Arithmetic Mean (UPGMA) (Sneath and Sokal, 1973) was used to analyze and compare the population and generates phenogram using NTSYS- pc version 2.02 (Rohlf, 2000). The Neighbor Joining (NJ) method (Saitou and Nei1, 987; Studier and Keppler, 1988) was used to compare individual genotypes and evaluate patterns of genotype clustering using Free Tree 0.9.1.50 Software (Pavlicek *et al.,* 1999).

To further examine the patterns of variation among individual samples on 3D, a principal coordinated analysis (PCO) was performed based on Jaccard's coefficient (Jaccard, 1908). The calculation of Jaccard's coefficient was made with PAST software version 1.18 (Hammer *et al.,*2001). The first three axes were used to plot the three dimensional PCO with STATISTICA version 6.0 software (Hammer *et al.,* 2001; statistica soft, Inc.2001). Nei's (1978) unbiased genetic distance coefficient was also used to estimate the genetic relationships among populations.

5.2 Results

ISSR Polymorphism

Among the twelve screened ISSR primers, four primers, $(AG)_8T$, $(AG)_8YC$, $(CA)_8RG$ and $(TC)_8RG$ with dinucleotide repeat motifs produced clear, reproducible and polymorphic bands and hence selected for genetic diversity analysis (Table 5.3). The other ISSR primers were excluded from the analysis as they gave either smeared, failed to give amplification products, poor or non-reproducible bands.

Table 5.3: Summary of genetic diversity of *M. stenopetala* using ISSR markers

S.no.	ISSR Primer name	Primer sequence (5' to 3')	Tm of primer (°C)	Range of marker size (bp)	Polymorphic bands/total no. of bands	% polymorphism
1	807	(AG)$_8$T	48	200-1000	10/17	58.82
2	835	(AG)$_8$YC	48	200-800	8/9	88.88
3	848	(CA)$_8$RG	48	200-1000	7/8	87.50
4	854	(TC)$_8$RG	48	200-1500	6/7	85.71
Average					31/41	75.61

Single-letter abbreviations for mixed base positions: R = (A, G) Y = (C, T)

The four primers selected generated a total of 41 scoreable and reproducible bands in 150 individuals. The highest numbers of polymorphic bands (10) were generated with primer ISSR-807. None of the bands was unique to a particular individual or population. Representative ISSR finger prints obtained using primer (AG)$_8$ T are shown in figure 5.3.

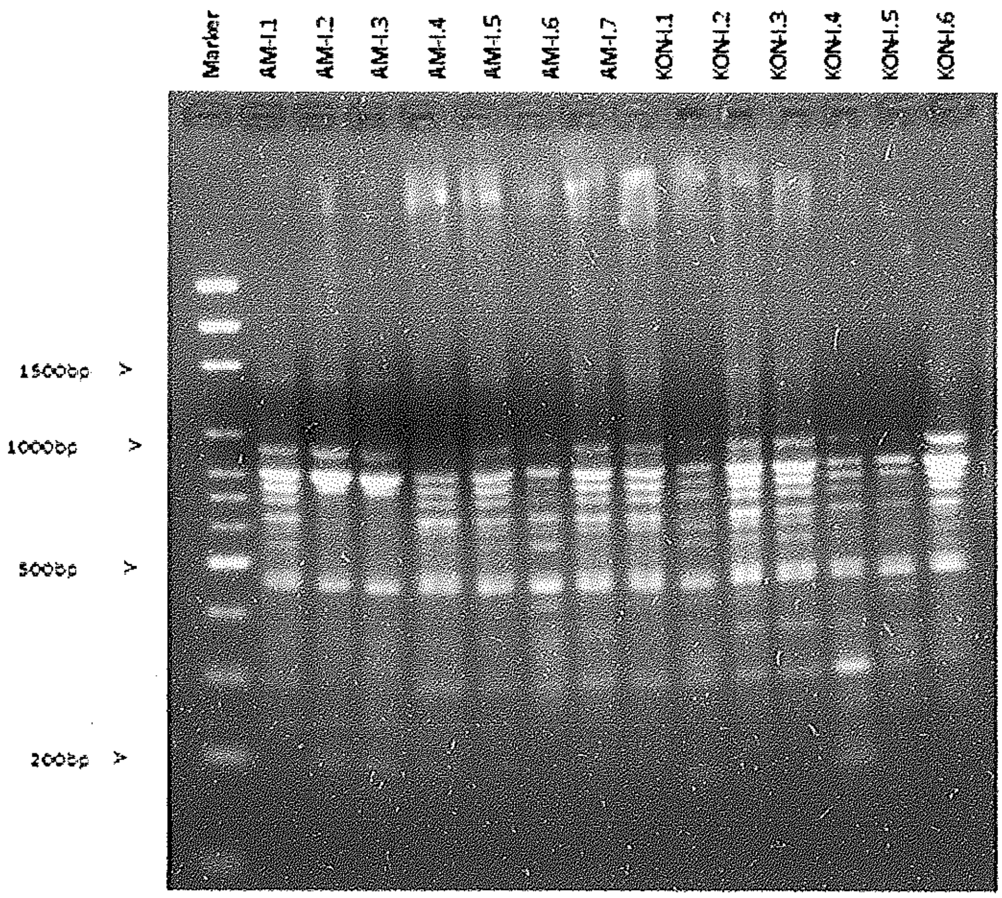

Figure 5.3: Gel picture of ISSR bands generated from individuals collected from Arba Minch Zone-Merab Abaya area (AM-I) and Konso town (KON-I) using ISSR-807 primer

Level of genetic diversity

Out of the total 41 bands, 31 fragments were polymorphic which accounts for 75.61%, with only 10 loci being monomorphic (Table 2). Based on aggregate data from all the four ISSR primers, the highest level of percent polymorphism (53.66%) was obtained from samples of Arba Minch (AM-III population), followed by KON-I, DER, AM-II, AM-IV,D.Omo-II and SR (51.22%). The least level of polymorphism was obtained from individuals in Arba Minch (AM-I population) that had only 19 polymorphic loci which accounted for 46.34% polymorphism. The gene diversity (*h*) index of the fourteen populations of *M. stenopetala* with overall primers analysis (Table 5.4), were calculated and the values ranged from 0.179 for D. Omo-I to 0.216 for KON-I populations.

Table 5.4: The Number of polymorphic loci (NPL), Percent polymorphic loci (PPL), gene diversity (h) and Shannon's information Index (I), using data generated from all the four primers

Populations	NPL	PPL (%)	h±SD	I±SD
WS-I	20	48.78	0.199± 0.217	0.290 ± 0.310
WS-II	20	48.78	0.197 ±0.215	0.288 ± 0.308
WS-III	20	48.78	0.215 ± 0.225	0.308± 0.322
AM-I	19	46.34	0.181 ± 0.208	0.266± 0.299
AM-II	21	51.22	0.185± 0.200	0.277 ±0.289
AM-III	22	53.66	0.215± 0.210	0.315 ± 0.303
AM-IV	21	51.22	0.198± 0.205	0.293± 0.297
D.Omo-I	19	46.34	0.179± 0.201	0.266± 0.294
D.Omo-II	21	51.22	0.210± 0.216	0.306 ±0.310
SR	21	51.22	0.202± 0.210	0.297± 0.303
DER	21	51.22	0.205± 0.215	0.300± 0.307
KON-I	21	51.22	0.216± 0.217	0.313± 0.313
KON-II	20	48.78	0.196± 0.212	0.287± 0.305
SD	20	48.78	0.203± 0.216	0.295± 0.311
Over all	31	75.61	0.403	0.740

Analysis of Molecular Variance (AMOVA)

To assess the overall distribution of genetic diversity within and among populations, the AMOVA analysis was carried out using the generated ISSR bands. The AMOVA results

(Table 5.5) revealed highly significant genetic differences ($P<0.001$) among the fourteen populations of *M. stenopetala* samples collected from Ethiopia. The genetic variation at population level accounted for 21.47% of the total variation, while the within populations component accounted for 78.53% and this revealed a higher within population variation than among populations.

