IN VITRO REGENERATION, MORPHOLOGICAL AND MOLECULAR CHARACTERIZATION OF *MELIA VOLKENSII* (GÜRKE)

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DECLARATION

This thesis is my original work and has not been submitted for award of a degree in any other University.

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DEDICATION

This thesis is dedicated first to the memory of my late parents Mr. Ben Cheye Sagwa and Mrs. Dorcas Isigi Sagwa, who really cherished education and would have been immensely proud of this work. It is also dedicated to my dear wife Carolyne Mukhaya Mulanda, my daughter Valerie Ajami Mulanda and son Melvin Cheye Mulanda, for their understanding, support and encouragement.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF PLATES

LIST OF ABBREVIATIONS AND ACRONYMS

ABA: Abscisic Acid

AFLP: Amplified fragment Length polymorphism

ANOSIM: Analysis of Similarities

ANOVA: Analysis of variance

ASAL: Arid and semi-arid land

BAP: 6-Benzyl aminopurine

BGF: Better Globe Forestry

CBO: Community Based Organization

CPPU: 1-(2-chloro-4-pyridyl)-3-phenylurea, also known as Forchlorfenuron

CTAB: Hexadecylytrimethyl ammonium bromide

DNA: Deoxyribonucleic acid

2,4-D: 2,4-Dichlorophenoxyacetic acid

EDTA: Ethylenediaminetetraacetic acid (disodium salt)

FAA: Formalin-Acetic acid-Alcohol mixture

FAO: Food and Agriculture Organization of the United Nations

GA_3: Gibberellic acid-3

GPS; Global positioning system

HSD: Tukey's honestly significant difference post-hoc test

IAA: Indole-3-acetic acid

IBA: Indole-3-butyric acid

ICRAF: International Centre for Research in Agroforestry, but now known as World Agroforestry Centre)

ISSR: Inter-simple sequence repeats

KEFRI: Kenya Forestry Research Institute

KFS: Kenya Forestry Service

MS: Murashige and Skoog medium

NAA: 1-Naphthaleneacetic acid

NALEP: Kenya's National Agriculture and Livestock Extension Programme

NGO: Non Governmental Organization

PAST: PAleontological Statistics software

PCOORDA: Principal coordinates analysis

PCR: Polymerase chain reaction

PEG: Polyethylene glycol

PGR: Plant growth regulator

RAPD: Random amplified polymorphic DNA

SCAR: Sequence-characterized amplified region

SDS: Sodium dodecyl sulphate

SIMPER: Similarity Percentages

SNP: Single nucleotide polymorphism

SSR: Simple sequence repeats

TBPT: Tree Biotechnology Programme Trust

ISSR: Inter-simple sequence repeats

TAE buffer: Tris-Acetate-EDTA buffer

TDZ: Thidiazuron or (1-Phenyl-3-(1,2,3-thiadiazol-5-yl)urea)

TE buffer: Tris-EDTA buffer

TRIS: Tris (hydroxymethyl) aminomethane

USD: United States of America dollar

UV: Ultraviolet light

v/v: Concentration expressed in volume of solute in 100 cm^3 volume of solution

w/v: Concentration expressed in mass of solute in 100 cm^3 volume of solution

WPM: McCown's woody plant medium

ABSTRACT

Melia volkensii Gürke (Meliaceae; mahogany family), is a drought-tolerant, multipurpose, fast-growing, hardwood tree species endemic to East Africa's arid and semi-arid lands. Utilization of its huge economic, ecological and biodiversity value is constrained by difficulties in propagation via seed and vegetative methods. Alternative propagation via tissue culture has received little attention. This study investigated high-frequency regeneration of the species through tissue culture and assessed the extent of morphological and genetic similarity between tissue-cultured and normal seedlings of same parent trees. Callus induction and regeneration potentials of ten explant types were investigated using Murashige and Skoog (MS) and Gamborg's B5 media containing 0.05 – 4 mg/l of the cytokinins 6-Furfurylaminopurine (Kinetin), 6-Benzyl-aminopurine (BAP) and 1-Phenyl-3-(1,2,3-thiadiazol-5-yl)urea [TDZ]). Modes of regeneration were studied morphologically and histologically. Influences of polyethylene glycol (PEG) and amino acids on regeneration response were also investigated, as were the effects of type and concentration of plant growth regulators on shoot elongation and rooting. Morphological characterization used morphometric and meristic traits while genetic characterization used random amplified polymorphic DNA (RAPD) molecular markers. Extents of morphological and genetic similarity were asessed using multivariate analysis involving principal coordinate analysis, cluster analysis, analysis of similarities (Anosim) and similarity percentages analysis (SIMPER). Cotyledons and zygotic embryos were the most suitable explants, with 100% regeneration frequency. Regeneration was indirect (callogenic) in zygotic embryos but direct (non-callogenic) in cotyledons.The best medium for callus induction and regeneration from zygotic embryos was ½ MS + 0.05mg/l TDZ. That for direct regeneration in cotyledons was ½ MS + 0.25 mg/l TDZ. Presence of 20 or 30% (m/v) polyethylene glycol-6000 in medium had beneficial effect on regeneration in zygotic embryos but not in cotyledons. In zygotic embryos, 50 or 100 mg/l of L-glutamine and L-tryptophan significantly enhanced (p<0.001) somatic embryo induction and shoot regeneration. In cotyledons, 50 or 100 mg/l of L-tryptophan and L-proline had no significant increase in the percent of explants with somatic embryos but significantly enhanced (p<0.001) the percent of explants with shoots. Optimal elongation of microshoots occurred in ½ MS + 0.1 mg/l BAP + 0.01 mg/l IAA. The best rooting occurred in ½ MS + 0.1 mg/l NAA + 0.1 mg/l IBA. Unconventional, low-cost agrochemical TDZ gave a high efficacy of regeneration (100%), outperforming both BAP (76%) and Kinetin (36%). The maximum mean yield of transplantable shoots obtained using TDZ was 12 shoots per explant. Phenotypically normal shoots of 3 to 6 cm height were obtained within 3.5 to 4 months and successfully acclimatized and transferred to soil. Regenerants had significantly reduced (p<0.05) taproot length, internode length, shoot height and number of lateral roots in comparison with normal seedlings. However, RAPD markers showed high genetic similarity between regenerants and their parents, with an overall average genetic similarity estimate of 85.32%. This indicates that observed morphological differences could be epigenetic or physiological. Cotyledons gave plants that were morphologically and genetically closer to the normal seedlings than those from zygotic embryos. These findings are the first indication of close genetic proximity between tissue-cultured *M. volkensii* and parent trees. The study also offers two new, simple and highly reproducible protocols for regeneration of *M. volkensii* plants. These could be applied in large scale *in vitro* cloning of elite *M. volkensii* mother trees for germplasm conservation and commercial forestry for supply of hardwood timber and botanical pesticides.

CHAPTER ONE: INTRODUCTION

1.1 Background

Melia volkensii Gürke (Meliaceae; mahogany family) is a drought tolerant multipurpose hardwood timber tree that is native to the East African arid and semiarid lands (ASALs). The species has vast but underutilized potential for greening and afforestation of the East African ASALs. However, combined effects of over-exploitation for its use as a source of valuable timber, firewood, animal fodder, herbal medicine and botanical pesticides, coupled with habitat fragmentation now limit its natural occurrence to sparsely distributed solitary trees or small clusters (Muok *et al.*, 2010). This has put the natural populations of the species at a high risk of disappearance.

M. volkensii is reported to be the most prized tree in the arid and semi-arid lands (ASALs) of eastern and coastal Kenya (Muchiri and Mulatya, 2005). It outperforms other tree species found in these ASALs, and has a higher profitability than two other alternative species, *Eucalyptus camaldulensis* and *Grevillea robusta*, when grown in the same environment (Wekesa *et al.,* 2012). Out of the three, the Kenya Forestry Service recommends *Melia volkensii* and *Grevillea robusta* for on-farm commercial forestry in eastern Kenya (Mfahaya *et al.,* 2013).

