DEVELOPMENT OF LOWCOSTMEDIA ALTERNATIVES FORMICROPROPAGATIONS OF MORINGA (*Moringa stenopetala*)

MSc. Thesis

By

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Jimma, Ethiopia

DEVELOPMENT OF LOW COST MEDIA ALTERNATIVES FOR MICROPROPAGATIONS OF MORINGA (*Moringa stenopetala*)

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Decision/Suggestion of Department Graduate Council (DGC)

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, love and
my life.

STATEMENT OF AUTHOR

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BIOGRAPHICAL SKETCH

Endris Seid was born on 1 September 1990 G.C., from his father, Seid Muhe, and his mother, W/o Fatuma Idris, at Worer 01 Kebela, Amibara Woreda in Afar Regional State. He attended elementary and Secondary School at Worer from 1998-2007 G.C. After successful completion of his secondary school, he joined Awash 7 killo Lemi Lefen Preparatory School in 2008 G.C.

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LIST OF ABBREVIATIONS

JUCAVM	Jimma University, College of Agriculture and Veterinary Medicine
BAP	6- benzyl Amino Purine
IBA	Indol - 3- Butyric acid
MS	Murashige and Skoog (1962)
PGRs	Plant Growth Regulators

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DEVELOPMENT OF LOW COST MEDIA ALTERNATIVES FOR MICROPROPAGATIONS OF MORINGA (*Moringa stenopetala*)

ABSTRACT

*Moringa (*Moringa stenopetala*) belongs to the family Moringaceae and grown for food, pharmaceutical use and various purposes. In tissue culture, the major media costcontribution isfrom agar and sucrose. Enset('bulla') powder and table sugar are cheap and easily available as gelling agents and carbon source, respectively. This study was mainly undertaken to evaluate low cost alternative media gelling agent and carbon source for micropropagation of moringa. Culture was initiated from shoot tips on MS-basal medium and multiplied using different concentrations of 'bulla'(40g, 60g, 80g, and 100g), a mixture of agar and 'bulla' (1g+70g, 2g+60, 3g+50g and 4g+40g)as gelling agent and same concentration of table sugar and sucrose(15g, 30g, 45g, and 60g) as carbon source. The result showed significantly different in shoot initiation. The highest percent of shoot initiation (100%) recorded in 60g, 80g and 100g 'bulla' media and non-significantly different with 40g 'bulla' (86.68%) but significantly different with liquid medium (33.33%).However, there was a significant difference among 'bulla' concentrations in shoot multiplication and rooting. The highest mean number of shoots per explants (6.946), shoot length (4.88) and leaf number (17.1) were obtained on medium containing 80g'bulla' as alternative gelling agent. MS media supplemented with 30 g/l sucrose showed the highest number of shoots (6.41),shoot length (6.41)andleaf number (15)which was not significantly different from medium containing 30 g/l table sugar (6.35 shoots, 4.5cmshoot length and 15 leaves per shoot). The highest root number (9.09) was observed on MS medium with 8 g/l agar but did not show significant difference with 2g agar + 60g 'bulla' (8.76),60g 'bulla' (8.51)and 80g 'bulla' (8.41).However,the highest root length (6.41) was recorded in media supplemented with 60g 'bulla'.Based on the result on all parameters, 80 g/l 'bulla' and 30g/l table sugar were found to be better. Moreover, 70.97% of the media cost of gelling agent and 98% carbon source cost was minimized by substituting 80 g/l 'bulla 'asa gelling agent and 30g table sugar ascarbon source. During acclimatization, 83.34% plantlets were survived.*

Keywords/Phrases: *'Bulla', Moringa,Table sugar*

1. INTRODUCTION

Moringa is a softwood perennial tree that belongs to the monogeneticfamily Moringaceae(OrdermCapparales)(Olson, 2002). It is a diploid plant andits chromosome number is 2n= 28 (Mendioro*et al*., 2005). India is the major producer of moringa with an annual production of 1.1 to 1.3 million tons of tender fruits from an area of 380 $km^2$and the production information of moringa in other countries wasscarce (Leone*et al*., 2015). Among 13 species of the genus moringa, *M. stenopetala* (Baker. c. cufodontis) is native to southern Ethiopia, northern Kenya and Eastern Somalia. It is the second most important domesticated moringa species after *M. oleifera* (Leone*et al*., 2015; Nor-bert*et al*., 2002). Moringa is known by different vernacular names in Ethiopia like shiferaw andhaleko (Abuye*et al., 2003*).

All plant parts of moringa traditionally used for different purposes, but the leaves are generally the most used part of the tree (Popoola and Obembe, 2013; Sivasankari*et al*., 2014). Leaves can be used or eaten in afresh as salad, cooked or after drying in the form of powder (as tea), for many months without refrigeration and loss of nutritional value. It is a good source of food in the tropics during shortages or scarcity of other food (Fahey, 2005). Moringa seeds have an oil content of up to 30%–40%, with a high quality fatty acid and the leaf is a potential plant protein supplement up to 6% of the diet of the grower to substitute expensive conventional protein sources (Rashid *et al*., 2008; Aberra*et al*., 2011). Fresh leaves of moringa approximately contain 6.6–6.8 mg/100 g of β-carotene (Ferreira *et al*., 2008; Kidmose*et al*., 2006) and 200 mg/100 g of vitamin C (Ramachandran *et al*., 1980).Moringa is a great fodder for cattle. The weight of livestock increased up to 32% through moringa feed and their milk yield of cows increased by 43%. The seeds yield 38–40% edible oil and its powder clarifies dirty water (Patel *et al*., 2010). Moringa is a drought tolerant plant that can be grown in diverse soils (Abdul, 2007).

The leaves of *M. stenopetala* are traditionally used for the treatment of diabetes mellitus in Ethiopia (Mussa*et al*., 2008) and it is also important for antimicrobial activity (Biffa, 2005). In the modern era, despite tremendous advancements in synthetic chemistry,

several human health problems are dominantly solved by herbal drugs and possess no or rare side effects. With the increase in world population, the uses of trees for different purposes were increased (e.g. for nutrition and anthapogenic activities) and it leads rapid demolishing of natural ecosystems. Due to this, precious and useful medicinal plants were dwindling and endangering (Kamboj, 2000).

Among 13 species of genus moringa,many are in danger of extinction including *M. stenopetala* (Stephenson and Fahey, 2004). There is a great concern in the conservation of the moringa species from biodiversity, ethno-botanical, dietary and pharmacological perspectives (Sanchezet*et al*., 2006).

Propagation is usually done by growing cuttings of 3-4 feet shoot or through seed (Noumane*t al*., 2012). Sexual propagation of some of these species is tedious and not even be possible without having enough individual plants for cross-pollination. Thus, seedgermination is low while conventional propagation system through cuttings of the mother plant limbs which ultimately cause the death of the mother plant. Micropropagation is one way to exploit the properties of this valuable tree and satisfy the demand for its planting material (Marla, 2015).

Tissue culture techniques are based on the totipotency concept of cells and play important role in plant micropropagation, and conservation of plant materials (Jaskani *et al*., 2008). In addition to these, tissue culture techniques are important tools in the fundamental and appliedresearch. These are helpful in the conservation of genetic resources, understanding of gene structure, function in molecular biology and plant improvement through transgenic technology. Since flowering of a number of the large tree species of moringa do not even commence until a critical size is attained, highly unlikely to occur with multiple trees in a greenhouse, tissue culture may be the only practical way to cultivate these trees out-side the tropics. Amplification of these rare individuals by tissue culture propagation would make them more widely available and less likely to become lost to cultivation.Thus, developing tissue culture methods for this genus is urgently required (Stephenson and Fahey, 2004).