Table 5.5: Analysis of Molecular Variance (AMOVA) among and within fourteen populations of *M. stenopetala*

Source of variation	d.f	Sum of squares	Variance components	Percentage of variation	Fixation indices	P-value
Among populations	13	257.230	1.381	21.47	0.214	$p<0.005$
Within populations	136	684.181	5.052	78.53		$p<0.005$
Total	149	937.747	6.383			

Cluster analysis

Dendrograms (Figures 5.4 - 5.6) were constructed by using UPGMA algorithm and neighbor joining (NJ) methods cluster analysis based on Jaccard's similarity coefficient matrices. The dendrogram constructed from UPGMA clearly identified three major clusters (I, II and III) using Jaccard's similarity coefficient of around 0.65.

The first cluster, with the second larger number of members, was composed of 45 individuals collected mainly from Arba Minch and Debub Omo zones. The second major cluster, with the least number of members, consisted of 30 individuals which were collected mainly from Sidama zone and Shewa Robit (Seimen shewa zone). The third major cluster, with the largest number of members, was composed of 75 individuals containing small sub-clusters mainly collected from Konso, Debub Omo, Derashe and Wolaita zone (Fig 5.5).

Moreover, NJ tree also generated similar numbers of major and sub- clusters like that of UPGMA with similar distribution over groups (Fig 5.6). However, individuals collected from Debub Omo and Shewa Robit zone formed new major cluster and associated to group I. The second major cluster was also composed of individuals collected from Debub Omo, Sidama and Shewa Robit. In addition, the third major cluster in NJ tree was composed of individuals collected from Arba Minch, Debub Omo and Konso.

It was also found that Jaccard's similarity coefficient for populations ranged from 0.601 to 0.749, with the highest similarity value (0.749) was being between Konso-I and Konso-II populations and the least similarity value (0.601) was between Sidama and Arba Minch-IV(Gofa wereda) populations (Table 5.6).

Table 5.6: Pair wise Jaccard's similarity coefficient based comparisons among fourteen populations of *M. stenopetala* collected from Ethiopia

Popn.	WS-I	WS-II	WS-III	AM-I	AM-II	AM-III	AM-IV	D.omo-I	D.omo-II	SR	DER	KON-I	KON-II	SID
WS-I	1													
WS-II	0.745	1												
WS-III	0.730	0.706	1											
AM-I	0.636	0.623	0.657	1										
AM-II	0.614	0.613	0.638	0.714	1									
AM-III	0.633	0.630	0.650	0.710	0.748	1								
AM-IV	0.615	0.618	0.619	0.726	0.709	0.713	1							
D.omo-I	0.656	0.649	0.643	0.608	0.687	0.665	0.677	1						
D.omo-II	0.661	0.673	0.654	0.659	0.666	0.685	0.683	0.729	1					
SR	0.639	0.639	0.647	0.646	0.630	0.650	0.642	0.656	0.651	1				
DER	0.692	0.698	0.688	0.650	0.621	0.643	0.626	0.654	0.660	0.606	1			
KON-I	0.677	0.685	0.689	0.684	0.649	0.633	0.661	0.652	0.656	0.621	0.684	1		
KON-II	0.674	0.664	0.673	0.651	0.658	0.640	0.632	0.653	0.644	0.614	0.656	0.749	1	
SID	0.645	0.667	0.665	0.659	0.604	0.629	0.601	0.669	0.662	0.668	0.618	0.637	0.652	1

Key: WS-I Wolaita zone (Bele wereda), WS-II Wolita zone (Humbo Wereda Humbo kebele),WS-III Wolita zone (Humbo wereda, Abela kebele), AM-I Arba Minch zone (Merab Abaya wereda), AM-II Arba Minch zone (Arba Minch town), AM-III Arba Minch zone (Beto Wereda), AM-IV Arba Minch zone (Gofa wereda, Sawla town), D. Omo-I Debub Omo zone (Jinka town), D.Omo-II Debub Omo zone (Male wereda), DER Derashe, KON-I Konso town, KON-II Around Konso town- Degatu, SID Sidama zone Hawassa town and SR Semien Shewa zone, Shewa Robit town.