Among the strategies proposed for conservation of this valuable endemic species is domestication through on-farm forestry and replanting of depleted natural stands in the ASALs. However, shortage of planting material arising from difficulties in seed germination and conventional vegetative propagation greatly hampers these efforts. This problem can be overcome through use of tissue culture, a non-

controversial form of plant biotechnology that is applicable in mass propagation of such threatened plant species.

In plant tissue culture, cells, tissues or organs are surface disinfected and cultured aseptically in gelled or liquid plant culture medium containing nutrients and plant growth regulators. The technique is based on the fundamental property of totipotency where plant cells that have already committed to a particular developmental path can de-differentiate and return to the meristematic or pre-embryonic state before regenerating whole plants (Bhojwani and Razdan, 1996; Smith, 2012). Often, high frequencies of plant regeneration are attained, making the technique ideal for mass propagation of plants.

Successful plant tissue culture depends on many factors such as nutrient composition of culture media, types and concentrations of plant growth regulators, the culture environment, genotype, explant type, developmental and physiological status of the explant (Li *et al.*, 2002). Careful manipulation of these factors is required to produce optimized regeneration protocols tailored for each plant species. Work on tissue culture of *Melia volkensii* is still rudimentary. The only previous report of *in vitro* propagation of the species (Indieka *et al.*, 2007) was constrained by low frequency of shoot regeneration (30 to 60%) and low rooting of plantlets (33%).

Despite its attractiveness as a means of rapid multiplication of plants, the tissue culture process has been found to induce genetic and epigenetic changes in some somaclones (Larkin and Scowcroft, 1981; 1983; Kaeppler *et al.*, 1998; Li *et al.*, 2010; Bairu *et al.*, 2011; Wang and Wang, 2012). The most common tissue culture-related changes are chromosome re-arrangements, DNA methylation and gene mutations. Both beneficial and harmful changes have been observed. Some of the

tissue culture-induced somaclonal variations could be used in genetic improvement of the respective plants (Larkin and Scowcroft, 1981; 1983; Karp, 1995; Tang, 2005).

In view of this, there is need for detailed morphological and genetic analysis of tissue-cultured plants to assess the extent of somaclonal variation among the regenerants. One of the techniques available for detection of genetic changes in genomic DNA is random amplified polymorphic DNA (RAPD).

1.2 Problem Statement

Extension of *M. volkensii* plantations has been constrained by difficulties in propagation via both seed and vegetative methods. The main problems encountered in propagation through seed are tedious extraction of seeds from the woody endocarps where they are entrapped, mechanical and physiological dormancy, and high post-germination seedling mortality. The main problem in vegetative propagation is poor rooting of stem cuttings. The extent of the problem is indicated by inclusion of *M. volkensii* in the Royal Botanic Garden Kew list of species whose seeds are difficult to handle, store and germinate (Royal Botanic Garden website, 2012).

Germination rates attained by farmers are usually about 5% (Orwa *et al.*, 2009). Some studies have reported 60 to 80 % germination rates under controlled laboratory conditions requiring high humidity and constant temperatures of $\geq 30\ ^{o}C$ **(**Milimo, 1989a; Mwamburi *et al.*, 2005; Kimondo and Kiamba, 2005). However, attempts to replicate these results in the field have not been successful. Some efforts have produced 25-55% seed germination rates in the field, but this is still too low for mass propagation of this important tree species (Muok *et al.*, 2010; Mfahaya, 2011; Better Globe Forestry, 2012).Vegetative propagation through cuttings is also difficult

with reports of up to 33% rooting (Stewart and Blomley, 1994; Indieka and Odee, 2005).

Somaclonal variations arising during the tissue culture process can produce undesirable off-types which can also be a problem in clonal propagation of elite genotypes where a high level of morphological and genetic similarity is desired. This calls for detailed assessment of phenotypic and genetic variability or fidelity of tissue-cultured plants.

1.3 Justification

This study was an attempt to address these problems. Tissue culture may offer an alternative means of overcoming the difficulties in propagation of this multipurpose tree species. Mass propagation via tissue culture is increasingly being used in clonal multiplication of elite plant genotypes (Chaturvedi *et al.*, 2007; Hussain *et al*., 2012). Regeneration protocols produced in this study could be applied in commercial and non-commercial mass production of *M. volkensii* seedlings. This could accelerate the supply of planting material and assist in rescuing this threatened indigenous medicinal, pesticidal and timber tree from imminent extinction.

Although some success in tissue culture of *M. volkensii* has been reported in cotyledons using BAP and IAA combinations (Indieka *et al.,* 2007), there is still need for further study into the efficacy of other plant growth regulators to determine if these could result in a higher frequency of regeneration. There is also need to identify plant growth regulators that could elicit regeneration in mature zygotic embryos, which failed to regenerate in the previous study by Indieka *et al.* (2007), and to investigate the regenerative abilities of other explant types, with a view to determining the best explants for *in vitro* culture.

The study also evaluated, for the first time, the morphological and random amplified polymorphic DNA (RAPD) marker characteristics of tissue culture regenerated *M. volkensii* plants in comparison with normal seedlings. Such studies are essential since the tissue culture process is capable of inducing genetic changes in the regenerated plants. A determination of the ability of a regeneration protocol to maintain genetic fidelity is important before it is applied for mass production of planting material.

Mass cultivation of native fast-growing sources of hardwood timber such as *M. volkensii* could reduce the acute shortage of hardwood timber in East African countries which now depend on timber imports from the Democratic Republic of Congo (DRC) (Chevallier and duPreez, 2012). Several factors combine to give *M. volkensii* vast yet untapped potential to reduce Kenya's dependence on imported timber and to create sustainable livelihoods for the inhabitants of the arid and semi-arid lands (ASALs). The tree has highly valued timber, is adapted to the ASALs, it has been recommended for on-farm commercial forestry in eastern Kenya by Kenya Forestry Service (KFS) (Mfahaya *et al.,* 2013), and Kenyan ASALs have huge, under-utilized tracts of land that can be used for tree plantations.

Muok *et al.* (2010), estimated the gross income from sawn timber per ha of a 10-15 year *Melia volkensii* plantation at Ksh. 3,000,000 (then USD 35,714). This figure does not take into account additional income from off-cuts; saw dust and firewood or even potential income from pesticidal and medicinal compounds.

1.4 Hypotheses

1. High-frequency regeneration of *Melia volkensii* plants can be achieved through tissue culture.
2. *In vitro*-regenerated *Melia volkensii* plants have the same morphological and genetic characteristics as seed-derived plants.

1.5 Objectives

1.5.1 Overall Objective

To determine optimized tissue culture regeneration protocols for propagation of *Melia volkensii* and the morphological and genotypic fidelity of the regenerated plants.

1.5.2 Specific Objectives

1. To determine protocols for rapid and optimized *in vitro* regeneration and propagation of *M. volkensii* through tissue or organ culture.
2. To determine the pathway(s) of regeneration.
3. To evaluate the morphological similarity of the tissue culture derived regenerants in relation to seed-derived seedlings using morphometric and meristic characters.
4. To assess the extent of genetic similarity between the regenerants and seed-derived seedlings using RAPD molecular markers.

1.6 Research Questions

The study was based on the following research questions:

1. Which explants, ecotype, type and strength of culture media, type and concentration of plant growth substances can elicit optimal regeneration response in *M. volkensii*?

2. What is the pathway of regeneration?

3. Are morphological and RAPD characteristics of *in vitro*-raised seedlings similar to those of seed-derived seedlings?

CHAPTER TWO: REVIEW OF LITERATURE

2.1 Taxonomy, Ecological Distribution and Economic Importance of *Melia volkensii* Gürke

2.1.1 Taxonomy

The genus *Melia* belongs to the family Meliaceae (Mahogany family) and has eight species, most of which are indigenous to Asia and Australia. Three species, *M. volkensii* Gürke., *M. bambolo* Melw., and *M. azedarach* L., are found in Africa, with *M. azedarach* L. being an exotic species (Moore, 1981). Only *M. volkensii* Gürke is native to East Africa (Dale and Greenway, 1961; Mabberley, 1984).