Different alternatives of plant tissue culture media routinely incorporate adding inorganic and organic nutrients, growth regulators, a carbon source, and frequently a gelling agent, agar, to support and orient the tissue (Hazel *et al.*, 1994). The properties of agar that make it the gelling agent of choice are stability, high clarity and resistance to metabolism during cultivation (Jain and Babbar, 2002). It is the most frequently used solidifier in plant tissue culture media (Afrasiab and Jafar, 2011) and the most expensive component used in plant tissue culture media (Daud*et al.*, 2011). Gelling agents such as agar contributes 60- 70% of the total cost of the media (Prakash *et al*., 2004; Kuria*et al*., 2008 and Puchooa, 1999). Because of the high price of tissue culture grade agar, attempts have been made to identify suitable alternatives like cassava starch (Moses *et al.,* 2004), gum katira (Jain and Babbar, 2002) guar gum (Jain *et al.,* 2005). However, despite a distinct cost advantage over agar, none is likely to be used as routinely as agar due to some inherent drawbacks such as unstable slant formation and remaining as a viscous liquid (Jain and Babbar, 2011). Therefore, the search for other alternative gelling agents is mandatory whereby Enset flour, 'bulla', could be an alternative (Ayenew*et al*., 2012)

Enset (*Enseteventricosum*) is one of the widely cultivated Ethiopian indigenous crops producing different starchy food products such as kocho, 'bulla' and amicho (Tsegaye and Struik, 2002). 'Bulla' is a water-insoluble starchy product, which is processed by squeezing and decanting the liquid followed by drying. 'Bulla' is considered as the best quality enset food obtained from fully matured enset plant (Pijls*et al*., 2006). Unfortunately, there is no work done for the potential of this crop as an alternative gelling agent for in *vitro* propagation*of M. stenopetala.* Due to the quality and starchy nature of the product, 'bulla' powder seems to be a possible alternative gelling agent for *M. stenopetala.*

Sugars are required for *in vitro* due to the observed phenomenon of heterotrophy/mixotrophy of cultured cells, to replace the carbon, which plants normally fix from the atmosphere by photosynthesis *in vivo* for growth and development. Carbon is an essential element for *in vitro* micropropagation of plants and it is an essential factor for the completion of the life cycle of the plant and its absence may lead to the death of

the plant (Kumar, 2009). In general, most of the tissue culture studies are performed using sucrose as the sole carbon source due to its efficient uptake across the plasma membrane, but it is high cost tissue culture media component (Swamy*et al.*, 2010).Recently, the use of high cost energy sources (sucrose) has been replaced by a cheaper and locally accessible carbohydrate sources such as table sugar, juices and plant extracts and the result showed promising responses (He et al., 2003). Table sugar has also found to be a suitable alternative low cost medium component for *in vitro* micro-propagation due to its high energy and easily accessible in the market (Demo *et al.*, 2008). The use of table sugar for micropropagation of different plants like cassava and potato, which reduced 97.1% of media cost compared with sucrose (Ombori*et al*., 2012; Ogero*et al*., 2012).

According to Kodym and Zapata (2001), it is possible to reduce the cost of medium up to 90% by replacing the standard tissue culture grade carbon source (sucrose) and the gelling agent (agar) in the medium with locally available commercial sugar and gelling agent. The study for *Vanilla planifolia* by substitution of agar with 'bulla' (8%) saved about 72.5% of the media gelling cost (Ayelign *et al*., 2012).This work was done to solve this problem by using 'bulla'and table sugar as low cost alternative gelling agent and carbon source respectively for *in vitro* multiplication of *M. stenopetala*.

1.1 Objectives

1.1.1. General objective

- Todevelop alow cost media alternativesfor *M. stenopetala*

1.1.2. Specific objectives

- To determine the optimum concentration of 'bulla'on initiation, multiplication and rooting as an alternative source of gelling agent.
- To determine optimum concentration of table sugar as an alternative source of carbon.

2. LITERATURE REVIEW

2.1. Botanical Classification:

Taxonomic classification of *Moringa stenopetala* is**Kingdom** -*Plantae,* **Subkingdom** – *Tracheobionta,* **Super Division**- Spermatophyta, **Division -** *Magnoliophyta,* **Class** – *Magnoliopsida,* **Subclass** – *Dilleniidae,* **SuperOrder –** *Violanae,* **Order** – *Brassicales,* **Family** – *Moringaceae,* **Genus** – *Moringa,* **Species** – *stenopetala*(Leone*et al*., 2015; Garima, 2011). Moringa belongs toMoringaceae family and it consists of a single genus (*Moringa*) (Verdcourt, 2000). Moringa genus consists ofapproximately 13 different species (Paliwal, 2011). Commonly known species are *M. oleifera* L., *M. arborea, M. borziana, M. longituba, M. rivae, M. ruspoliana,* and *M. stenopetala* (Stephenson and Fahey, 2004).

2.2. Morphology and Physical Characteristics

Moringa is a fast growing soft deciduous tree about 10 to 12 m long and 30cm in diameter. Leaves are alternate bi or trip innate compounds, feathery, 3-5cm long and dark green in color. White to cream color flowers are lightly fragrant and straight stems are poorly formed. The barks are white thick corky and sticky (Mishra *et al*., 2011). Fruits are rounded green in color, 15-45cm long with 5 - 20 rounded or trilobite capsule shaped seeds (Hsu *et al.*, 2006). The seeds are round with a brownish semi-permeable seed hull. The hull itself has three white wings that run from top to bottom at 120-degree intervals. Each tree cans produce between 15, 000 and 25,000 seeds /year. The average weight of the seed is 0.3g (Morton JF, 1991).

2.3. Propagation of Moringa

Moringa adapted to a type of self-pollination (geitonogamy) and crossing pollination (xenogamy) with larger fruit set, seed set and fecundity in the latter mode (Jyoth*et al*., 1990). The flowers produce both pollen and nectar with bees as the main pollinators (Muluvi, 2004;Jyoth*et al*., 1990). Traditional propagation of moringa tree is by direct seedling. This method takes between 3-14 days and results in one plant per seedling. Moringa trees obtained from seeding vary in genotypes and hence in their phenotypes

leading to variation in fruit (pod) production and nutritional values. Selected characteristics of plants may be maintained via *in vitro*propagation (Steintz*et al*., 2009).

2.4. Low Cost Gelling Agent

Enset is one of the widely cultivated Ethiopian indigenous crops producing different starchy food products such as *kocho*, 'bulla' and *amicho* (Tsegaye and Struik, 2002).'bulla' is the small amount of water-insoluble starchy product of enset that may be separated from mixture of the decorticated (scarped) leaf sheaths and grated corm (underground stem base), kocho, during processing by squeezing and decanting the liquid (Atlabachew and Chandravanshi, 2007) to be used as gelling agent. So far, Hirose *et al*.(2010) studied enset starch for food and industrial uses and found a characteristic of high gelatinization property.

Enset flour, 'bulla', has been tried in the study for Pineapple (*Ananascomosus* VAR. Smooth cayenne) *in vitro* propagation the first time to substitute agar and showed no significant difference for shoot number, root number, shoot height, leaf number and an associated fresh weight of the plantlets besides better gelling ability than agar. Subsequently, 80 g/l 'bulla' was gelled well and gave 11.8 shoots with 0.95g fresh weight and 13.33 roots having 1.37 cm length. This rate can also save up to 76 % of gelling cost and significant difference was found for root length of 3.23 cm with agar, 8 g/l. This indicates further study on biochemical and/ or hormonal activity and across crop genotypes to use 'bulla' as a cheap alternative commercial gelling agent (Biruk*et al*., 2012).

The study on*Vanilla planifolia* by substitution of agar with 'bulla' (8%) could save about 72.5% of agar cost per a litter media. Generally, replacing agar with 6-10% 'bulla' could decline the cost of agar by about 50-72.5% per a liter media. However, 'bulla' resulted in poor media clarity that cause problem in contamination detection and enhanced root proliferation during multiplication phase. Hence, 'bulla' seems to be more preferable in rooting phase that needs short growth period and improved the plantlets growth (Ayelign *et al*., 2012). The use of an alternative gelling agent has saved costs of pineapple tissue culture significantly at both pure 'bulla' (with different concentrations) and in mixture

with agar up to 76% cost of gelling agent is saved by the use of ‘bulla’ without compromising shoot multiplication and rooting (Biruk*et al*., 2012).

Other alternative gelling agents like potato starch, rice flour, cassava flour and corn reduced the cost of tissue culture media by 60-90% (Daud*et al*. 2011). The nutritional supplements of ‘bulla’ should be studied by different scientists and it consists of high concentration of macro and micro nutrients like K, Ca, Na, Mg, Fe, Zn, Co, Cu and Mn which is constituent of MS basal media (Debebe, 2006; Atlabachew and Chandravanshi, 2007). Cassava starch as a gelling agent for potato *in vitro* multiplication minimizes cost of the media.The use of 10% cassava and composite 8% ‘bulla’ + 0.25% agar reduced 43% and 28% of media gelling cost respectively (Kuria*et al.,* 2008). Banana *in vitro* multiplication on isabgol alternative gelling media 100% cultures survived, which indicated that significantly higher than that on agar media (79–83%). The total cost of media was reduced by 59% when using isabgolwas used as an alternate gelling agent for banana *in vitro* propagation (Anuradha*et al*., 2010).