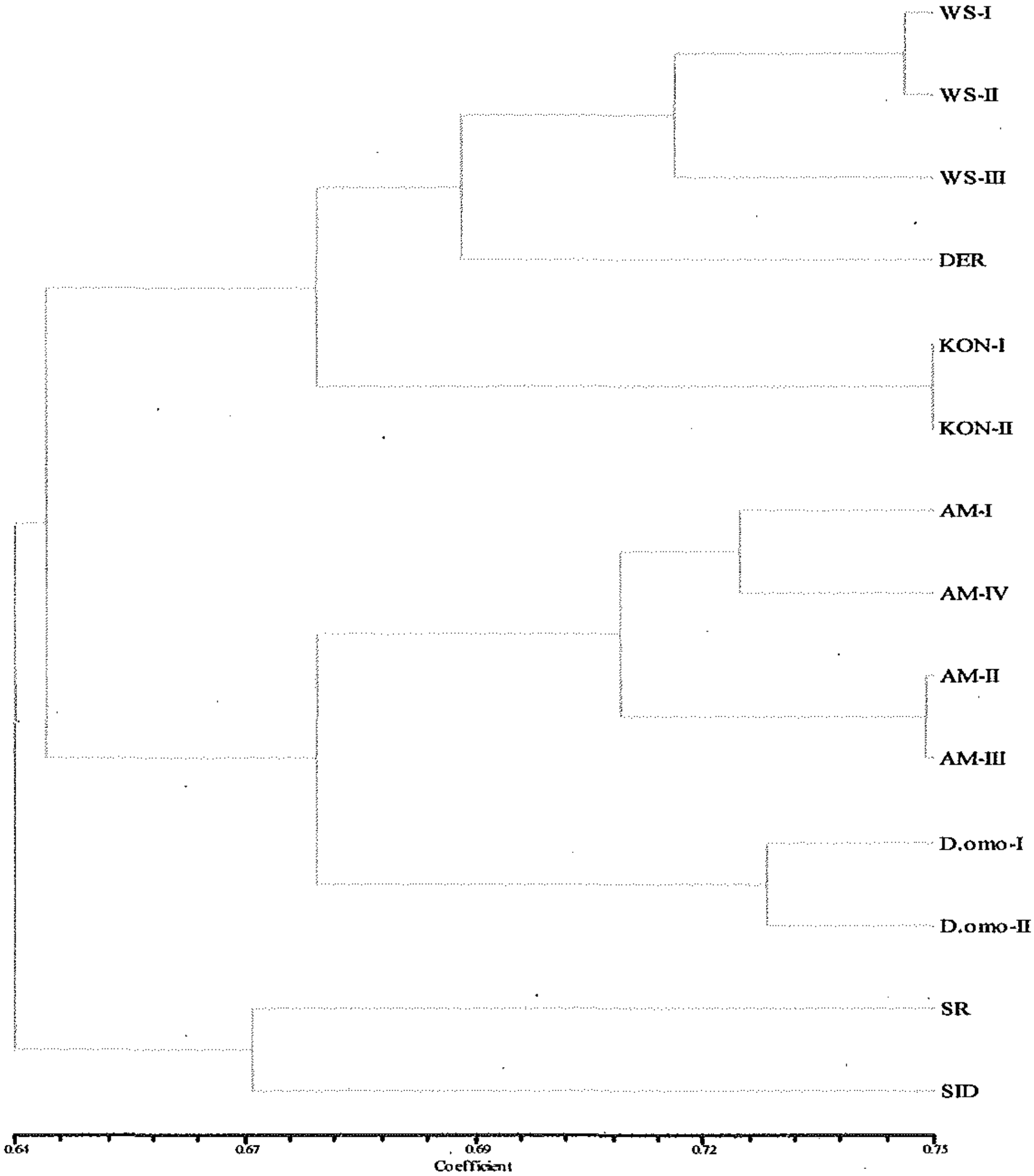

Figure 5.4: UPGMA based dendrogram for fourteen populations of *M. stenopetala* collected from Ethiopia. Key: WS-I Wolaita zone (Bele wereda),WS-II Wolaita zone (Humbo Wereda Humbo kebele),WS-III Wolaita zone (Humbo wereda Abela kebele), AM-I Arba Minch zone (Merab abaya wereda), AM-II Arba Minch zone (Arba Minch town), AM-III Arba Minch zone (Beto Wereda), AM-IV Arba Minch zone (Gofa wereda, Sawla town), D.omo-I Debub omo zone (Jinka town), D. Omo-II Debub Omo zone (Male wereda), DER Derashe, KON-I Konso town, KON-II Around Konso town- Degatu, and SID Sidama zone Hawassa town, SR Semien shewa zone Shewa Robit town.

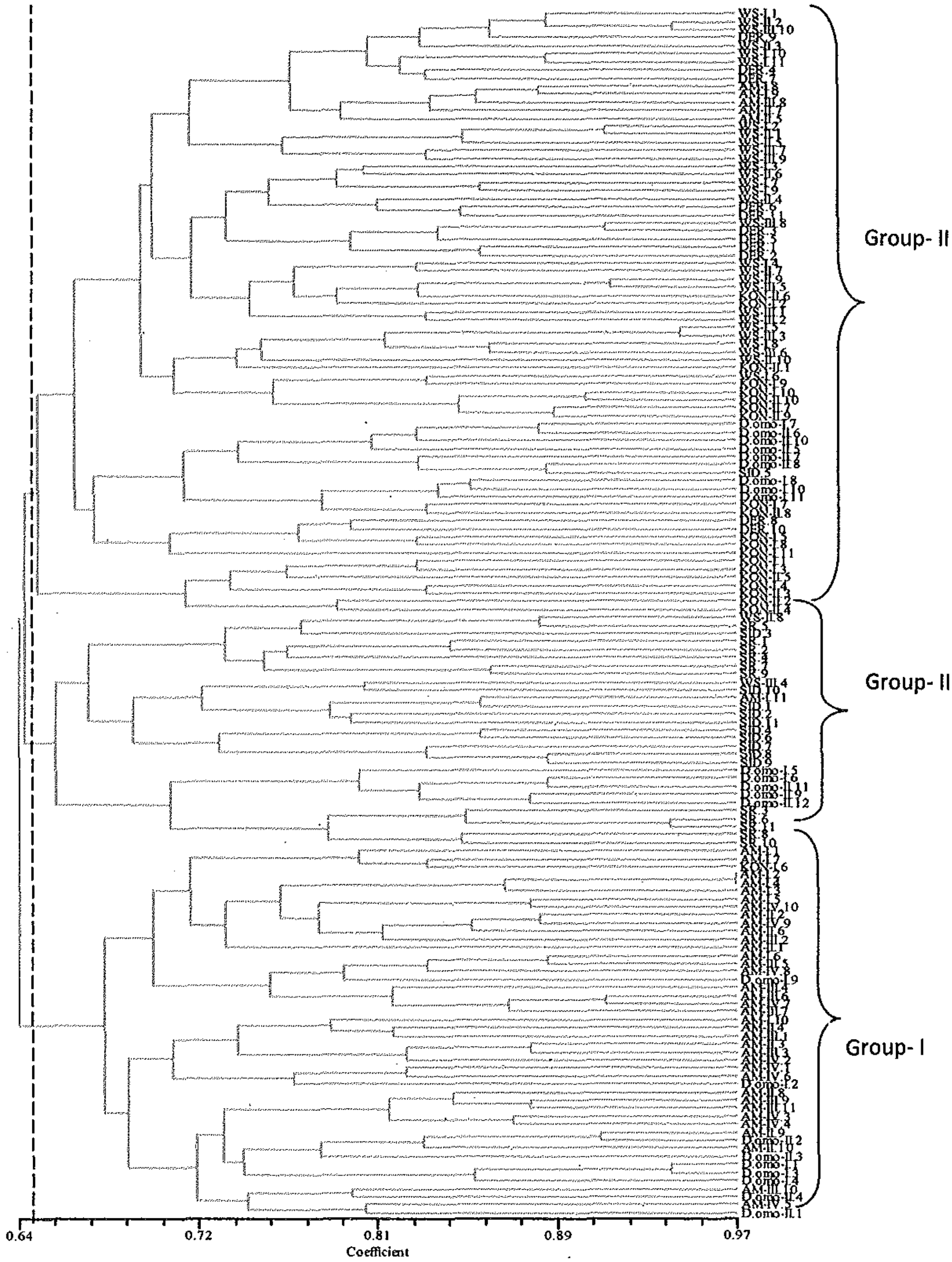

Figure 5.5: UPGMA based dendrogram for 150 individuals of fourteen populations of *M.stenopetala* using 4 ISSR primers. Key: WS-I Wolaita zone (Bele wereda), WS-II Wolaita zone (Humbo Wereda Humbo kebele),WS-III Wolaita zone (Humbo wereda, Abela kebele), AM-I Arba Minch zone (Merab Abaya

wereda), AM-II Arba Minch zone (Arba Minch town), AM-III Arba Minch zone (Beto Wereda), AM-IV Arba Minch zone (Gofa wereda, Sawla town), D. Omo-I Debub Omo zone (Jinka town), D.Omo-II Debub Omo zone (Male wereda), DER Derashe, KON-I Konso town, KON-II Around Konso town- Degatu, and SID Sidama zone Hawassa town, SR Seimen Shewa zone Shewa Robit town.