2.1.2 Ecological Distribution

Melia volkensii is a drought-tolerant tree whose distribution extends from the arid and semi-arid lands of Ethiopia to southern Somalia, through eastern Kenya and down to northern Tanzania (Orwa *et al*., 2009**)**. It is a deciduous and laxly branched tree that can grow up to 20 m tall with a stem diameter of 25 cm. In Kenya, it is found in the dry lands of eastern and northern regions (Figure 1) where mean annual precipitation is 300 – 800 mm. Its local names include Mukau in Kamba, Embu and Mbeere; Kirumbutu in Taita, Mukowe in Taveta, Bamba in Borana, Maramarui in Samburu and Boba in Somali (ICRAF, 1992).

2.1.3 Economic Importance

M. volkensii is a fast-growing tree with multiple uses. It is a source of hardwood timber, wood fuel, animal fodder, bee forage, botanical pesticides and medicines, with use for timber being the most important. The trees can be coppiced, enabling the same tree to be cropped severally. Its timber is highly durable and termite-resistant **(**ICRAF, 1992; Orwa *et al*., 2009).

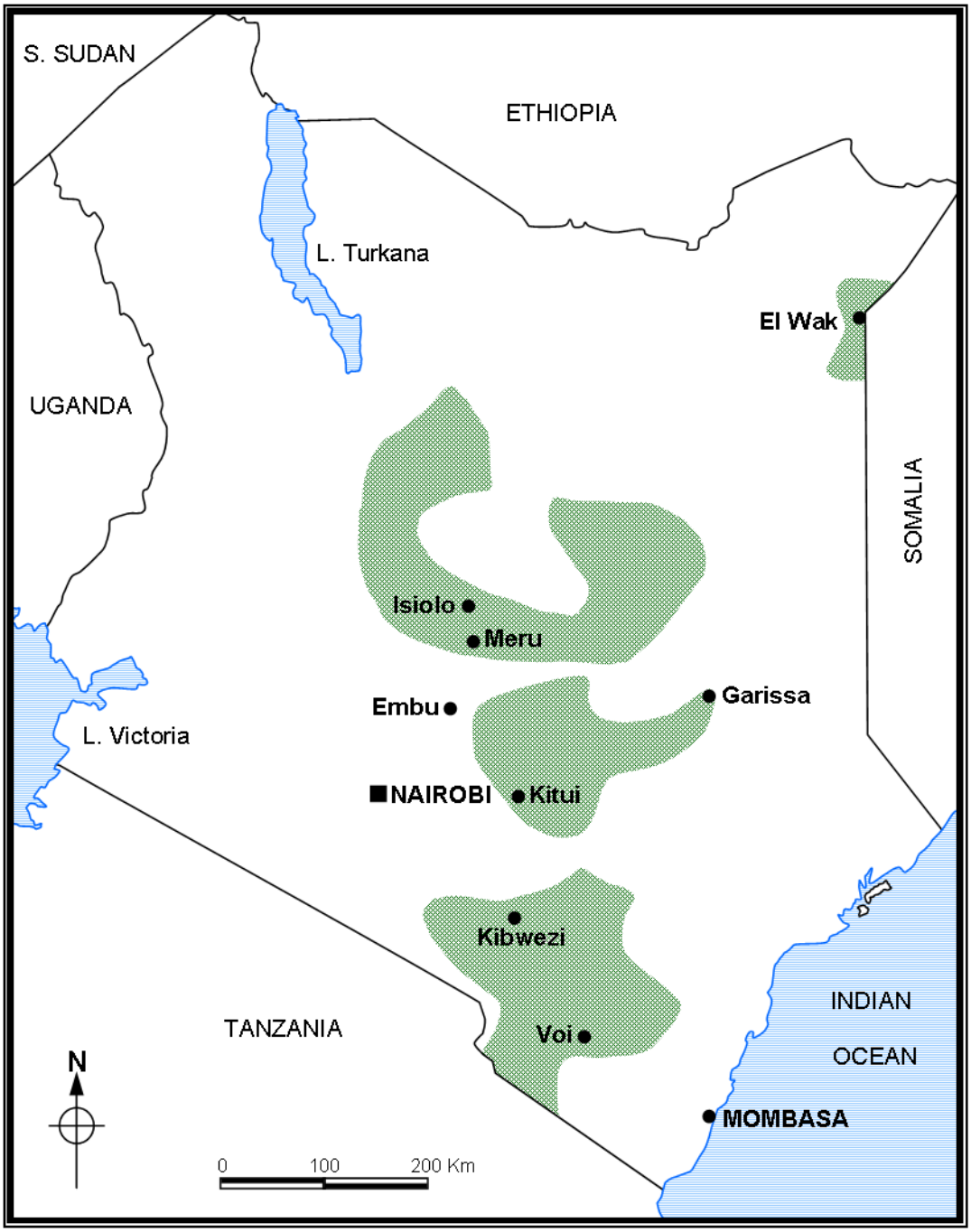


Figure 1: Distribution of Melia volkensii in Kenya:-Adapted from KEFRI

2.1.3. 1 Timber

M. volkensii is a hardwood timber tree whose properties and value are very similar to those of other highly prized hardwood species such as Camphor (*Ocotea usambarensis)* and Meru Oak *(Vitex keniensis)* (ICRAF, 1992; Muchiri and Mulatya,

2005). The tree has high quality mahogany timber (Kimondo and Kigwa, 2008; Orwa *et al.*, 2009). Its wood is easy to work and shape, making it suitable for making furniture, acoustic drums, containers and mortars (ICRAF 1992, Muok and Kyalo, 2005). The wood is also durable with termite- and decay-resistance, properties that make it an alternative source of fencing posts in place of the increasingly scarce cedar posts.

2.1.3.2 Wood Fuel

After chopping off, tree branches are left to dry in the field before being used for firewood (Orwa *et al.*, 2009). However, the tree produces poor quality charcoal and its firewood produces an unpleasant smoke. These two undesirable qualities could be useful deterrents to wanton destruction of its population especially when it is considered that felling of trees for direct use as wood fuel and processing into charcoal are a major cause of deforestation in rural parts of sub-Saharan Africa (Food and Agriculture Organization, 2006).

2.1.3.3 Anti-insect Properties

Melia volkensii and other members of the Meliaceae family such as *Azadirachta indica* A. Juss (Neem tree) and *Melia azedarach* L. (Persian Lilac tree), have been identified as potential sources of botanical pesticides, owing to the presence of limonoid triterpenes (Arnason *et al*., 1987; Champagne *et al*., 1992; Isman *et al*., 2002). The anti-insect limonoids found in *M. volkensii* fruit and seed extracts include volkensin, meliavolkenin and salannin (Rajab and Bentley, 1988; Zeng *et al*., 1995; Akhtar and Isman, 2004; Isman, 2006).

The fruit extract is toxic to a broad range of insects including dipterans, lepidopterans and coleopterans (Mwangi and Rembold, 1988; Rembold and Mwangi,

2002; Akhtar and Isman, 2004). In addition, seed extracts have potent anti-feedant and growth inhibitory properties against army worms (*Sporodeptera frugiperda* and *Sporodeptera litura*) (Rajab and Bentley, 1988; Champagne *et al.,* 1989), locusts (*Schistocerca gregaria* and *Locusta migratoria*) (Mwangi, 1982; 1997; Diop and Wilps, 1997; Kabaru and Mwangi, 2002), larvae of the cabbage looper (*Trichoplusia ni*), the lepidopteran *Pseudaletia unipuncta* (Akhtar and Isman, 2004; Akhtar *et al.,* 2008), larvae of several species of mosquitoes (*Aedes aegypti, Culex pipiens molestus, Anopheles gambiae)* (Mwangi and Rembold, 1988; Kishore *et al.,* 2011) and the green stink bug (Mitchell *et al.,* 2004). *M. volkensii* could thus be a useful source of botanical pesticides.