2.5. Low Cost Carbon and Energy Source

Low costmedia protocol optimization on potato *in vitro* multiplication used table sugar as an alternative carbon source. Table sugar not only enhanced micro propagation but also significantly lowered the production input costs by 34 to 51% when compared with the analytical grade sucrose (Demo *et al*., 2008). The use of table sugar (30 g/l) was led to 97.1% cost savings in regard to the source of carbon alternative to sucrose for *in vitro* potato multiplication (Ogero*etal*., 2012). Perennial medicinal plant*Tylophoraindicain vitro* multiplication on MS media with 8g/l of agar and different alternative carbon sources (AR grade sucrose, white refined sugar (table sugar), unrefined brown sugar, jiggery and sugarcane juice) highly minimized media cost. Alternative carbon sources such as white refined sugar (table sugar), sugarcane juice, unrefined brown sugar and jiggery were reduced carbon source media cost by 94.8%,76.8%,73.8%and 67.6% respectively (Rajavel and Stephan, 2014).

Cost reduction by61.4% from the nutrients used in media preparation for *in vitro* multiplication of banana by replacing sucrose with 30 g/l table sugar by making 30

g/lsucroseas a control in 8g/l agar media, which is not significant difference with 30 g/l table sugar (Dhanalakshmi and Stephan, 2014). Cassava *in vitro* multiplication using 30g/l table sugar as an alternative source of carbon and 2 g/l Easygro vegetative fertilizer as source of both macro and micro nutrients were reducedby 97.1% and 92.2% of media cost respectively (Ombori*et al*., 2012).

Different concentrations of carbon source optimized for different plants. For optimal growth and multiplication, 2–4% sucrose is optimal for any propagation system based on the genotype or plant species (George and Sherrington, 1984). Patchouli (*PogostemoncablinBenth*.) *in vitro* multiplication with different concentrations of table sugars (1%, 2% and 3%) as an alternative carbon sources on MS medium resulted in shoot number of 50.57±0.49, 55.77±0.36 and 33.80±0.60 respectively, shoot length 3.47±0.45, 4.20±0.10 and 2.20±0.46 respectively, fresh weight of the shoots (g) 4.53±0.31, 4.81±0.12 and 3.47±0.16 respectively (Swamy*et al.*, 2010). Maximum shoot length (11.0 ± 0.28cm) for *Solanumnigrumin vitro* propagation was achieved on MS medium supplemented with 4% sucrose (Sridhar and Naidu, 2011). Buah*et al*. (2011) used 5 and 10% sugarcane juice for *in vitro* cultures of *Musa sp*. to compare it with laboratory grade sucrose and found 5% juice as a better alternative for sucrose. Beech *in vitro* regeneration was achieved with four concentrations of glucose (1, 2, 3 and 4%) and four concentrations of sucrose (1, 2, 3 and 4%) resulting in 4% sucrose and 3% glucose media showed the longest shoot but in 3 and 4% glucose media instead of longest shoot which resulted highest shoot number (Cuenca and Vieitez, 2000).*In vitro* multiplication of *Dendrobium* Hybrid CV.*Sonia*by alternative carbon source (white sugar) at 20 g/l was no significant difference with commonly used carbon source which is sucrose (Ramart*et al*., 2010).*In vitro* shoot multiplication of *Nicotianatabacum* L. on different alternative carbon source media (1.5 – 4%) like sucrose, glucose andjaggery with 0.8% agar and 3% sucrose as a control, which resulted maximumshoot multiplication and growth in media contained highest concentration of sucrose and glucose (4%) (Joshi, 2009).

2.5. Shoot Initiation and Multiplication

Moringa is a large perennial tree and due to its high nutritional value for a lot of people use it as a diet because it is a rich source of essential human vitamins and minerals. It has the potential to help alleviate malnutrition worldwide. Experiments were conducted to develop a protocol for micropropagation of moringa. Shoot growth was optimal on shoot growth medium containing high concentrations of Drew and Smith macro- and micro-nutrients (DSH), MS vitamins and 1 µM kinetin (Drew and Smith, 1986). Experiments on the addition of various concentrations of kinetin, riboflavin and potassium were conducted in an attempt to optimize root growth and minimize callus production on *in vitro* shoots. Transfer of both apical and axillary bud explants to medium containing 1 µM kinetin and 10µM riboflavin after two days on medium containing 10 µM IAA, minimized callus formation. Increased potassium concentration decreased shoot growth, callus production, number of roots per shoot and root growth in the presence of DSH but it increased root growth in medium containing DSM (Shokoohmand and Drew. 2013). Bestmoringa propagations were achieved on MS media containing 8 g/l agar through shoot regeneration from the cotyledonary node by cutting tip of the seedlings. Next to that auxiliary shoot from single node shoot segments (Steinitz *et al*., 2009).

Juvenile shoots were obtained from mature year round fruit bearing plant of *M. oleifera* L. wascultured inMS nutrient media contained 3% sucrose and 0.8% agar with different concentrations of BAP and among them, 100% explants produced shoots in BAP 1.0 and 1.5 mg/l but maximum number of shoots (4.0±0.29) were observed in media contained 1.0 mg/l BAP. Number of shoots was much more increased when repeated subculture on the same BAP concentration (Shahina*et al.,* 2005). *In vitro* multiplications of *M. peregrine* explants were collected from *in vitro* germinated seed shoot tip on MS medium with 3 % sucrose, 0.8 % agar and different concentrations of BA or Kin (0.5, 1.0 and 2.0 mg/l) resulted in 100 % shootdevelopmentin all samples and the media contained 1.0 mg/l BA better in shoot formation and resulted in the highest mean number of shoots/explants (6.5), leaves (16) and shoot height (5cm) (Wesam*et al.,* 2013).Direct regeneration of hypocotyls and nodes were affected with different levels of growth regulators. Maximum shoot length (4.31±0.49 cm) was attained when on medium

containing 0.1 mg/lKinetinfrom hypocotyl explants. Maximum number of leaves (17.67±1.45) and number of shoots per explant (2.07±0.25) were obtained on MS medium supplemented with 1.0 mg/l BAP and 1.0 mg/l BA respectively.

2.6. Rooting

Moringa oliefera node and hypocotyl were cultured on MS medium containing1.0 mg/l IBA and IAAwith 8 g/l agar and 30 g/l sucrose and produced 3.22±0.43 and 3.56±0.38roots, respectively. The node explants cultured on half strength MS mediumcontaining 8 g/l agar produced4.67±0.33 where asfull strength MS medium produced 3.0±0.0. The hypocotyl explants were cultured on full strength MS medium supplemented with IAA, 8 g/l agar and 30 g/l sucrose and resulted in root length of 3.29±0.42 cm whereas roots produced on half strength MS medium produced 6.83±0.17cm(Umbreen*et al.*, 2014).

Auxiliary shoots were cultured on MS medium containing 3% sucrose and 0.8% agar with the interaction of 2.85 µM IAA and 4.92 µM IBA resulted in maximum number of roots (15±1.3) per shoot. The growth hormone IAA and IBA individually produced 6.3±0.6 and 9.6±0.57 root numbers per shoot respectively. Maximum root length was obtained on4.92µM IBA and resulted in 6.5±0.6 cm (Saini *et al.*, 2012). From micro-shoot root regeneration on MS media supplemented with 3% sucrose and 0.8% agar resulted in maximum number of roots (44) on 1.0 mg/l IBA and maximum root length was produced (13cm) on 0.5 mg/l IBA (Wesam*et al*., 2013).