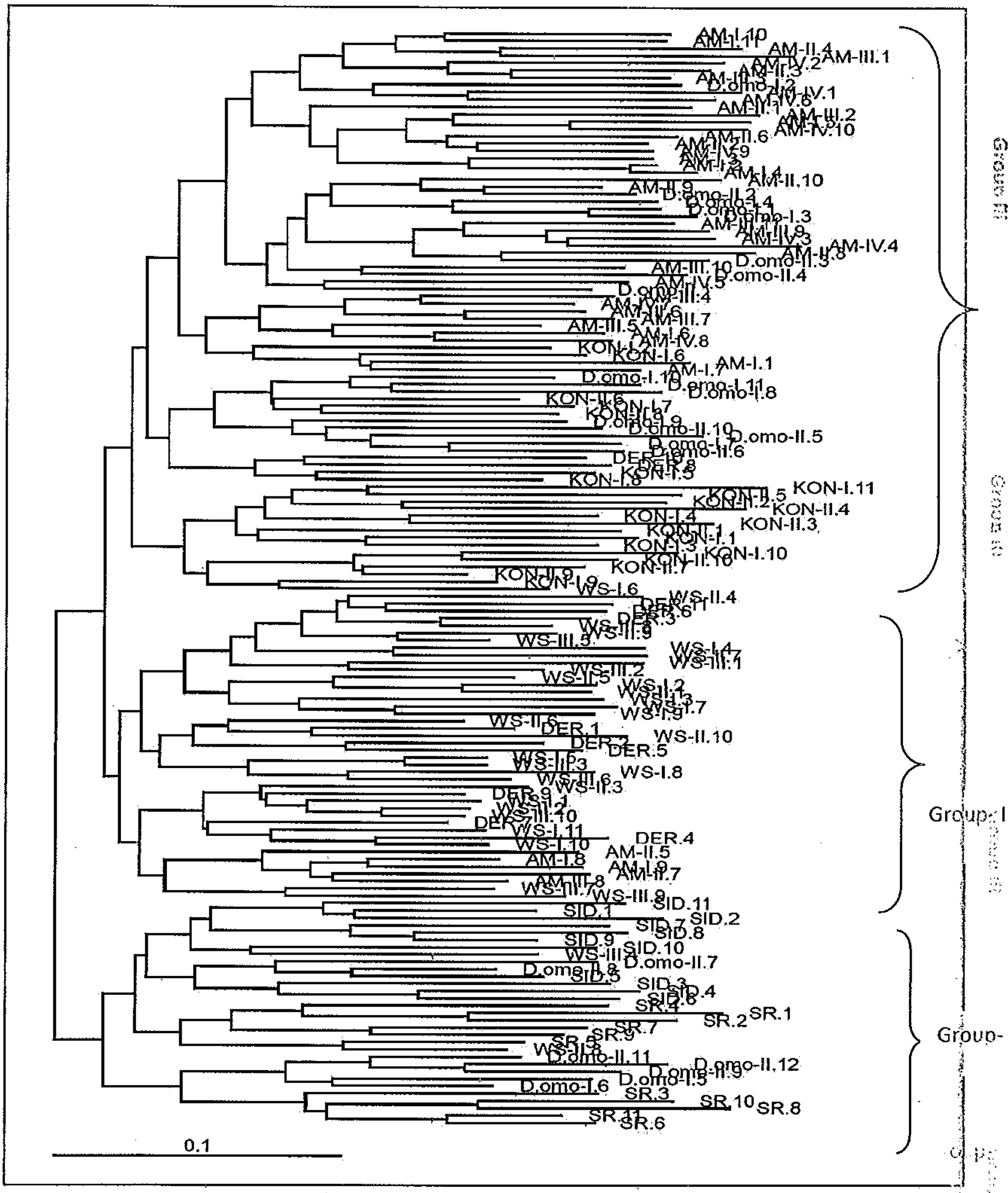

Figure 5.6: Neighbor-joining dendrogram based on Jaccard's coefficients for 150 individuals of 14 populations of *M. stenopetala* collected from Ethiopia using four primers. Key: WS-I Wolaita zone (Bele

wereda), WS-II Wolita zone (Humbo Wereda Humbo kebele),WS-III Wolaita zone (Humbo wereda, Abela kebele), AM-I Arba Minch zone (Merab Abaya wereda), AM-II Arba Minch zone (Arba Minch town), AM-III Arba Minch zone (Beto Wereda), AM-IV Arba Minch zone (Gofa wereda, Sawla town), D. Omo-I Debub Omo zone (Jinka town), D.Omo-II Debub Omo zone (Male wereda), DER Derashe, KON-I Konso town, KON-II Around Konso town- Degatu, and SID Sidama zone Hawassa town, SR Semien Shewa zone Shewa Robit town.

Principal Coordinate Analysis (PCO)

To plot the different individual genotypes of *M. stenopetala* on 2D and 3D coordinate planes, Principal coordinate analysis was carried out by employing Jaccard's coefficients of similarity based on the ISSR products (Fig 5.7 and 5.8). The first three coordinates of the PCO had Eigen values of 6.76, 5.01 and 4.57 with percentage of 8.08%, 5.98% and 5.46%, respectively. The analysis of this study showed that the different individuals examined did not strictly tend to form clusters based on their geographic origin. Likewise, the result of three coordinate analyses showed similar patterns, except few individual samples from Konso area that formed cluster on the 3D coordinate plane.