According to Isman (1997; 2006), global adoption and commercialization of botanical pesticides has been limited by three drawbacks: low sustainability of source material, difficulties in standardization of botanical extracts and lack of regulatory approvals. Production of botanical pesticides on a commercial scale requires that source plant biomass be available on an agricultural scale by being either abundant in nature or amenable to cultivation. Natural variations in the chemistry of the active principles of botanical pesticides across geographical zones, genotypes and seasons have also made the standardization of extracts difficult (Isman, 2006). These sustainability and standardization problems could be addressed through *in vitro* mass propagation. An efficient *in vitro* regeneration system could increase both the availability of planting material and uniformity of extracts by allowing mass cloning of elite trees.

2.1.3.4 Medicinal Properties

Actual and potential medicinal uses of *M. volkensii* are documented. The Taita people of coastal Kenya have traditionally used it as a cure for pain and body aches

(Kokwaro, 1993). The bark of the tree is boiled and very small amounts of the extract consumed as overdose can be fatal. Zeng *et al*. (1995) also reported significant cytotoxic effects of two new bioactive triterpenoids from *M. volkensii*, meliavolin and meliavolkin. These had a cytotoxicity potency similar to that of the drug adriamycin when used on human breast cancer cells. Rogers *et al*. (1998) also reported the isolation of a new compound called volkensinin from *M. volkensii*, which exhibited cytotoxicities against six human tumour cells lines.

2.2 Conservation Status of *Melia volkensii*

Melia volkensii is among the valuable semi-arid tree species in Kenya that are threatened at the population level due to over-exploitation for timber and woodfuel (Kigomo, 2001; Runo *et al.*, 2004; Hanaoka *et al.*, 2012). Habitat fragmentation and loss of the natural populations of the species are also on the increase as more of the fragile natural *Accacia-Commiphora* vegetation in which the species grows is converted into farms and settlements (Odee *et al.*, 2005). Consequently, the tree is sparsely distributed both in the natural habitat and on farms.

In eastern and coastal Kenya, selective cutting of trees has adversely affected the genetic composition of natural stands by removing good quality trees and leaving those with poor quality (Muchiri and Mulatya, 2005). Consequently, there has been a high erosion of genetic variability in the species (Kimondo and Kiamba, 2005). However, some superior trees still exist in areas that have escaped the destruction through inaccessibility, such as the national parks (Kimondo and Kigwa, 2008). Kimondo and Kiamba (2005) proposed that efforts be made to identify such trees for *ex-situ* conservation. Other workers have recommended *in situ* conservation through participatory nursery management, social forestry and domestication of the species on

farms (Milimo *et al.*, 1994; Kidundo, 1997; Kimondo and Kigwa, 2008; KEFRI Strategic Plan 2008-2012).

In a study of the genetic structure of Kenyan populations of *Melia volkensii* using RAPD markers, Runo *et al.* (2004) found a higher genetic diversity within farmland populations than in the natural populations, suggesting that domestication may indeed be a useful method of conservation of the species. The tree has also been reported to grow at a faster rate on farms than in the wild, lending more support to the strategy of conservation through domestication (Muchiri and Mulatya, 2005).

The Kenya Forestry Research Institute (KEFRI), in its efforts to promote the domestication of the species, has organized numerous farmers' extension workshops and prepared leaflets on seed collection, processing and cultivation of the species (KEFRI, 1992; Mulatya *et al.*, 2000; Mwamburi *et al.*, 2005, Muok *et al.*, 2010). In addition, the KEFRI 2008-2012 Strategic Plan highlighted the need for documentation and validation of indigenous knowledge on *M. volkensii* propagation and management techniques (KEFRI, 2008). The exploitation of indigenous knowledge in conservation of the species has also been suggested by Muniafu *et al.* (2008).

Several non-governmental organizations (NGOs) and community-based organizations (CBOs) have also joined in the creation of awareness on the importance of *Melia volkensii* and its conservation through engagement with women conservation groups. Notable among these is the NGO called Melia Project, which has partnered with the Department of Land Resource Management and Agricultural Technology, Faculty of Agriculture, University of Nairobi (Melia Project website, 2012). Its goal is to promote *Melia* propagation and field cultivation for utilization and conservation by raising and selling seedlings to farmers.

The Tree Biotechnology Programme Trust (TBPT), in partnership with KEFRI, is another initiative that is promoting conservation of local and exotic tree species for maintenance of gene pools and widening of the genetic base. However, their main focus is on *Prunus africana*, bamboo, pines, *Gmelina species* and *Eucalyptus urophylla* (TBPT Website, 2012), with *M. volkensii* receiving less attention.

From the above literature, there is need for techniques for enhanced seed germination and vegetative propagation, application of biotechnology for rapid propagation and genetic improvement, further characterization of the genetic diversity of *M. volkensii* within and between regions and strategies for *in situ* and *ex situ* conservation.

2.3 Potential of *M. volkensii* in Arid Land Agroforestry in Kenya

The role of tree species adapted to dry lands in rural economies and in combating desertification in arid and semi-arid parts of Africa is well documented (Food and Agriculture Organization, 1989; 1993; 2010). Dry land afforestation programs will rely on native species with adaptations for survival in arid and hyper-arid lands, such as *Melia volkensii*.

In the past decade, KEFRI has promoted on *M. volkensii* as dry land forestry species for eastern Kenya (KEFRI/NALEP, 2011). These promotion efforts have increased interest in the tree among peasant farmers in Kitui, Kibwezi, Mwingi and Makueni, which are some of the driest parts in eastern Kenya. However, low availability of seedlings remains a challenge. In an effort to address this problem, the KEFRI dry land forestry program at the Kitui research station is engaged in research on improved propagation and cultivation of the species, focusing on propagation via

seed (Kariuki *et al.*, 2008; Muok *et al.*, 2010). Techniques for mass propagation of the tree will lower seedling costs from the current Ksh. 100 and ensure adequate supply for rapid and large scale extension through social forestry programs.

Studies carried out in eastern and coastal Kenya have shown that *M. volkensii* outperforms most of the other dry land tree species in growth, thus demonstrating its suitability for cultivation in Kenyan ASALs (Mwamburi *et al.*, 2005; Wekesa *et al.*, 2012). According to Mutomo Maarifa Centre blogspot (2011), one successful farmer in Mutomo had attained a stable income from his ten-year old *M. volkensii* plantation. He was fetching around Ksh. 10,000 (nearly USD 101 at the time) per harvested tree, with additional income of Ksh. 100 (USD 1.18) per seedling sold.

The main objective of the integration of trees into farming systems in the ASALs is to improve the welfare of local communities through the introduction of tree species with multipurpose use: firewood, fruit, honey, gum, tannin, fodder and pharmaceutical products (Wood and Burley, 1991; Tengnas, 1994). Potential income from such trees serves as an incentive for farmers to plant the trees on their farms. Due to its multi-purpose nature, *M. volkensii* meets this criterion. Its use in arid land agroforestry systems can be an important means of achieving sustainable livelihoods in the East African ASALs.

There is also considerable interest in commercial farming of *M. volkensii* trees in Kenyan ASALs. Better Globe Forestry Ltd., (BGF), a company owned by Belgian investors, has set up tree farms at four sites (BGF website, 2012). These are at Kiambere site, a 100-ha farm on the fringes of Kiambere dam in Eastern Kenya, Sosoma site, a 60,000-ha arid and semi-arid land located along the Thika-Garissa road, Nyangoro site, a 32,000-ha ranch in Lamu County and Mukuyu farm, a 20-ha

commercial farm located in Kibwezi, along the Kibwezi river. BGF is engaged in growing of fruit trees, vegetables and multipurpose trees, mostly mukau (*Melia volkensii*), neem (*Azadirachta indica*) and *Acacia senegal* (BGF website, 2012).