2.7. Acclimatization

For any tissue culture experiment, after rooting medium it needs to acclimatize the plantlets in greenhouse to get survived seedlings. Due to that, different acclimatization methods were developed with different authors. According to Saini *et al*. (2010), by covering the pots with polythene bags for two weeks in shaded greenhouse condition before exposing to normal environment which is possible to get 80% well survived seedlings. Whereas, related result also reported by Mitiku (2013) for *in vitro* propagation of *M. stenopetala*, from 25 plantlets transferred to sterilized soil containing pot and covered with plastic bags for two weeks in greenhouse condition 78% seedlings were well survived.Wesam*et al*. (2013) also reported that 90% of the plantlets were survived after sterilized transfer to *ex-vitro* growth room.

3. MATERIALS AND METHODS

3.1. Experimental Materials

Moringa stenopetala seeds were obtained from Jimma Agricultural Research Center, Ethiopia. As the genotype was not developed and released by the research center, it was not possible to get information on it.

3.2. Stock Solutionand Media Preparation

MS basal medium (Murashige and Skoog, 1962) was used throughout the experiments. A stock solution of macronutrients, micronutrients, growth regulators and vitamins were prepared separately and stored at 4^oC for immediate use. MS basal medium was prepared by mixing the appropriate volumes of stock solutions and finally different concentrations and combinations of 'bulla' and agar were added accordingly. After dissolving all the components, except gelling agent, the pH was adjusted to 5.8 using 1N NaOH and 1N HCl. The mixture of 'bulla' with medium was made carefully since it precipitates at the bottom and forms aggregates. So, 'bulla' powder was first mixed with the medium and heated on stove with continuous agitation followed by dispensing of the media in Jam jars. Then the dispensed media were autoclaved at 1.06 kg/cm^2 and 121^oC for 15 minutes (Ayelign *et al*., 2012).Growth regulators such as 6-Benzyl Amino Purine (BAP) for shoot initiation and shoot multiplication and Indol-3-Butyric Acid (IBA) for rooting were used in this study. The preparation of growth regulators was done by 1mg/1ml. The powder dissolved by a few drops of 1N NaOH and the required volume was adjusted by distilled water. Then it was kept in a refrigerator at 4°C.'bulla' was first mixed into half MS media and then dispensed gradually in to heated media on stove with continuous agitation and dispensed in to jars and autoclaved with 121^oC for 15 minutes (Ayelign *et al*., 2012).

3.3. Preparation of Explants

The seeds were removed from pods and washed under tap water with a drop of liquid detergent throughout and then rinsed with distilled water. Then they were surface

sterilized by washing with 70% ethanol for 2 min and then berekina (NaOCl) 1.5% active chlorine for 15 min, followed by rinsing five to ten times in sterile distilled water.

After sterilization, seeds were cultured aseptically in Petri dish containing sterile distilled water with cotton support until sprouted out its radicle. Then seed coats were removed aseptically to permit direct contact of seed tissue with the culture medium and to reduce contamination, then the seeds were transferred in to growth regulator free medium (MS + 3% sucrose + 0.8% agar). In this medium, the seedlings were kept until the first pair of leaves emerged and then shoot tips were taken as explant source (Kumari and Singh, 2012).

3.4. Culture Condition

The culture was placed at a temperature of25 ± 2^{o}Cand light intensity of 2,000-Lux produced from cool white fluorescent tubes for 16 h photoperiod. The time was adjusted manually from 6 pm up to 10 am white florescent light was on and from 10 am up to 6 pm the florescent light switched off.

3.5. Experiments

3.5.1. Experiment 1. The effect of different concentrations of *'bulla'* on shoot initiation

The sterilized shoot tips (1.5cm) were cultured on basal MS medium supplemented with various concentrations of 'bulla' (liquid medium, 40, 60, 80, 100 g/l) and 8 g/l agar (control) with 1.0 mg/l BAP. The experiment was replicated five times in Completely Randomized Design (CRD). Three explants were used per jar. Subculture was done over21day. Data onpercentages of explants showing shoot initiation were collected.

3.5.2. Experiment 2. The effect of different concentrations of 'bulla' on shoot multiplication

From initiated shoots 1.5 cm explants were taken and cultured on basal MS medium supplemented with various concentrations of 'bulla' (liquid medium, 40 g/l 'bulla', 60 g/l 'bulla', 80 g/l 'bulla', 100 g/l 'bulla', 1g/l agar + 70 g/l 'bulla', 2 g/l agar + 60 g/l 'bulla',

3 g/l agar + 50 g/l ‘bulla’ and 4 g/l agar + 40 g/l ‘bulla’) and 8 g/l agar (control) with 1.0 mg/l BAP. The experiment was replicated five times in Completely Randomized Design (CRD). Three explants were used per jar. Data on number of shoots, shoot length and number of leaves were collected after 4 weeks of culture.

3.5.3. Experiment 3. The effect of different concentrationsof table sugar and sucrose on shoot multiplication

From experiment two (multiplication) 1.5 cm long explants were taken and cultured on basal MS medium supplemented with various concentrations of sucrose (15, 30, 45,60 g/l) and table sugar (15, 30, 45, 60 g/l) with1.0 mg/l BAP. Optimum concentration of gelling agent (80 g/l ‘bulla’) obtained from experiment two was used. The experiment was replicated five times in Completely Randomized Design (CRD). Three explants were used per jar. Data on number of shoots, shoot length and numbers of leaves were collected after 4 weeks of culture.

3.5.4. Experiment 4. The effect of different concentrations of ‘bulla’ on rooting

After one week on growth hormone free media, 3 cm sized explants were taken for rooting and cultured on basal MS medium supplemented with various concentrations of ‘bulla’ (40 g/l, 60 g/l, 80 g/l and 100 g/l ‘bulla’) and two media used as a control (8 g/l agar and 2 g/l agar + 60 g/l ‘bulla’) with 1.0 mg/l IBA. The experiment was replicated four times in Completely Randomized Design (CRD). Three explants were used per jar. Data on number of shoots that produce root, number of roots per shoot and length of roots were collected after three weeks of culture.

3.6. Acclimatization

The plantlets obtained after rooting were carefully separated from the medium and the roots were washed with tap water to remove the gelling agent adhering to them. Then 30plantlets were transplanted in to 10 cm diameter nursery pots containing a mixture of sun sterilized soil, sand and compost at 2:1:1 ratio and transferred to sun light accessible

room which was Jimma university tissue culture laboratory used as hardening off before transferred to greenhouse up to four week. Then the seedlings were transferred to greenhouse. The plantlets were covered with plastic for one week to create shade and watered frequently. The plastic cover gradually removed after one week and watering frequency reduced, after three weeks two times per day was watered. The survived percentage of plantlets was recorded on week four.

3.7. Data Analysis

Data was subjected to statistical analysis using SAS 9.2 software (SAS, 2008). Least significant difference (LSD) was used to the significant differences among means and significances were determined at $p< 0.01$. The data recorded from all experiments in all parameters except from experiment two (number of shoot and shoot length) were transformed.

Cost of Gelling Agent and A carbon Source Calculation

A cost benefit analysis of gelling agents and carbon sources for moringa micro-propagation was made. The cost of gelling agents and carbon source was calculated per a liter of medium and per a kilogram of gelling agents and carbon source. Then, the total cost would be saved using 'bulla' and table sugar per a litter of media was estimated using the following mathematical formula: (Ayelign et al., 2012).

1. Cost saved (%) = [100 − ('bulla' cost/agar cost) ×100]

- For pure 'bulla' calculation

2. Cost saved (%) = [100 - (table sugar cost/sucrose cost) ×100]

- For table sugar calculation

3. Cost saved (%) = [100- ('bulla' cost + agar cost/ agar cost) ×100]

- For composite calculation

4. Overall cost saved = [100- ('bulla' cost + table sugar cost/ agar cost + sucrose cost) ×100]

- For overall cost saved by 'bulla' and table sugar calculation

4. RESULT AND DISCUSSION

4.1. Effect of ‘bulla’ on Shoot Initiation

*M. stenopetala*shoot tips cultured on MS media with different concentrations of ‘bulla’ resulted 100% of shoot initiation in four concentrations (8 g/l of agar and, 60 g/l, 80 g/l and 100 g/l of ‘bulla’) and no significant different with 40 g/l ‘bulla’ (86.68%) but there were significantly different with liquid media (33.33%) (Table1).The amount of ‘bulla’ bellow 60g/l affected or reduced the gelling strength and anchoring capacity of *in vitro*explants. The anchoring quality of ‘bulla’ gelling agent was increased when ‘bulla’ was added above 60g/l but the gel become strong when above 80g/l of ‘bulla’. The medium gelled with 100 g/l‘bulla’was affected absorption of nutrients by explants, reduced water availability, and adventitious shoots regeneration also decreased (Casanova *et al.,* 2008). Similar result reported by Riyathong,*et al*. (2010) for *M. olieferain vitro* multiplication in MS medium containing 8g/l agar was produced 100% shoot formation. One of the problems in ‘bulla’ gelling agent was not possible easily identify media contamination. Mitiku (2013) also resulted a maximum of 100% *M. stenopetala* i*n vitro* shoot initiated on MS medium fortified with 8 g/l agar medium.