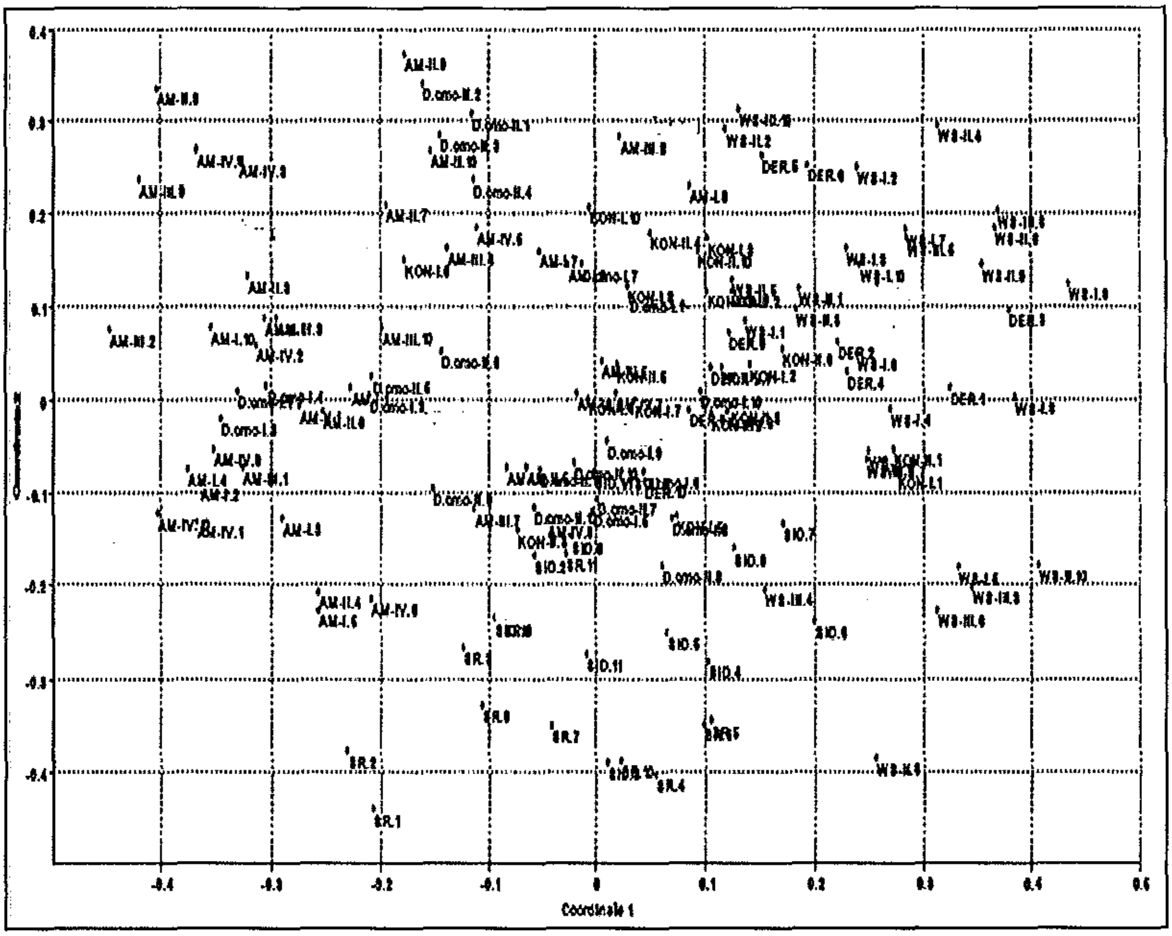

Figure 5.7: Two dimensional representations of *M. stenopetala* individuals based on Jaccard's similarity coefficients.

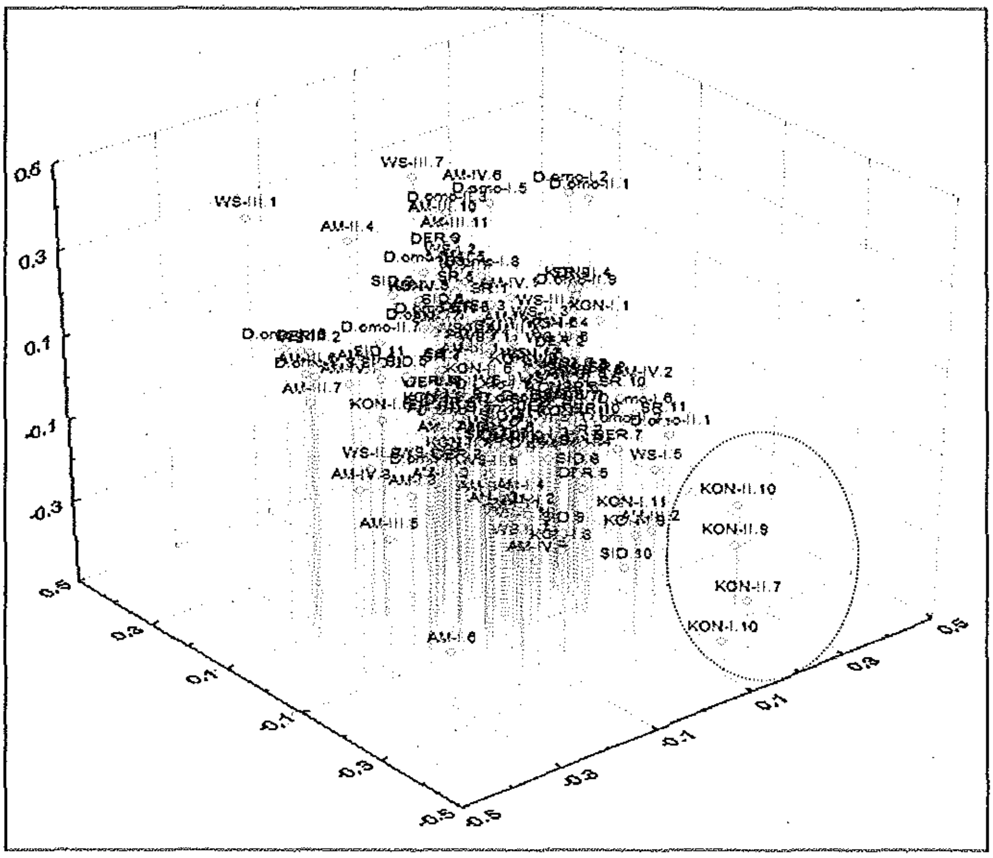

Figure 5.8: Three dimensional representation of principal coordinate analysis of genetic relationships among 150 individuals of *M. stenopetala* as revealed by ISSR marker.

5.3 Discussion

Genetic diversity

Genetic diversity studies using ISSR DNA markers in *Moringa species* are rare. However, some studies were made to examine the genetic diversity of Moringa species, especially *M.oleifera*, in many countries using RAPD markers. In the present study, 14 populations that were represented by 150 individuals were studied. A total of 4650 (31*150) polymorphic bands were scored from 4 primers. Thirty one polymorphic and reproducible bands with the average of 7.75 fragments per primer were produced. The genetic diversity of *M. stenopetala* species was found to be 75.61 percentages of polymorphic loci (PPL) and with 0.40 gene diversity index (h). However, the ISSR bands generated in this study identified the availability of a range of genetic diversity indexes at population level, with PPL values ranging from 46.34% for AM-I and D. Omo-I populations to 53.66% for AM-III population. The 'h' values also range from 0.18 for D. Omo-I to 0.22 for KON-I populations. So the present study indicated that *M. stenopetala* maintained a higher genetic diversity at the species level than at the population level.

The result obtained in the present study was virtually comparable with other previous studies made on *M. oleifera*. For example, the assessment of genetic diversity in some *M. oleifera* land races using RAPD markers were reported in some countries: the percentage polymorphic loci was found to be 89.6% in Tanzania from 96 accessions using 12 RAPD primers, 74% in North Nigeria from 75 accessions using 24 RAPD primers, 62% in Brazil from 16 accessions using 17 RAPD primers and 81.5% in West Nigeria from 10 accessions using 10 RAPD primers (Ojuederie *et al.*, 2013). In a study reported by Rufai *et al.* (2013), 15 accessions (one from USA, 10 from Thiland, 2 from India, one fromTanzania and one from Taiwan) using 12 RAPD primers, PPL values of 72.73% were found. In another study reported by Silva *et al.* (2012), 16 accessions from Brazil using 17 RAPD primers, PPL values of 62% and h value of 0.22 were found. The 17 primers also produced a total of 102 polymorphic fragments with an average of 6 fragments per primer. In addition, Muluvi *et al.* (1999) used AFLP analysis of genetic variation in *M. oleifera* from 150 individuals in 7 populations each with 20 individual samples (two populations from Southern India, one population from Southern Malawi and four populations from Kenya) and found PPL=66.5% and 'h'=0.067. Saini *et al.*(2013) also studied the genetic diversity of 8 cultivars of *M.oleifera* from India using RAPD, ISSR and cytochrome P_{450}-based markers together and found 48.68%, 48.57% and 40% PPL values respectively. In line with the above studies, the percentage polymorphic loci and Shannon index at species level in the present study are higher than the average values in related species.