2.4 Constraints in Propagation of *Melia volkensii* Through Seed

2.4.1 Seed Extraction

Melia volkensii seeds are trapped in woody endocarps of 3 to 4 mm wall thickness which is a major barrier to seed extraction (Appendix 1). Seed extraction involves tedious and delicate splitting of the endocarps along a median cross-sectional plane of weakness, often with a high rate of damage to the seeds. Farmers extract the seeds by placing endocarps, commonly referred to as stones, on a piece of wood and cutting them open using a machete. In an effort to to address the seed extraction dificulty, Kenya Forestry Research Institute (KEFRI) Kitui Regional Research centre developed a vice-like machine for use in seed extraction (Lugadiru, 2005; Muok *et al.,* 2010). However, the seed extractor is yet to be mass-produced and farmers have to rely on the few that are present at the research and field stations.

2.4.2 Seed Dormancy

In the meliaceae family, conventional propagation via seed is reported to be severely limited by recalcitrance, rapid loss of seed viability and low percentage seed germination rates, usually less than 10 % (Milimo, 1989a; Chaicharoen *et al.,* 1996; Murthy and Saxena, 1998; Shahin-uz-zaman *et al.*, 2008). Propagation of *M. volkensii* via seed has remained a challenge to foresters, farmers and other stakeholders (Kyalo, 2005). Failure to achieve mass production of seedlings has constrained the establishment of plantations and wider dissemination of *Melia volkensii* in the arid and semi-arid lands of East Africa. This has been due to difficulties in seed extraction,

complex mechanical seed dormancy, suspected physiological seed dormancy, and high post-germination mortality (Kyalo, 2005; Indieka *et al*., 2007).

M. volkensii seeds can stay dormant for 2-5 years before germinating naturally (Orwa *et al.,* 2009). In the absence of suitable pre-treatments, the rate of germination is as low as 5%. Germination can be speeded up using various pre-sowing scarification treatments. Some farmers scarify the seeds using the fast fire of dried grass or dung. Use of fire is usually achieved by spreading dry, naturally de-pulped endocarps across un-cleared land prior to burning and cultivation of the land. Such burning of the endocarps is reported to yield a germination rate of 5 -10% in 1-3 weeks after the onset of rains (Mwamburi *et al.,* 2005). Other farmers use seeds that have passed through the guts of goats where partial digestion helps to break seed coat-related mechanical dormancy (Joker, 2003; Mwamburi *et al.,* 2005). Goats egest the seeds into the litter of the goat pen, where the warmth of the decaying manure stimulates germination. The seedlings are then transplanted to the desired sites on the farm.

Research conducted by KEFRI over the last 15 to 20 years resulted in a pre-treatment method with field rates of germination of up to 64% (Muok and Kyalo, 2005). This method involves soaking the seeds in water for 6 - 12 hours at ambient temperature followed by nipping of the micropylar end and two to three longitudinal incisions through the integuments. The scarified seeds are then grown in polythene-covered mini-greenhouses called “propagators” which trap heat and moisture to provide ideal conditions for germination.

Using these pre-treatment and nursery methods, Kyalo (2005) reported germination rates of up to 80% at Tiva nursery, a KEFRI field station located in Kitui

County. However, there has been no reported replication of this success outside the station. With the same methods, the Belgian commercial tree-farming company Better Global Forestry Project Ltd only attained on-farm germination rates of 25 to 35 % (Vandenabeele, 2010). This prompted the company to seek ways of improving field germination rates to at least 50%, hence it approached the University College of Ghent in Belgium to research and develop a methodology for mass production of *M. volkensii* (Mukau) seedlings using *in vitro* techniques (Vandenabeele, 2010). The present study complements these efforts.

There is only one previous report on the usefulness of chemical scarification in breaking of mechanical seed dormancy in *M. volkensii* (Mfahaya, 2011). This study reported up to 53.33% germination using a combination of nipping of the micropylar ends and a 10 minute pre-treatment with 10% sulphuric acid. While this demonstrated the amenability of the seeds to chemical scarification, more work needs to be done for optimization of the germination rate before this approach can be adopted for commercial production of seedlings. Furthermore, the nipping process that preceded chemical scarification is laborious and time-consuming. Consequently, there is still need for stand-alone chemical scarification processes that can optimize the germination response and substitute the slow and tedious physical scarification methods currently in use.

2.5 Conventional Vegetative Propagation of *Melia volkensii*

The most commonly used method of vegetative propagation of *M. volkensii* on farms is the use of shoots that sprout from accidentally or deliberately damaged root cuttings (Joker, 2003; Orwa *et al.*, 2009). Most of the damage to roots occurs during digging of trenches on farms in the dry season ahead of the rains, for rain-water harvesting and prevention of soil erosion. However, data on the efficiency of on-farm

regeneration and propagation from root cuttings is scarce. Some data exists for root cuttings grown on station under nursery conditions, with Kyalo (2005) reporting 40% vegetative propagation success. However, many farmers do not prefer root cuttings as they result in unstable and morphologically off-type trees.

Vegetative propagation of *M. volkensii* from stem cuttings has largely remained difficult due to difficulty in rooting of the cuttings (Stewart and Blomley, 1994; Indieka *et al*., 2007). Milimo (1989b) reported 50% rooting of juvenile stem cuttings using the auxin Indole-3-butyric acid (IBA). However, Indieka and Odee (2005) attained only 33% rooting in leafy stem cuttings pre-treated with 8 g/l IBA. There is no literature on grafting or air-layering, suggesting that these methods have not been tried (Orwa *et al.*, 2009).

Despite its significance as a tree that can fulfill a variety of needs on farms in the ASALs, *M. volkensii* is yet to be vegetatively propagated at rates suitable for large scale dissemination (Indieka, 2008). Development of optimized vegetative propagation techniques may provide a cheaper means of mass propagation of this vital tree species.

2.6 *In vitro* Propagation of Selected Meliaceae

2.6.1 Shoot Organogenesis

Organogenesis is a regeneration process in which groups of cells become organized into unipolar structures having meristems from which either shoots or roots can develop (Sugiyama, 1999). Accordingly, it can be referred to as either shoot organogenesis or root organogenesis. Shoot organogenesis often results in multiple

shoot induction. *In vitro* regeneration via multiple shoot induction has been reported in several members of the meliaceae family.

2.6.1.1 Genus *Azadirachta*

In the genus *Azadirachta*, Liew and Teo (1998) reported multiple shoot induction from axillary buds of *Azadirachta excelsa* Jacobs. (Philippine mahogany or Sentang) using MS with 2 mg/l BAP and 0.5 µM NAA. Salvi *et al.* (2001) attained shoot regeneration from hypocotyl, epicotyl, cotyledonary node, root-shoot zone, cotyledon, leaves, and roots of 7-day-old seedlings of *A. indica* on MS with 8.88 µM BAP and 0.57 µM IAA. Foan and Othman (2006) reported direct organogenesis and plant regeneration from leaf explants of *Azadirachta excelsa* Jacobs on MS medium with a cytokinin combination of 8.88 µM BAP, 5.58 µM Kinetin and 6 mg/l Adenine Sulphate. Similar shoot organogenesis was attained from shoot tip and root tip explants of *Azadirachta indica* A. Juss. (Neem) on MS medium with 0.1- 0.6 mg/l BAP and 0.05 mg/l NAA (Shahin-uz-zaman *et al*., 2008). Srivastava *et al*. (2009) reported callus-mediated organogenesis from unfertilised ovaries of a mature *A. indica* tree using MS with 1 µM 2.4-D and 5 µM BAP.

2.6.1.2 Genus *Melia*

In the genus *Melia*, Handro and Floh (2001) reported shoot regeneration characterized by flower-bud formation from hypocotyl segments of *Melia azedarach* L. cultured on MS medium without plant growth regulators. Vila *et al*. (2002) attained shoot regeneration from *M. azedarach* shoot apical meristems cultured on MS medium supplemented with 0.5 mg/l BAP, 0.1 mg/l IBA and 0.1 mg/l GA_3. Vila *et al.* (2004) also attained organogenic shoot regeneration from leaf explants of *M. azedarach* on MS with 4.44 µM BAP, 0.465 µM Kinetin and 3 mg/l Adenine sulphate. In addition,

Vila *et al.* (2005) reported *M. azedarach* shoot bud regeneration on ¼ MS supplemented with 0.44 µM BAP and the development of these buds into shoots on MS containing 0.44 µM BAP, 0.46 µM Kinetin and 3.26 µM Adenine sulphate.