The result indicated that using a gelling agent for *M. stenopetala* shoot tip initiation cultured media (liquid media, 8g/l agar and ‘bulla’ 40g/l, 60g/l, 80g/l and100g/l) any one of gelling amount except liquid media and 40g/l‘bulla’ have resulted in good gelling quality to support and anchor the explants for shoot initiation. Pilot study was done on‘bulla’with concentrations of 10, 20, 30, 40, 50g/l and the result showed that below 40g/lbecome liquid. These three ‘bulla’ levels (60g/l, 80 g/l, and 100g/l) were not-significantly different from the control (8g/lagar). But 80 g/l of ‘bulla’ was better to get easily survived explants for multiplication media (Figure 1).

Table 1: Effect of different ‘bulla’ concentrationson *in vitro* shoot initiation

Gelling agent	Concentration g/l	Shoot initiation (%)
Liquid media	-	33.33[b]
Agar	8	100[a]
‘bulla’	40	86.68[a]
‘bulla’	60	100[a]
‘bulla’	80	100[a]
‘bulla’	100	100[a]
CV (%)	-	2.46

The same letter indicates non-significant difference in the same column.

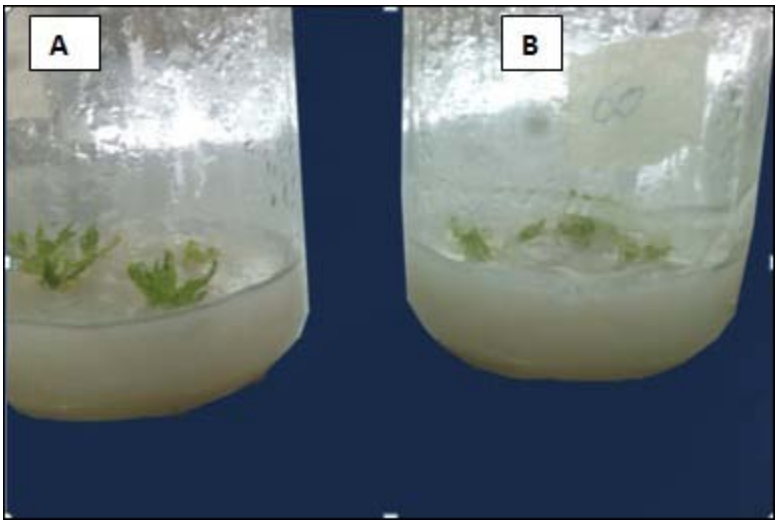


Figure 1: Effect of ‘bulla’as gelling agent for shoot initiation of *M. stenopetala*:

A) 80 g/l B) 60 g/l

4.2. Effect of ‘Bulla’ on Shoot Multiplication of *M. stenopetala*

The result of moringa multiplication on different concentrations of alternative gelling agents of ‘bulla’ and composites showed significant difference on number of shoots per explants, shoot length and number of leaves (Table 2).When the concentration of ‘bulla’ was lower than 80 g/l, it showed low gelling quality due to reduction of anchoring (supporting) capacity. Multiplication media needs long time compared with initiation and rooting media. Due to unknown reason MS medium containing 60 g/l ‘bulla’, resulted low gelling quality when culturing time was longer than three weeks. Composite(1g agar + 70g ‘bulla’ 2g agar + 60g‘bulla’, 3g agar with 50g ‘bulla’ and 4g agar + 40g ‘bulla’) were important to identify ‘media’ contamination compared with pure ‘bulla’ because agar reduces milky colors of pure ‘bulla’ and increased purity of the gel (Figure 2). When the amount of agar increased in a composite, media purity and contamination identification will be increase but increased the cost of gelling media.

4.2.1. Number of shoots per explant

Moringa shoot tip cultured on MS medium supplemented with 8g/l agar (control) produced maximum number of shoots (7.01±0.3) and not significant different with 80g/l‘bulla’ (6.94±0.26) and the composite 1g/l agar + 70g/l‘bulla’ (6.95±0.26) (Table 2).Related result reported by Wesam*et al*. (2013)from*in vitro* propagations of *M. oliefera* in MS media with 8g/l agar, and highest mean number of shoot/explant was6.5. This result was disagreed withUmbreen*et al.,* (2014) who observed that2.07±0.25 of shoots perexplants using MS media supplemented with BAP and agar as a gelling agent. This may be due to the difference of growth hormone and genotype.

4.2.2. Shoot length

The result of this experiment showed that different concentrations of gelling agent also had significant effect on shoot length. The maximum shoot length observed on MS medium supplemented with 8 g/l agar (4.90 cm) showed non-significant difference with 80 g/l ‘bulla’(4.88 cm).Similarly,Wesam*et al*. (2013) who worked on*M. peregrine* shoot tip culture (excised from *in vitro* germinated seedlings) on MS medium containing 8 g/l agar, resultedin shoot length of 5 cm.According to Umbreen*et al.,* (2014), the maximum shoot

length (4.31±0.49 cm) was attained when the hypocotyl explants were cultured on medium containing 8g/lagar. This result disagreed with the result reported by Mitiku (2013) (1.9±0.12 cm) for *M. stenopetala in vitro* propagation in 8 g/l agar and 1.0 mg/l BAP medium. This disagreement may be due to difference of gelling agent. 'Bulla' (80g/l) performed well in this experiment but with poor gel clarity which makes identification of contamination very difficult. However, better gel clarity was observed in the medium having a mix of agar and 'bulla'.

4.2.3. Number of leaves

The result showed that number of leaves in different concentrations of pure and composite gelling agents had significant effect. Moringa shoot tip culture revealed that the maximum number of leaves (17.07) was obtained on 80 g/l pure 'bulla' and it indicated that non-significant difference with the control 8g/l agar (16.81).The composites also produced 14.60 and 14.88 numbers of leaves when cultured on 1.0 g/l agar + 70 g/l 'bulla' and 3 g/l agar + 50 g/l 'bulla', respectively and there was no significant difference between them but there were significant difference between the control (8 g/l) agar and 80 g/l 'bulla'.

This experiment showed that maximum number of leaves (17.07) produced on media gelled with 80 g/l 'bulla 'disagreed with the result reported by Umbreen *et al.* (2014) and Wesam*et al*., (2013) on*M. oleifera in vitro* hypocotyls and shoot tip culture in 8 g/l agar media, whose maximum number of leaves (16.0±0.5 and 16,respectively)were reported. This may be because of the difference in gelling agent and the genotype of the explants.

Table 2: Effect of different concentrations of 'bulla' in shoot multiplication.

Gelling agent	concentration g/l	No. of shoots	Shoot length (cm)	No. of leaves
Liquid media	-	1.75±0.27^{e}	2.13±0.40^{e}	2.87±0.37^{h}
Agar	8	7.01±0.30^{a}	4.90±0.18^{a}	16.81±1.05^{a}
'bulla'	40	1.76±0.13^{e}	2.08±0.13^{e}	6.07±0.26^{g}
'bulla'	60	4.55±0.20^{d}	3.11±0.25^{d}	6.82±0.16^{f}
'bulla'	80	6.94±0.26^{a}	4.88±0.16^{a}	17.07±0.86^{a}
'bulla'	100	6.412±0.29^{c}	3.43±0.22^{c}	8.01±0.0.74^{e}
Agar + 'bulla'	1 + 70	6.95±0.26^{a}	4.73±0.19^{ab}	14.60±1.47^{b}
Agar + 'bulla'	2 + 60	6.47±0.38^{bc}	4.10±0.16^{b}	9.47±0.38^{d}
Agar + 'bulla'	3 + 50	6.82±0.16^{ab}	4.77±0.23^{ab}	14.88±1.00^{b}
Agar + 'bulla'	4 + 40	6.55±0.38^{bc}	4.70±0.21^{ab}	11.41±1.36^{c}
CV (%)		5.07	5.79	3.68

The same letter indicates non-significant difference in the same column.