Some studies have demonstrated that endangered and endemic species tend to possess low levels of genetic diversity based on ISSR data (Xiao *et al.*, 2004 and Li *et al.*, 2005). However, some other studies had shown contrary results (Ge *et al.*, 2003; Luan *et al.*, 2006; Li and Jin, 2007). Genetic diversity of a plant species could be affected by many factors such as distribution range, life form, breeding system and the way that its seeds disperse (Wang *et al.*, 2012). A species which has had a long life, a high frequency of gene flow and many seeds tends to have high genetic diversity (Hamrick and Godt, 1990).

In *M. stenopetala,* the seed pods are dehiscent and when the seed pods mature and dry the pods burst and release a large number of seeds which can further be dispersed by humans, wind and water. Schneemann (2011) estimated that 6 kg of seeds can be produced per tree under ideal conditions. In addition, *M. stenopetala* is a lifelong tree species and produce large numbers of seeds every year. These characteristics, therefore, provide more opportunity to accumulate mutant and may contribute to the high amount of diversity in this species.

Of all the studied populations, AM-III (Beto wereda) had the highest genetic diversity (*PPL* = 53.66%, *H* = 0.215) and AM-I (Arba Minch town) had the least genetic diversity (PPL=46.34, h=0.181, Table 5.4). The percentage of polymorphism, gene diversity and Shannon's information index were consistently higher for individuals from AM-III (Beto wereda) and KON-I (Konso town) as compared to those from other areas, indicating that a greater genetic diversity lies among individuals from AM-III and KON-I. In contrast, samples from AM-I (Mirab Abaya), Wolaita Sodo (Bele wereda, Humbo and Abela kebele in Humbo wereda) and SID (Hawassa) showed low genetic diversity.

Furthermore, in order to know the source of genetic variation for these *Moringa stenopetela* genotypes, ISSR profile was analyzed using AMOVA. This was aimed to partition all the sources of variation existing in the germplasm into two major groups. The result revealed that 78.53% of the total genetic variation occurred as a result of variation within the populations, while variation among the populations accounted for the remaining 21.47% of the total genetic variance (Table 5.5). Rufai *et al.* (2013) also reported high (95%) genetic variation within population and low (5%) genetic variation among populations of *M. oleifera* accessions from Malaysia and other countries using RAPD primers.

The high genetic variation within populations of *M. stenopetala* indicated in the present study can be due to the dioecius and outbreeding nature of the species and high level of heterozygosity due to the high rate of cross-pollinating nature of the plant which have been conserved by the propagation of clones through vegetative reproduction. The observed large genetic variability also reduces the chance of genetic drift and inbreeding depression and hence increases the survival chance of the population under various environmental stresses.

Genetic relationship of *Moringa stenopetala* populations

To better visualize the genetic relationship of individualss of *M. stenopetala*, cluster analysis (UPGMA, NJ, and PCO) were performed on the basis of Jaccard's similarity coefficient. A UPGMA-based dendrogram for 14 populations of *M. stenopetala* collected from Ethiopia showed clear clustering of genotypes of *M. stenopetala* on the basis of area of collection and respective populations (Fig 5.4). This UPGMA-based cluster analysis of the 14 sampled populations showed that WS-I, WS-II, WS-III, DER, KON-I and KON-II formed a tight cluster, thus grouping all six populations together. The six populations, AM-I, AM-II, AM-III, AM-IV and D. Omo-I and D. Omo- II formed a second cluster. SR and SID also formed the third cluster. The result revealed that the individuals collected from the same geographic region showed close genetic relationships and were grouped into the same cluster. Similarly, the UPGMA based dendrogram for 150 individuals of *M. stenopetala* formed 3 major clusters but with a slightly different pattern of cluster (Fig 5.5). For example, individuals from AM-I, AM-II, AM-III, AM-IV, D. Omo-I and D. Omo-II were clustered in one group. Individuals from SR, SID and D. Omo-I and D. Omo-II also formed the second cluster. Individual samples from WS-I and II, DER, AM-I, II, III, KON-I, II, D. Omo-I and II formed the third group. So some individuals from a single population could be clustered in different group indicating that some individual members of a population showed more similarity with other members of a population or groups. The UPGMA based dendrogram for 150 individuals of *M. stenopetala* was virtually similar with the Neighbor-joining dendrogram based on Jaccard's coefficients for 150 genotypes of the 14 populations of *M. stenopetala* (Fig 5.6).

Jaccard similarity coefficient-based pair wise comparisons of the 14 populations showed that KON-I and KON-II are the closest with 0.75, whereas SID and AM-IV as well as SID and AM-II are the most distant populations with a similarity coefficient of 0.60 (Table 5.6).

Besides, PCO analysis based on two and three coordinates showed none of the populations clearly formed their own clusters and tended to scatter on the 2D and 3D space except few individual samples from Konso area formed cluster on the 3D coordinate plane. The results suggest that there were clear divergence among individuals of the 14 populations that should be conserved and exploited for breeding via heterosis effect (Gallais, 1988; Schnable and Springer, 2013).

6. Conclussion and Recommendations

In depth study and analysis was carried out to improve use value and productivity of this multipurpose tree: *M. stenopetala*. This study has scientific and practical significance in propagation, conservation and improvement of *Moringa stenopetala.* It also contributes to maximize the potential use of *M. stenopetala.* Based on the result of this study, the findings obtained in this study can be used for:

- Mass propagation of disease free planting materials within relatively short period
- Solving the demand of plantlets both in quality and quantity
- Food security of both humans and cattle
- Regeneration of *M. stenopetala* plantlets from callus.
- Production of secondary metabolites and isolation of pharmaceuticals of interest from *M. stenopetala* callus.
- Conservation and genetic improvement of *M. stenopetala*
- Increasing source of income for local residents

The following recommendations are also drawn from the findings of this study :

- Additional explant sources should be examined for culture initiation and shoot multiplication.
- More subculturing should be done to examine the subculture stage at which maximum shoot multiplication occurs and the stage when the multiplication rate starts to decline.
- Using the developed protocol , *M. stenopetala* plantlets should be multiplied in quality and quantity and distributed to farmers.

- Promotion activities should be done to familiarize *M. stenopetala* in different part of the country.
- Additional DNA markers such as AFLP and SSR markers should be used to determine the molecular diversity of *M. stenopetala* in Ethiopia.