Sharry and Silva (2006) reported shoot organogenesis from cotyledons of *M. azedarach* using a two-step approach; callus induction on MS medium with 4.44 µM BAP plus 2.69 µM NAA and 1 g/l casein hydrolysate, followed by shoot proliferation on MS medium with 4.44 µM BAP, 200mg/l casein hydrolysate, 40 mg/l adenine and 80.5 mg/l putrescine. Husain and Anis (2009) also induced multiple shoots from nodal segments of *M. azedarach* on MS with 5 µM BAP, 0.5 µM IAA and 30 µM Adenine Sulphate. Shoot organogenesis is yet to be reported in *Melia volkensii*.

2.6.1.3 Genus *Swietenia*

Lee and Rao (1988) regenerated *Swietennia macrophylla* (Honduran mahogany) shoots from nodal segments of *S. macrophylla* on MS medium supplemented with 2 or 5 mg/l BAP. Cerdas *et al.* (1998) reported low-frequency shoot regeneration from adventitious buds of *S. macrophylla* using MS with 4-10 mg/l of the cytokinin BAP. Tacoronte *et al.* (2004) attained optimal shoot induction from axillary buds of *S. macrophylla* on ½ MS with 1.94 mg/l BAP and 0.38 mg/l NAA.

2.6.1.4 Genus *Cedrella*

Nunes *et al.* (2002) attained efficient micropropagation of *Cedrella fissilis* using nodal segments cultured on MS medium supplemented with 1.25 to 5.0 µM BAP. Pena-Ramirez *et al.* (2010) reported shoot regeneration from hypocotyl segments of the Central American cedar *Cedrela odorata* using full-strength Woody Plant medium (WPM) (Lloyd and McCown, 1980) plus half-strength MS vitamins with 20%

coconut water as the sole plant growth regulator. Diaz-Quichimbo *et al.* (2013) reported shoot regeneration from apical buds and nodal explants of white cedar (*C. montana*) on MS medium with 2.0 mg/l BAP.

2.6.1.5 Genus *Khaya*

Danthu *et al.* (2003) reported shoot multiplication from nodal explants in *Khaya senegalensis* using MS with 0.5 mg/l BAP and 0.26 µM of IBA. Okere and Adegeye, (2011) also reported shoot organogenesis from mature embryos of the West African native tree *Khaya grandifoliola* C. Dc., using MS supplemented with 1.0 mg/l BAP and 0.01 mg/l NAA.

2.6.2. Somatic Embryogenesis

Somatic embryogenesis is the process by which diploid somatic cells give rise to bipolar embryos capable of maturation and germination (Cheliak and Rogers, 1990; Adams *et al.*, 1994). *In vitro* regeneration via somatic embryogenesis has been reported in the Meliaceae.

2.6.2.1 Genus *Azadirachta*

There are many reports of somatic embryogenesis in *Azadirachta indica* A. Juss. (Neem). Shrikhande *et al.* (1993) reported indirect somatic embryogenesis from immature cotyledonary tissues of *A. indica* on MS medium with 0.5 -3.0 mg/l BAP, 0.5 mg/l IAA and 1000 mg/l casein hydrolysate. Akula *et al.* (2003) reported direct somatic embryo induction from root and nodal explants after 8 weeks on MS medium supplemented with 100 mg/l adenine sulphate. However, leaf explants cultured on the same medium required the addition of 2.3 – 4.5 µM Thidiazuron (TDZ) and 0.5 µM 2,4-D to the medium for induction of somatic embryos via callus. Rout (2005) induced somatic embryos from immature zygotic embryos on MS medium with 1.11

µM BAP and 4.52- 6.78 µM 2,4-D. Gairi and Rashid (2004) induced somatic embryos directly from immature cotyledons cultured on MS medium with 0.5 µM TDZ. Although mature cotyledons were less responsive to 0.5 µM TDZ, response improved when TDZ was increased to 1 µM, with 50% of the cotyledons forming somatic embryos (Gairi and Rashid, 2005).

Singh and Chaturvedi (2009) obtained cyclic somatic embryogenesis from immature zygotic embryos on MS medium with 0.1 µM TDZ and 4 µM ABA. Das (2011) also reported somatic embryogenesis from immature zygotic embryos on MS medium with 2,22 µM BA and 6.79-9.05 µM 2,4-D. Morimoto *et al*. (2006) induced somatic embryos in leaf and petiole explants of *Azadirachta excelsa* Jacobs on MS medium with 5 µM BAP. The embryos germinated into plantlets when transferred to ½ MS without plant growth substances.

2.6.2.2 Genus *Melia*

There are fewer reported studies of somatic embryogenesis in the genus *Melia*. Vila *et al.* (2003; 2007; 2010) reported somatic embryogenesis from immature embryos of *Melia azedarach* L. using MS supplemented with 0.45, 4.54 or 13.62 µM TDZ. Sharry and Silva (2006) obtained somatic embryos from cotyledons of *M. azedarach* L. using MS with 3 mg/l NAA, 1 mg/l BAP with or without 5 mg/l gibberellic acid (GA_3). Indieka *et al.* (2007), in the only prior study of *in vitro* propagation of *Melia volkensii* Gürke, attained regeneration via direct somatic embryogenesis in 60% of cotyledonary explants cultured on MS medium supplemented with combinations of 0.5 to 4.0 mg/l BAP and 0.2 mg/l 2,4-D. However, in the same study mature zygotic embryos of *M. volkensii* failed to regenerate on this medium.

2.6.2.3 Genus *Swietenia*

Brunetta *et al.* (2006) reported somatic embryo induction in epicotyl explants of *Swietenia macrophylla* (Big-leaf mahogany) on MS medium with 1 mg/l BAP and 0.25 mg/l NAA. Gonzalez-Rodriguez and Pena-Ramirez (2004) induced embryogenic cultures from leaf explants of *S. macrophylla* on Woody Plant medium with 13.6 µM Dicamba and 5% coconut water. Maruyama (2009) induced somatic embryos from cotyledons on MS medium containing 18.1 µM BAP followed by embryo development and maturation on half-strength medium with 4.4 µM BAP.

2.6.2.4 Genus *Cedrella*

In the genus *Cedrella*, Vila *et al.* (2009) induced somatic embryos from zygotic embryos of *Cedrela fissilis*by transferring embryogenic callus from MS with 22.5 µM 2,4-D and 0.4 µM BAP to MS medium with 4.5 µM 2,4-D. Cameron (2010) reported somatic embryogenesis from cotyledons of *C. odorata* using Driver and Kuniyuki (DKW) (1984) medium with 5µM BAP and 50 µM 2,4-D. Pena-Ramirez*et al.* (2011) attained somatic embrys by culturing immature zygotic embryos of *C. odorata* on MS medium with 13.57 µM Dicamba.

2.7 Morphological Characterization Using Morphometric and Meristic Traits

The term morphometrics refers to the methods and techniques used to quantify the body form or morphology of an animal or plant (Bookstein, 1991). It is concerned with features for which measurements of size can be obtained and is used to quantify size and shape variation within and among samples in relation to developmental changes or evolutionary trends (Rohlf and Marcus, 1993). The data is usually subjected to multivariate statistical analysis methods such as principal component

analysis and cluster analysis. These traits tend to be allometric in nature and are easily influenced by the environment.

Meristic traits are counts of the number of organs or other parts of the body (Hyam, 1997; Kellog *et al*., 2004; Lawing *et al*., 2008). Although they share some properties with morphometric characters, these traits are usually fixed early in development and are less influenced by the environment (Lawing *et al.,* 2008). They are also amenable to multivariate statistical tests.