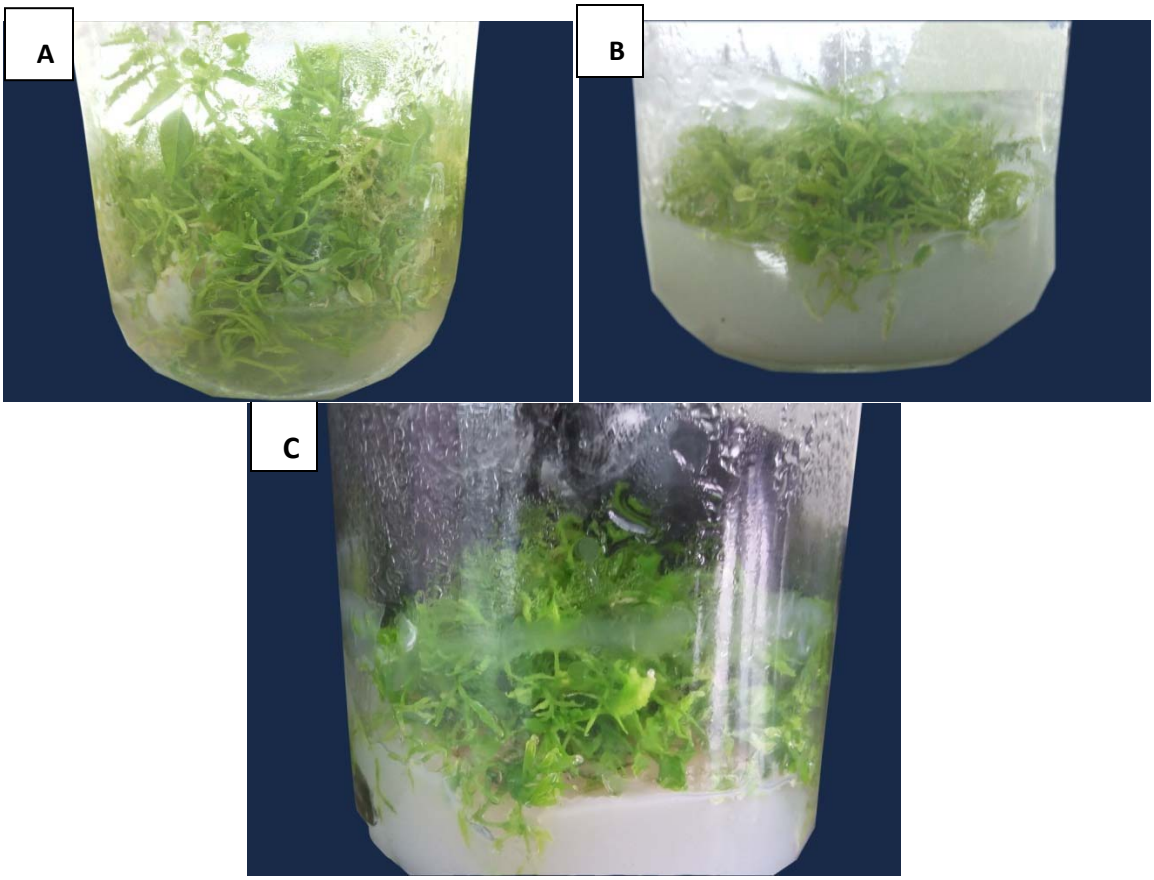


Figure 2: Effect of gelling agent in shoot multiplication of *M. stenopetala*;A. pure agar (8 g/l), B. composite (1 g/l agar + 70 g/l 'bulla') and C. pure 'bulla' (80 g/l).

4.3. The Effect of Different Concentrations of Table Sugar and Sucrose on Shoot Multiplication

Different concentrations of table sugar and sucrose had significant effect on *M. stenopetalain vitro* multiplication. The result showed that maximum shoot number (6.41), shoot length (4.61 cm) and leaf number (15.14) were obtained in MS media supplemented with 30 g/l sucrose but showed no significant difference with 30 g/l table sugar which resulted in 6.35shoot per explant, 4.5 cm shoot length and 15leaves per plant(Table 3, Figure 3).

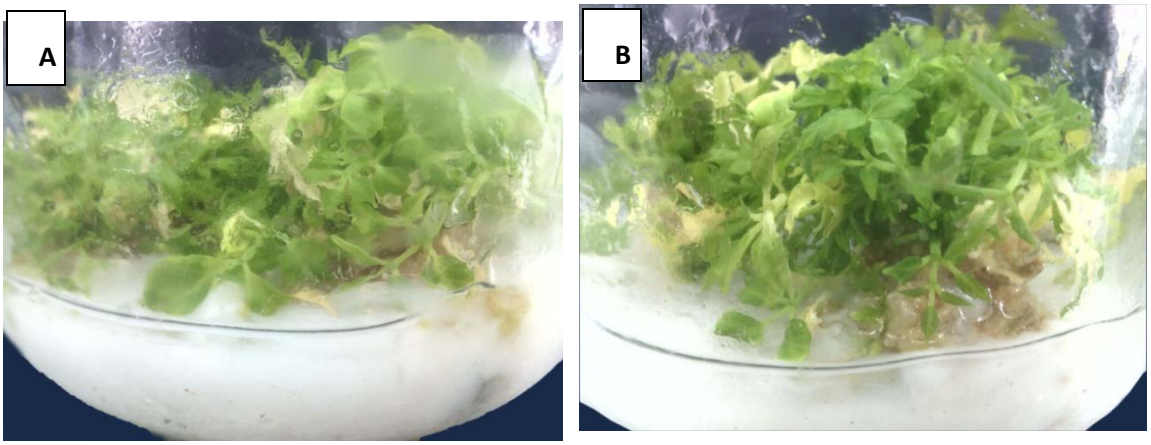
When the concentrationof both sucrose and table sugar increased to 45 g/l, therewasnon-significantly different with 30 g/l sucrose and table sugar on shoot number and leaf number but significantly different on shoot length. When the concentration of both table sugar and sucrose were increased to 60 g/l, it induced large and maximum number of roots. Similar result was reported by Wesam*et al*., (2013) on 30 g/l sucrose, highest shoot numbers (6.5), shoot length (5cm) and leaf number (16) for *M. oliefera in vitro* multiplication.

Different researchersreportedthat optimization of *in vitro* carbon source for different plant species were differed in concentrations and type of carbon source. Carbon source optimization for *Nicotianatabacum* L. using 40 g/l sucrose and glucose produced maximum shoots and growth (Joshi, 2009). *In vitro* propagation of *Solanumnigrum*was achieved on MS medium supplemented with 4% sucrose (Sridhar and Naidu, 2011). *Dendrobium* Hybrid CV.*Soniain vitro* multiplication of 2% white sugar medium was optimum and non-significant difference with sucrose (Ramart*et al*., 2010). For patchouli (*Pogostemoncablin*Benth.)*in vitro* multiplication, 20 g/l table sugar resulted optimum concentration (Swamy*et al*., 2010). All the above experimentsby different authors showed that optimum concentration of carbon source differed due to different plant species. However, in this experiment 30 g/l table sugar with 80 g/l ‘bulla ‘resultedin better *in vitro* multiplication of *M. stenopetala*.

Table 3: Effect of carbon sources on shoot multiplication of *M. stenopetala*

Treatments	Conc. g/l	Shoot number	Shoot length (cm)	Leaf number
Sucrose	15	3.95±0.26^{de}	4.10±0.23^{bc}	8.81±1.22^{b}
	30	6.41±0.44^{a}	4.61±0.20^{a}	15.14±0.28^{a}
	45	5.81±0.63^{a}	4.42±0.27^{ab}	14.74±0.83^{a}
	60	4.50±0.45^{c}	3.76±0.34^{d}	8.75±0.65^{b}
Table Sugar	15	3.70±0.24^{e}	4.07±0.23^{bc}	8.6±1.01^{b}
	30	6.35±0.35^{a}	4.50±0.37^{a}	15.00±0.74^{a}
	45	6.10±0.25^{a}	4.43±0.36^{ab}	14.81±0.30^{a}
	60	4.35±0.35^{cd}	3.82±0.20^{cd}	8.74±1.01^{b}
CV (%)		4.94	4.44	3.51

The same letter indicates non-significant difference in the same column



A **B**

Figure 3: Effect of sucrose and table sugar on shoot multiplication of *M. stenopetala*: A) 30 g/l sucrose B) 30 g/l table sugar

4.4. The Effect of Different Concentrations of ‘bulla’ on Rooting

The alternative gelling agent and their concentration individually had the significant effect on the root formations of moringa. Minimization of *invitro* rooting cost by replacing commonly used *in vitro* gelling agent (agar) with alternative gelling agent had significant effect on rooting media cost.