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Appendex 1

List of Acronyms

2D	Two dimentional
3D	Three dimentional
A	Adenine
AC	Acetate
AFLP	Amplified fragment length polymorphisim
AMOVA	Analysis of Molecular Variance
B5	Gamborg *et al* Medium
BA	Benzyladenine
BAP	6- Benzylaminopurine
BM	Basal medium
C	Cytosine
CTAB	Cethyl trimethyl ammonium bromide
CWS	Church World Service
DF	Degree of freedom
ECHO	Educational Concerns foe Hunger Organization
EST	Expressed sequence tags
G	Guanine
GA	Gibberellins
GA3	Gebberrelic acid
H	gene diversity
I	Shannon's information Index

IAA	Indoleacetic acid
IBA	Indole-3- butyric acid
ISSR	Inter simple sequence repeat
KN	Kinetin
MS	Murashige and Skoog (1962)
NAA	α-Naphthalene Acetic Acid
NJ	Neighbor joining methods cluster analysis
NPL	Number of polymorphic loci
PCO	Principal Coordinate Analysis
PCR	Polymerase chain reaction
PGR	Plant growth regulator
PPL	Percent polymorphic loci
PVP	Polyvinylpyrrolidone
RAPD	Random amplified polymorphic DNA
RPM	Round per minute
SCAR	Sequence-characterized amplified region
SNNPR	Southern Nations, Nationalities and Peoples Region
SPSS	Software Programme for Social Sciences
SSR	Simple sequence repeat
T	Thiamine
TBE	Tris/Borate/EDTA buffer
TDZ	Tidiazuron
TE	Tris-Hcl and EDTA buffer
UPGMA	Unweighted Pair Group Method with Arithmetic Mean
WPM	Woody plant media

Appendex 2

Nanodrop Results

Sample ID	Nucleic Acid Conc.	Unit	A260	A280	260/280	260/230	Sample Type
AM-I	578.5	ng/µl	11.569	6.229	1.86	1.16	DNA
AM-I	235.5	ng/µl	4.711	2.796	1.68	0.63	DNA
AM-I	427.6	ng/µl	8.552	4.676	1.83	0.9	DNA
AM-I	929.6	ng/µl	18.592	9.706	1.92	1.91	DNA
AM-I	81.8	ng/µl	1.636	0.951	1.72	0.43	DNA
AM-I	172.9	ng/µl	3.458	2.315	1.49	0.84	DNA
AM-I	312.6	ng/µl	6.252	3.662	1.71	0.81	DNA
AM-I	60.6	ng/µl	1.212	0.84	1.44	0.69	DNA
AM-I	645	ng/µl	12.9	6.821	1.89	1.49	DNA
AM-I	459.7	ng/µl	9.194	5.246	1.75	1.04	DNA
AM-I	1440.2	ng/µl	28.804	13.685	2.1	2.06	DNA
AM-II	105.3	ng/µl	2.105	1.974	1.07	0.42	DNA
AM-II	257.1	ng/µl	5.141	2.684	1.92	1.7	DNA
AM-II	900.5	ng/µl	18.01	8.595	2.1	2.03	DNA
AM-II	1027.2	ng/µl	20.545	9.735	2.11	2.17	DNA
AM-II	387.5	ng/µl	7.751	4.033	1.92	1.68	DNA
AM-II	1094.5	ng/µl	21.891	10.641	2.06	1.6	DNA
AM-II	569.2	ng/µl	11.383	5.573	2.04	2.03	DNA
AM-II	1242.3	ng/µl	24.846	12.021	2.07	2.14	DNA
AM-II	505.4	ng/µl	10.108	5.06	2	1.76	DNA
AM-II	177.8	ng/µl	3.556	1.952	1.82	1.41	DNA
AM-III	1585.5	ng/µl	31.711	15.191	2.09	2.12	DNA
AM-III	635.7	ng/µl	12.714	6.264	2.03	2.05	DNA
AM-III	1354.8	ng/µl	27.096	12.873	2.1	2.17	DNA
AM-III	1097.4	ng/µl	21.948	10.677	2.06	2.12	DNA
AM-III	525.2	ng/µl	10.504	5.345	1.97	1.98	DNA
AM-III	434.1	ng/µl	8.681	4.379	1.98	1.72	DNA

AM-III	2019.7	ng/µl	40.394	19.688	2.05	1.96	DNA
AM-III	93.8	ng/µl	1.877	0.985	1.91	1.61	DNA
AM-III	1153.2	ng/µl	23.064	11.008	2.1	2.03	DNA
AM-III	1752.5	ng/µl	35.05	17.816	1.97	1.63	DNA
AM-III	623	ng/µl	12.46	8.964	1.39	1.21	DNA
AM-IV	62.7	ng/µl	1.254	0.99	1.27	0.42	DNA
AM-IV	3023.7	ng/µl	60.473	28.956	2.09	2.01	DNA
AM-IV	2104.9	ng/µl	42.098	20.595	2.04	1.41	DNA
AM-IV	2943.9	ng/µl	58.879	19.363	3.04	1.66	DNA
AM-IV	3652	ng/µl	73.041	35.244	2.07	2.07	DNA
AM-IV	752.3	ng/µl	15.046	7.389	2.04	1.71	DNA
AM-IV	2617.1	ng/µl	52.343	24.858	2.11	1.69	DNA
AM-IV	4240.8	ng/µl	84.816	41.517	2.04	1.96	DNA
AM-IV	2721.3	ng/µl	54.427	26.142	2.08	1.94	DNA
AM-IV	2665.8	ng/µl	53.315	32.037	1.66	1.6	DNA
DER	9740.5	ng/µl	194.809	93.821	2.08	1.95	DNA
DER	3215	ng/µl	64.3	30.748	2.09	1.89	DNA
DER	4596.1	ng/µl	91.923	53.985	1.7	1.16	DNA
DER	1520.4	ng/µl	30.409	15.465	1.97	1.24	DNA
DER	912.7	ng/µl	18.255	9.276	1.97	1.92	DNA
DER	14744.6	ng/µl	294.893	148.325	1.99	1.99	DNA
DER	623	ng/µl	12.46	6.987	1.78	1.56	DNA
DER	1999.4	ng/µl	39.988	20.277	1.97	1.86	DNA
DER	4053.1	ng/µl	81.061	40.195	2.02	1.97	DNA
DER	1687.5	ng/µl	33.749	17.62	1.92	1.8	DNA
DER	1810.4	ng/µl	36.208	19.249	1.88	1.92	DNA
D.Omo-I	2022.5	ng/µl	40.451	20.519	1.97	1.74	DNA
D.Omo-I	1418.6	ng/µl	28.372	14.897	1.9	1.85	DNA
D.Omo-I	1936.4	ng/µl	38.727	20.864	1.86	1.57	DNA
D.Omo-I	1022.9	ng/µl	20.457	14.752	1.39	1.01	DNA
D.Omo-I	513.5	ng/µl	10.27	5.698	1.8	1.61	DNA
D.Omo-I	4381.1	ng/µl	87.621	44.462	1.97	1.96	DNA
D.Omo-I	1793	ng/µl	35.86	17.684	2.03	2.03	DNA