Barciela and Vieitez (1993) applied morphometric analysis in the study of morphological variation in somatic embryos of *Camellia japonica* L. Pratta *et al*. (2000) used stem perimeter at the base, middle and top, and number of flowers per cluster as morphometric and meristic traits in the assessment of phenotypic stability of *in vitro* regenerated *Lycopersicon* plants. Principal components analysis showed significant phenotypic stability. Sheidai *et al.* (2008a) found significant variations in the length of shoots and number of leaves among tissue-cultured cotton. On their part, Vantu and Gales (2009**)** found no significant difference in the density of glandular and non-glandular leaf hairs between *in vitro* regenerated and parent chrysanthemum plants.

The list of studies where morphometric and/or meristic traits have been used on tissue cultured plants has been growing. Kumar *et al.* (2012) used shoot length, bulblet diameter and bulblet length as morphometric parameters in evaluation of phenotypic stability of micropropagated *Trigridia pavonia* (L.f) DC. Gamburg and Voinikov (2013**)** used stem length and thickness, internode length and number of internodes for morphometric and meristic comparison of somaclonal variation in *in vitro* regenerated poplar plants (*Populus berolinensis* Dipp). Similar parameters were

used by Haque and Ghosh (2013) to assess true-to-type conformity in two-year old field grown *in vitro*-regenerated *Aloe vera*.

It is evident that there has been a growing use of morphometrics and meristics in characterization of morphological variation of plants. However, there are no reports on the use of either of these traits in assessment of morphological or phenotypic variability of *in vitro* regenerated or natural populations of *Melia volkensii.* This study was an attempt to address this gap and to determine the regeneration system that can produce *in vitro* plants that are morphologically close, if not identical, to the wild seedlings.

2.8 Molecular Characterization Using DNA Markers

In recent years several types of PCR-based techniques have produced a variety of molecular markers for use in DNA fingerprinting, gene mapping, analysis of phylogenetic relationships and genetic diversity (McGregor *et al.*, 2000). Some of the most common PCR-based markers are random amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), simple sequence repeats (SSR), inter-simple sequence repeats (ISSR), sequence-characterized amplified region (SCAR) and single nucleotide polymorphism (SNP).

Random amplified polymorphic DNA (RAPD) (Welsh and McClelland, 1990; Williams *et al.,* 1990) is a simple and quick DNA fingerprinting technique that uses a single decamer primer of arbitrary nucleotide sequence and hence does not require prior knowledge about the genomic DNA being amplified. It is therefore ideal for species with scanty or no previous elucidation of their genomic DNA, such as *M. volkensii*. The observed polymorphism is due to variations in nucleotide sequences of template DNA regions randomly selected by the primers, as a result of nucleotide

insertion, deletion or substitution (McGregor *et al.*, 2000). However, RAPD markers have the disadvantage of not being able to distinguish between homozygous and heterozygous fragments, implying that they are dominant markers.

Amplified fragment length polymorphism (AFLP) (Vos *et al.,* 1995) is a DNA fingerprinting technique that uses restriction enzyme digestion followed by the selective amplification of the resulting fragments. The basis of observed polymorphism is nucleotide variations arising from mutations in the restriction site sequences or in sequences complementary to the adaptor and selector nucleotides (Matthes *et al.,* 1998). AFLPs are more reliable than RAPD but require more time and labour. They are dominant biallelic markers and can reveal variations at many loci (Vos *et al.,* 1995). Like RAPD markers, these can also be used with unknown genomic DNA.

Simple sequence repeats (SSR), also known as microsatellite polymorphism, is a technique that relies on amplification of tandemly repeated sequences that are polymorphic and widespread in plant genomes (Cregan, 1994; Morgante and Olivieri, 1993; Varshney *et al*., 2005). Polymorphism of the fragments is due to variations in fragment length arising from a variable number of repeat units. SSRs can distinguish heterozygotes, implying that they are co-dominant markers. Although they are highly reproducible and species-specific they are costly to develop and require prior information about the sequences to be amplified (Jones *et al.,* 1997). This latter aspect made them unsuitable for this study.

The inter-simple sequence repeats (ISSR) technique uses primers with a 5'-anchor to amplify simple sequence repeats without the need for prior sequence information (Zietkiewicz *et al.,* 1994; Kantety *et al.,* 1995). These markers are more

reliable than RAPD and generate more polymorphic information per primer as they target variable regions in the genome. ISSRs are also quicker and less costly than SSRs and AFLPs, but they are also dominant. However, with a longer 5'-anchor they can become codominant markers (Fisher *et al.*, 1996).

In sequence-characterized amplified region (SCAR) technique (Paran and Michelmore, 1993) the RAPD fragment which is linked to a particular trait is eluted from the gel and sequenced. The sequence is then used to design a 15-30 base pair primer for specific amplification of the locus associated with that particular trait. SCARS are highly reproducible and can be used as co-dominant markers.

In single nucleotide polymorphism (SNP) (Ota *et al.*, 2007), specific amplification of a DNA region is carried out using a forward and reverse primer. The PCR product is then sequenced. Sequences of different individuals may show variations in a single nucleotide.

Several studies on genetic diversity in plants have been carried out using one or more of these markers. RAPD markers have been used to detect somaclonal variation in the following tissue-cultured plants; the woody dicot *Picea abies* (Heinze and Schmidt, 1995), date palms (Saker *et al.*, 2000), sugarcane (Zucchi *et al.*, 2002; Pandey *et al*., 2012), the aromatic tree *Cinnamomum* species (Soulange *et al*., 2007) potato callus tissue (Ehsanpour *et al.*, 2007), cotton (Sheidai *et al.*, 2008a), banana (Sheidai *et al.*, 2008b; Abdellatif *et al.*, 2012), strawberry (Sutan *et al.*, 2009) and the medicinal herb *Withania somnifera* L., (Shetty and chandra, 2012). Lakshmanan *et al.* (2007) used both RAPD and ISSR markers to characterize somaclonal variation in long-term tissue cultures of banana.

2.9 Use of DNA Markers in *Melia volkensii*

Prior to the present study, there are only two previous reports on DNA marker-assisted analysis of genetic variation in natural populations of *M. volkensii* but no report of application of these techniques to tissue-cultured *M. volkensii* plants. In the first study, Runo *et al.* (2004), using RAPD markers in the study of genetic structure of *M. volkensii*, found that trees growing on farms had a higher genetic diversity than those in the wild habitats. They also found significant genetic differentiation between eastern and coastal populations of *M. volkensii* in Kenya. In the second study, Hanoaka *et al.* (2012) developed 15 novel microsatellite (SSR) markers for *M. volkensii*, revealing high polymorphism and heterozygosity in three natural Kenyan populations of the species.

2.10 Identified Information Gaps and Research Needs

From the literature review, the following gaps and research requirements were identified and will need to be addressed to enable massive planting of *M. volkensii*:

(i) Current mass propagation methods using seeds appear to be reliable only in research stations and specialized nurseries. There is need for simpler and more reproducible procedures for optimization of seed germination and seedling establishment rates on farms.

(ii) *M. volkensii* has been shown to be amenable to vegetative propagation via stem cuttings but erratic and low frequency of rooting (33%) of the cuttings remains the major obstacle. There is urgent need for optimized rooting treatments before propagation via stem cuttings can be commercially viable.

(iii) High susceptibility to fungi and associated seedling mortality in the nursery is another constraint. There is need for studies on identification and control of the fungal pathogens involved.

(iv) One previous study has shown *M. volkensii* to be amenable to propagation through tissue culture. That study tested cotyledon and zygotic embryo explants and attained regeneration in 60% of cotyledon explants using BAP and 2,4-D, with low frequency rooting of shoots (33%). No regeneration was attained in zygotic embryos. There is need for further studies testing a wider range of explant types and plant growth regulators for attainment of higher frequencies of shoot and root formation before adoption of this technology in mass propagation of the species.

(v) There are no previous reports on morphological characterization of *in vitro* regenerated *M. volkensii* for assessment of the extent of phenotypic conformity or non-conformity to the true-to-type condition. There is need for this knowledge as a pre-requisite to the adoption of tissue culture derived seedlings.