4.4.1. Percentage of root formation

The result indicated that Percent of root formation showed non-significant difference between treatments (8g/l agar, 40g/l‘bulla’, 2g/l agar + 60g/l‘bulla’, 60g/l‘bulla’, 80g/l‘bulla’ and 100g/l‘bulla’) and 100% shoots produced root in all MS media with 30g table sugar and 1mg/l IBA except treatment two (40g/l‘bulla’) (Table 3). In this experiment, percent of root initiation was higher and dissimilar with the highest root percent (60%) produced for *M. oliefera in vitro* multiplication on MS media with 8g/l agar (Thidarat*et al.,* 2012). This disagreement on percent of root formation may be due to the result of genotype difference. The amount of alternative gelling agent (‘bulla’) becomes reduced below 60g/l. In rooting media 60g/l and 80g/l‘bulla’had better rooting capacity and also showed non-significantly different with the control (8g/l) agar and the composite (2g/l agar + 60g/l‘bulla’).

4.4.2. Number of roots

‘Bulla’had significant effect on the number of roots. The medium that treated or gelled with 8 g/l agar (control) resulted in highest root number (9.09) but not significantly differentfrom2 g/l agar + 60 g/l ‘bulla’ (8.415), 60 g/l ‘bulla’ (8.51) and 80 g/l ‘bulla’ (8.41).Reports of other authorsindicated that the node explants produced more roots on 8g/l agar (4.67±0.33) (Umbreen*et al.,* 2014) and also medium contained 8g/l agar and 1mg/l IBA resulted in 6.3±0.6 roots (Saini *et al.,* 2012). This indicated that dissimilarity was the result of gelling type and due to the difference in source of explants and genotype. The composite was important to identify contamination compared to media that contained pure‘bulla’ (Figure 4).

4.4.3. Root length

Different gelling agent concentrations had effects on root length. The highest root length was obtained in 8 g/l agar medium (6.51 cm) and it showed as non-significant difference with 60 g/l ‘bulla’ (6.41 cm) and composite 2 g/l agar + 60 g/l ‘bulla’(6.025 cm) (Table 4). The result indicated thatalternative gelling agent of 80g/l‘bulla’ (5.51) was significantlydifferent with the control (8g/l agar) and composite (2g/l agar + 60g/l‘bulla’). The MS media with 80g/l‘bulla’ had a better gel quality to anchoring of explants but 100g/l‘bulla’ resulted in hard gel and it reduced easy root elongation and proliferation. Similarly, Saini *et al.* (2012) reported on *M. oleiferain vitro*multiplication in 8 g/l agar medium showed root length of 6.5±0.6 cm. The result of root length in this experiment disagreed with Umbreen*et al.* (2014) who reported the root length (3.29±0.42 cm) on *M. oleiferainvitro*propagation.It may be due to difference in media composition. This experiment indicatedthat the time required for rooting media was long, the gelling quality to support the explants was reduced especially the amount of ‘bulla’ reduced from 60 g/l.

Table 4: Effect of different concentrations of gelling agent on root initiation

Gelling agent	Concentration g/l	Rooting (%)	No. of root	Root length (cm)
Agar	8	100[a]	9.09±0.73[a]	6.51±0.49[a]
'bulla'	40	66.7[b]	1.51±0.21[c]	2.60±0.44[d]
agar + 'bulla'	2 + 60	100[a]	8.76±0.56[a]	6.02±0.45[ab]
'bulla'	60	100[a]	8.51±0.21[a]	6.41±0.54[a]
'bulla'	80	100[a]	8.41±0.41[a]	5.51±0.43[b]
'bulla'	100	100[a]	2.16±0.19[b]	3.21±0.28[c]
CV (%)	–	4.11	4.95	5.60

CV= coefficient of variation, the same letter indicates non-significant difference in the same column

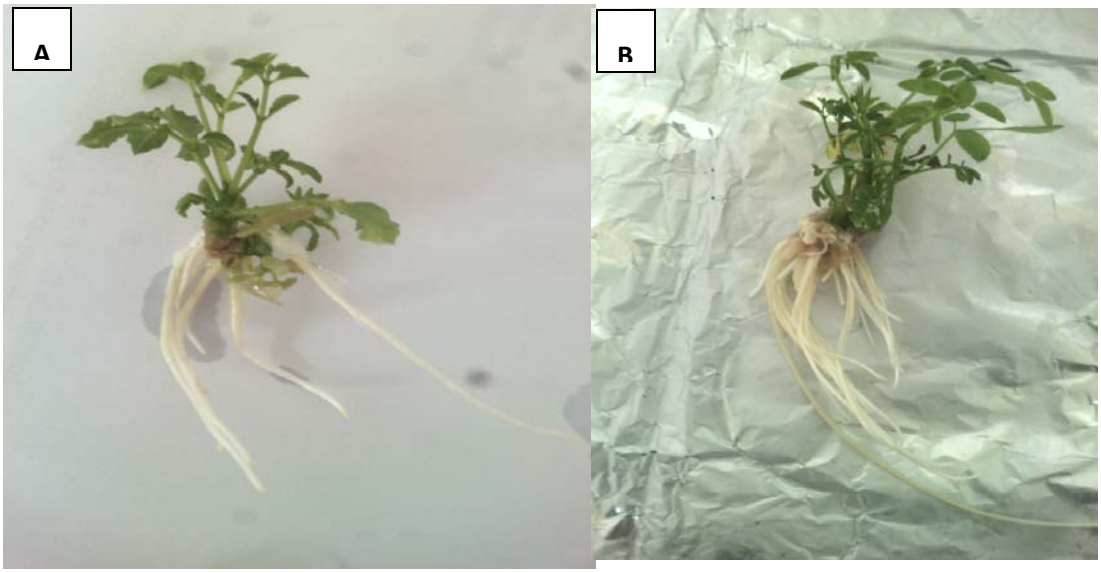


Figure 4: Effect of gelling agent on rooting: A) 8 g/l agar: and B) 60g 'bulla'

4.5. Cost Reduction Summary

4.5.1. Using different concentrations of 'bulla' as gelling agent

The alternative gelling agent effectively minimizes the cost of tissue culture media (Ayenew*et al*., 2012; Daud*et al*., 2011). A gram of agar costs3.1 birr maximum it compared with a gram of 'bulla' (0.09 birr). In this experiment, the use of alternative gelling agent saved cost of *M. stenopetala in vitro* propagation media significantly both in pure and composite form. Substitution of agar gelling agent with 60 g/l, 80 g/l and 100 g/l of 'bulla' reduced the gelling cost by 78.23%, 70.97% and 63.7% respectively. Both initiation and multiplication media by using 80 g/l 'bulla' reduced the cost of gelling media up to 70.97%. The composites 1 g/l agar + 70 g/l 'bulla', 2 g/l agar + 60 g/l 'bulla' and 3 g/l agar + 50 g/l 'bulla' also reduced the multiplication media gelling cost by 62.10%, 53.23% and 44.35% respectively. According to this experiment, rooting media using 60g/l and 80g/l'bulla'as alternative gelling agent were better to minimize *in vitro* rooting media cost.

Different alternative gelling agents were optimized to reduce the cost of tissue culture media. The use of pure 10% cassava and composite 8% cassava + 0.25% agar reduced media gelling cost by 43% and 28%, respectively (Kuria*et al.*, 2008).Anuradha*et al*. (2010) reported that 100% of banana explants survived on both initiation and multiplication media when isabgol was used as alternative gelling agent. Whereas only 79-83% of explants survived on medium containing agar. The total cost of medium was reduced by 59% whenisabgolwas used as an alternate gelling agent for banana *in vitro* propagation.