D.Omo-I	1821.2	ng/μl	36.425	17.948	2.03	2.03	DNA
D.Omo-I	1065.8	ng/μl	21.315	10.68	2	2.01	DNA
D.Omo-I	1869.9	ng/μl	37.397	18.518	2.02	1.9	DNA
D.Omo-I	3636.3	ng/μl	72.727	35.292	2.06	2.14	DNA
D.Omo-II	608.9	ng/μl	12.179	6.585	1.85	1.63	DNA
D.Omo-II	4848.3	ng/μl	96.965	49.161	1.97	1.85	DNA
D.Omo-II	561.2	ng/μl	11.224	6.457	1.74	1.16	DNA
D.Omo-II	1033.7	ng/μl	20.673	11.871	1.74	1.16	DNA
D.Omo-II	765.3	ng/μl	15.306	8.809	1.74	1.39	DNA
D.Omo-II	1665.7	ng/μl	33.314	16.584	2.01	2.07	DNA
D.Omo-II	581.6	ng/μl	11.633	5.947	1.96	1.93	DNA
D.Omo-II	4504.4	ng/μl	90.088	44.619	2.02	2.05	DNA
D.Omo-II	2033.9	ng/μl	40.677	20.418	1.99	1.93	DNA
D.Omo-II	2798.7	ng/μl	55.973	27.642	2.02	1.96	DNA
D.Omo-II	2961.5	ng/μl	59.23	29.371	2.02	1.92	DNA
D.Omo-II	2356.5	ng/μl	47.13	23.166	2.03	2.08	DNA
KON-I	4683.7	ng/μl	93.674	45.825	2.04	2.13	DNA
KON-I	1263.4	ng/μl	25.269	14.512	1.74	1.36	DNA
KON-I	548.6	ng/μl	10.972	6.034	1.82	1.4	DNA
KON-I	284.9	ng/μl	5.697	4.077	1.4	0.74	DNA
KON-I	895.5	ng/μl	17.91	9.535	1.88	1.66	DNA
KON-I	878.4	ng/μl	17.567	9.052	1.94	1.66	DNA
KON-I	687	ng/μl	13.741	7.325	1.88	1.92	DNA
KON-I	424.5	ng/μl	8.49	4.92	1.73	1.4	DNA
KON-I	1114.5	ng/μl	22.29	11.528	1.93	2	DNA
KON-I	289.8	ng/μl	5.797	3.33	1.74	0.91	DNA
KON-I	144.7	ng/μl	2.893	3.725	0.78	0.17	DNA
KON-II	534.1	ng/μl	10.683	6.026	1.77	1.57	DNA
KON-II	2682.8	ng/μl	53.656	28.157	1.91	2.15	DNA
KON-II	2109	ng/μl	42.179	22.144	1.9	2.14	DNA
KON-II	754.6	ng/μl	15.092	8.158	1.85	1.76	DNA
KON-II	1133.2	ng/μl	22.664	12.073	1.88	2	DNA
KON-II	1887.1	ng/μl	37.741	20.312	1.86	2.05	DNA

KON-II	1601.7	ng/µl	32.035	17.273	1.85	1.98	DNA
KON-II	3188.7	ng/µl	63.774	33.704	1.89	2.08	DNA
KON-II	3357.7	ng/µl	67.153	35.063	1.92	2.16	DNA
KON-II	1443.9	ng/µl	28.877	15.302	1.89	2.05	DNA
SID	1544	ng/µl	30.88	16.698	1.85	1.89	DNA
SID	626.5	ng/µl	12.53	6.118	2.05	1.17	DNA
SID	570.3	ng/µl	11.406	5.789	1.97	1.1	DNA
SID	3045.8	ng/µl	60.915	30.115	2.02	1.78	DNA
SID	502.1	ng/µl	10.043	5.605	1.79	1.21	DNA
SID	1041.9	ng/µl	20.837	10.595	1.97	1.42	DNA
SID	1224.8	ng/µl	24.497	11.893	2.06	1.42	DNA
SID	464.3	ng/µl	9.286	5.546	1.67	1.02	DNA
SID	379	ng/µl	7.581	3.801	1.99	1	DNA
SID	1317.7	ng/µl	26.354	12.437	2.12	2.03	DNA
SID	355.2	ng/µl	7.104	3.694	1.92	1.01	DNA
SR	2299.6	ng/µl	45.992	23.042	2	1.96	DNA
SR	1406.9	ng/µl	28.138	13.74	2.05	1.98	DNA
SR	1343.6	ng/µl	26.873	13.179	2.04	2.01	DNA
SR	1350.1	ng/µl	27.003	13.319	2.03	1.93	DNA
SR	1471.9	ng/µl	29.439	14.466	2.04	2.03	DNA
SR	3509.4	ng/µl	70.188	34.252	2.05	2.01	DNA
SR	4138.4	ng/µl	82.768	41.267	2.01	1.9	DNA
SR	2341.4	ng/µl	46.828	23.263	2.01	1.96	DNA
SR	4475.1	ng/µl	89.502	45.567	1.96	1.77	DNA
SR	596.4	ng/µl	11.929	6.41	1.86	1.56	DNA
SR	2015.5	ng/µl	40.31	19.992	2.02	1.98	DNA
WS-I	1795.5	ng/µl	35.911	18.417	1.95	1.74	DNA
WS-I	1435.6	ng/µl	28.712	13.963	2.06	1.95	DNA
WS-I	4882.8	ng/µl	97.656	49.948	1.96	1.94	DNA
WS-I	4295.4	ng/µl	85.909	43.946	1.95	1.76	DNA
WS-I	14059.3	ng/µl	281.185	139.604	2.01	1.99	DNA
WS-I	2175.7	ng/µl	43.513	21.265	2.05	2.01	DNA
WS-I	3386.5	ng/µl	67.73	34.445	1.97	1.73	DNA

WS-I	1932.9	ng/µl	38.658	19.46	1.99	1.93	DNA
WS-I	1257	ng/µl	25.141	12.605	1.99	1.81	DNA
WS-I	942.6	ng/µl	18.852	10.083	1.87	1.42	DNA
WS-I	653.1	ng/µl	13.061	6.917	1.89	1.79	DNA
WS-II	1449.2	ng/µl	28.984	14.708	1.97	1.66	DNA
WS-II	1568.7	ng/µl	31.375	15.408	2.04	1.9	DNA
WS-II	1600.7	ng/µl	32.014	16.044	2	1.98	DNA
WS-II	747.9	ng/µl	14.959	7.764	1.93	1.62	DNA
WS-II	1692.6	ng/µl	33.852	17.117	1.98	1.95	DNA
WS-II	503.4	ng/µl	10.069	6.354	1.58	1.14	DNA
WS-II	424.9	ng/µl	8.499	5.086	1.67	1.02	DNA
WS-II	532.6	ng/µl	10.652	5.964	1.79	1.23	DNA
WS-II	3817.4	ng/µl	76.347	38.46	1.99	1.84	DNA
WS-II	665.8	ng/µl	13.315	7.39	1.8	1.83	DNA
WS-III	1292	ng/µl	25.84	13.283	1.95	1.93	DNA
WS-III	1393.5	ng/µl	27.871	13.906	2	1.85	DNA
WS-III	324.2	ng/µl	6.483	4.59	1.41	0.69	DNA
WS-III	3518.8	ng/µl	70.375	35.062	2.01	2.02	DNA
WS-III	1100.5	ng/µl	22.01	11.528	1.91	1.84	DNA
WS-III	1908.9	ng/µl	38.179	18.816	2.03	2.06	DNA
WS-III	1495.1	ng/µl	29.903	14.81	2.02	1.99	DNA
WS-III	2116.3	ng/µl	42.326	20.886	2.03	2.05	DNA
WS-III	1689.1	ng/µl	33.781	16.265	2.08	2.16	DNA
WS-III	424.9	ng/µl	8.499	5.086	1.67	1.02	DNA