4.5.2. Using different concentrations of table sugar and sucrose as carbon source

Substitution of sucrose with table sugar for *M. stenopetala in vitro* propagation highly reduced media cost.A gram of sucrose (1.16 birr) was higher than the cost reviled by table sugar (0.024 birr). In this experiment, the result showed that using 30 g/l table sugar instead of 30 g/l sucrose as a carbon source reduced *in vitro* media cost by up to 98%.

When low cost media protocol optimization on potato *in vitro* multiplication used table sugar as an alternative carbon source, result showed that table sugar not only enhanced

micropropagation but also significantly lowered the production input costs by 34 to 51% when compared with the analytical grade sucrose (Demo *et al*., 2008). The use of table sugar (supplemented with 30 g/L) led to 97.1% savings in regard to the source of carbon alternative to sucrose for *in vitro* potato multiplication (Ogero*et al*., 2012). *Tylophoraindicain vitro* multiplication with alternative carbon sources white refined sugar (table sugar) (94.8%), sugarcane juice (76.8%), unrefined brown sugar (73.8%) and jiggery (67.6%) reduced carbon source media cost (Rajavel and Stephan, 2014). Cassava *in vitro* multiplication using 30g/l table sugar as an alternative source for carbon reduced the cost by 97.1%. *In vitro* multiplication of banana by replacing sucrose with 30 g/l table sugar reduced media cost by 61.4% (Dhanalakshmi and Stephan, 2014). All the experiments with different authors showed that alternative carbon source highly reduced media cost due to that in this experiment 30 g/l table sugar recommended for low cost *in vitro* multiplication of *M. stenopetala.*

4.6. Acclimatization

The plantlets obtained after rooting were carefully separated from the medium to avoid damaging and washed with tap water to remove the galling agent adhering to them. Then thirty plantlets were transplanted in to 10 cm diameter nursery pots containing a mixture of sun sterilized soil, sand and compost in 2:1:1 ratio and transferred to sun light accessible room which was Jimma University Tissue Culture Laboratory used as hardening off before transferred to greenhouse up to four weeks that helps survival of plantlets when transferred to greenhouse. The plantlets were covered with plastic for one week to create shad and watered frequently. After one week,the plastic covers were removed and watering frequency reduced gradually, after three weeks two times per day was watered. The exposure time to sun light was increased daily until four week according to Saini *et al.* (2012) and data for the survived plantlets were recorded.From 30plantlets were shifted to the hardening off room 83.34% successfully survived (figure 5). Related results were reported by Umbreen*et al.* (2014) andMitiku(2013) from all plantlets transferred to acclimatization room 85%and 78% of them survived, respectively.

Figure 5: Acclimatized seedling stages;A) at the first week and B)after four week

5. SUMMARY AND CONCLUSION

Tissue culture *in vitro* multiplication helps to produce large number of plants within a short period of time and it is important for commercial production. In this experiment, seedling derived shoot tips were used as explants for *in vitro* multiplications of *M. stenopetala* in MS medium. One of the problems for *in vitro* multiplication was the cost of culture media constituent's specially gelling agent and carbon source. Optimization of these two media components with alternative gelling agent and carbon source (agar with 'bulla' and sucrose with table sugar) highly minimizes the cost of tissue culture media.

In this experiment with different concentrations of gelling agent,'bulla' and carbon source table sugar were optimized for low cost alternatives for *M. stenopetalain vitro* multiplication. In initiation media, 60 g/l and 80 g/l 'bulla' resulted in best gelling quality but the initiation culture took time. 'Bulla'80 g/l is recommended for its longer stability. These two levels of 'bulla' (60 g/l and 80 g/l) reduced the media gelling cost by 78.23% and 70.97% respectively. Pure 'bulla' gelling agent looks like a milky color and difficult for easy identification of media contamination.Therefore, composite gelling agents (agar + 'bulla') were essential to solve this problem. In multiplication media, the composites of agar with 'bulla' (1 g/l + 70 g/l, 2 g/l + 60 g/l, 3 g/l + 50 g/l, and 4 g/l + 40 g/l) and pure 'bulla' (80 g/l) were a good gelling agents and reduced the cost of tissue culture gelling media. 'Bulla'80 g/l is recommended for multiplication media and reduced media gelling cost by 70.97%. Nonetheless, for identification of media contamination, the composite 1 g/l agar + 70 g/l 'bulla' and 2 g/l agar + 60 g/l 'bulla'is recommended and reduces the gelling media cost by 62.10% and 53.23%, respectively. In rooting media, 60 g/l and 80 g/l 'bulla' gelled media resulted in best performance of root proliferation and reduced gelling media cost by 78.23% and 70.97%. Duration of rooting media takes more than three weeks and80 g/l 'bulla' isrecommended for its gelling stability.

Optimization of table sugar highly reduced the cost of tissue culture media. The experiment showed, in 30g/l table sugar did not show significant difference with commonly used carbon source (30g/l sucrose). The use of 30g/l table sugar as an alternative carbon source, which reduced by 98.6% of carbon source cost and it

recommended. In this experiment, by using alternative gelling agent 80g/l‘bulla’ and 30g/l table sugar as carbon source minimized 86% of the overall cost of the media. Alternative carbon source optimizations for different plant species or genotypes resulted in different concentrations from the range of 1g/l up to 50g/l. The concentrations also differed based on the type of carbon source. In this experiment, 30 g/l table sugar together with 80 g/l ‘bulla’ resulted in better *M. stenopetala in vitro* propagation. From all explants transferred to acclimatization room 85% survived.

For the future;

Based on the present study the following future line recommendations are forwarded:

- Interested researchers are also recommended to work on other genotypes

- For further research working on cheap and easily accessible substitutes of macro and micro nutrients and vitamins required for micropropagation of different moringa genotypes are recommended.

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8. APPENDICES

Appendices 1: ANOVA table for the effect of 'bulla' on percent of shoot initiation

Source of variation	DF	Mean Square
		PI
Treatment	5	0.96**
Error	20	0.01
CV		2.46

Note: ** Highly significant at P ≤ 0.01, CV= Coefficient of Variation, DF= Degree of Freedom, PI; Percent of shoot initiation.

Appendices 2: ANOVA summery for the effect of 'bulla' on shoot multiplication

Source of variation	DF	Mean Square		
		SN	SL	LN
Treatment	9	22.25**	6.52**	1.60**
Error	36	0.08	0.05	0.01
CV		5.07	5.79	3.68

Note: ** Highly significant at P ≤ 0.01, CV= Coefficient of Variation, DF= Degree of Freedom; SN; shoot number, SL; shoot length and LN; leaf number

Appendices 3: ANOVA table for the effect of carbon source on shoot multiplication

Source of variation	DF	Mean Square		
		SN	SL	LN
Treatment	7	0.25**	0.03**	0.42**
Error	28	0.01	0.004	0.01
CV		4.94	4.44	3.51

Note: ** Highly significant at P ≤ 0.01, CV= Coefficient of Variation, DF= Degree of Freedom; SN; shoot number, SL; shoot length and LN; leaf number

Appendices 4: ANOVA table for the effect of 'bulla' on root initiation

Source of variation	DF	Mean Square		
		PI	RN	RL
Treatment	5	0.15**	2.70**	0.64**
Error	15	0.03	0.01	0.01
CV		4.11	4.95	5.60

Note: ** Highly significant at P ≤ 0.01, CV= Coefficient of Variation, DF= Degree of Freedom, PI; Percent of root initiation, RN; Root number and RL; Root length.

Appendices 5: MS Media compositions

Constituents	Concentration (g/l)
NH_4NO_3	16.50
KNO_3	19.00
CaCl2.2H2O	4.40
$MgSO_4.7H_20$	3.70
KH_2PO_4	1.70
$ZnSO_4.4H_2O$	0.86
H_3BO_3	0.62
$MnSO_4.4H_2O$	2.23
$CuSO4.5H_2O$	0.03
KI	0.86
$Na_2MoO_4.2H_2O$	0.25
$CoCl_2.6H_2O$	4.40
Na-EDTA	3.73
$FeSO_4.7H_2O$	2.23
Nicotinic acid	0.50
Thiamin (B1)	0.50
Pyridoxine (B6)	0.50
Glycine	2.00
Sucrose	30.00
Agar	8.00
Myo-inositol	0.10