(vi) There are no previous reports on molecular assessment of genetic variability in tissue-cultured *M. volkensii* plants. There is need for this knowledge to inform present and future efforts on the use of tissue culture technology as a viable means of mass propagation of the species.

The present study is an attempt to address the last three of these knowledge gaps and research needs.

CHAPTER THREE: MATERIALS AND METHODS

3.1 *In Vitro* Regeneration

3.1.1 Plant Materials and Collection Sites

Collection of plant materials was done in four randomly selected agro-ecological zones of arid and semi-arid eastern Kenya (Figure 2). These were:

1. Machanga village in Mavuria ward, Mbeere South, Embu County (0^o 46.379'S, 37^o 39.308'E).

2. Mbathani village in Katulani, Mulango ward, Kitui Central, Kitui County (1^o 28.528'S, 37^o 58.052'E).

3. Kikoneni village in Nguutani ward, Mwingi West, Kitui County (1^o 3.734'S, 37^o 55.014'E).

4. Kakuuni village in Kyua, Katangi ward, Machakos County (1^o 25.310'S, 37^o 46.715'E).

The materials (fresh twigs, inflorescences and mature fruits) were collected from wild *M. volkensii* trees found in the natural vegetation and from domesticated trees growing on farms. The collection sites were geo-referenced in longitude, latitude and altitude using an Android global positioning system. The geo-coordinates were then mapped using Googlemaps (Figure 2). However, fruit supply was erratic at the Kakuuni site in Machakos and consequently, this site were excluded in the later stages of the study.

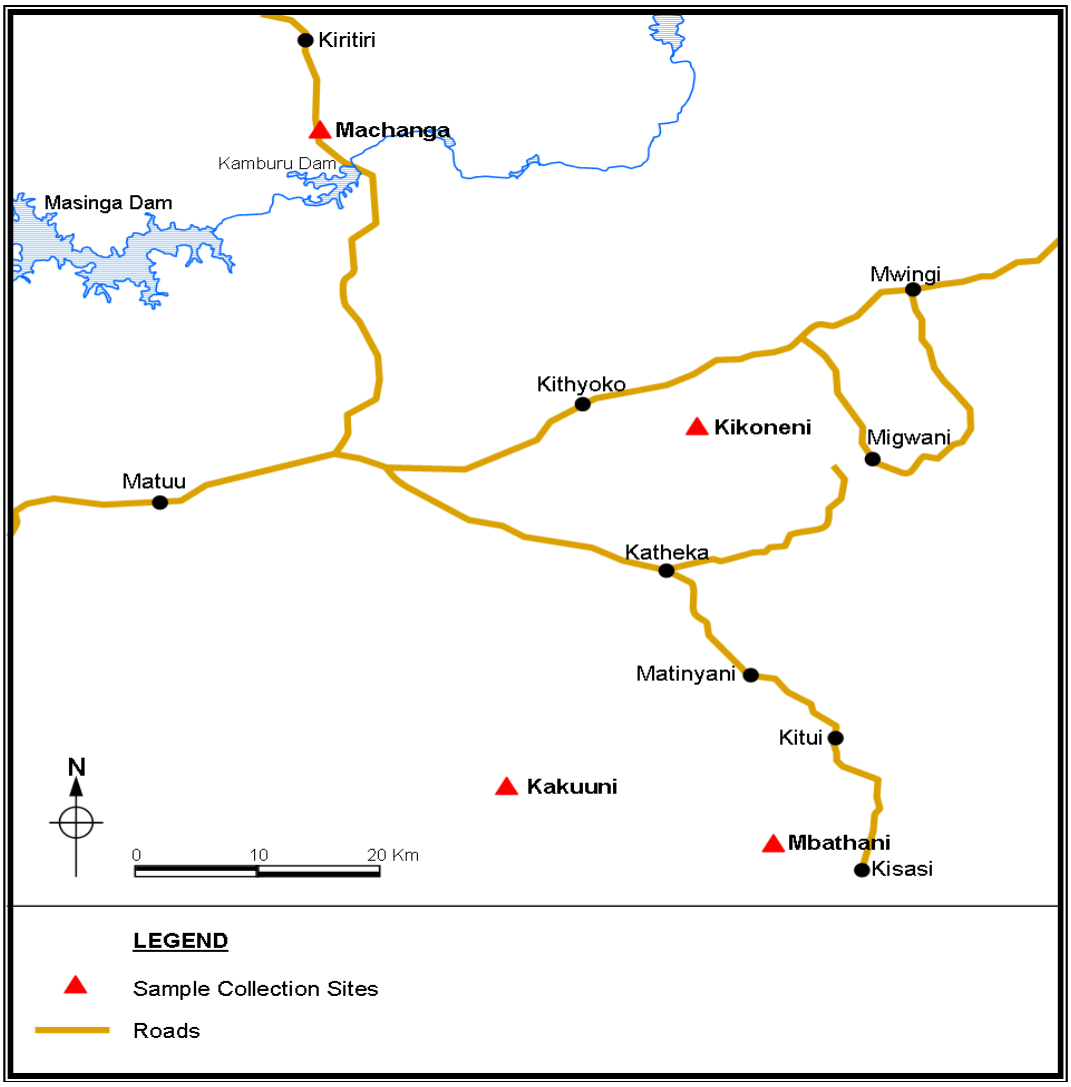


Figure 2: Map of parts of Eastern Kenya where samples were collected; the four collection sites (shown by red triangles) are Machanga in Mavuria (Mbeere South), Kikoneni in Nguutani (Mwingi West), Kakuuni in Kyua (Machakos) and Mbathani in Katulani (Kitui).

Fresh twigs were used as sources of leaves, rachis, petiole and axillary bud explants, inflorescences with opened flowers were the sources of carpel explants while immature and mature fruits were used as sources of seeds, zygotic embryos and cotyledon explants. Except for mature fruits, the freshness of samples was preserved during collection and transit to the laboratory by placing them in polybags humidified with moist cotton wool. Further short-term preservation was attained in the laboratory

by storing perishable samples at 5 ^{o}C for another four to five days, after which senescence set in and samples not used were discarded.

To avoid this problem of perishability, collection in the later stages of the study was limited to mature fruits from which seeds were extracted, for supply of mature zygotic embryo and cotyledon explants. Mature fruits were harvested in the month of August from the same parent trees for three consecutive years; 2011, 2012 and 2013. Two field indicators of fruit ripeness were used. These were a pale yellow coloration with some grey and a fruit length of approximately 4 cm, as recommended by Joker (2003) and Orwa *et al.* (2009).

3.1.2 Seed Extraction and Germination for Supply of Explants

Fruits were thrashed for de-pulping and extraction of endocarps. The stony endocarps were sun-dried for 7 days and then stored at room temperature in unsealed polybags. Endocarps were cracked open to extract seeds as and when the seeds were needed, to prevent loss of viability and fungal infections which occur quickly in extracted seeds (Joker, 2003). Each batch of seeds was only used over an 8 month period from the date of collection, to avoid significant differences in the viability of the seeds.

Some of the seeds were germinated in the laboratory using a slight modification of the method described by Muok *et al*. (2010). Seeds were scarified before germination by nipping of the micropylar ends and slitting of the seed coats. The scarified seeds were then treated for 30 minutes with 1% (m/v) Bavisitin® fungicide, followed with three rinses with sterile (autoclaved) water. This was followed by a 48-hour soak in sterile water at ambient temperature, with a change of water after 24 hours, for imbibition and to get rid of phenolics and other inhibitors. The seeds were then germinated on autoclaved moist vermiculite in 400-ml culture

bottles with foil tops. These were incubated at 29.8 ± 0.8^{o}C under cool-white fluorescent light of a 16:8 hour (light: dark) photoperiod. The resulting seedlings were also used as sources of explants.

3.1.3 Preliminary Experiments

3.1.3.1 Selection of Explants

The preliminary study tested the callogenic and regeneration potential of ten explant types namely immature fruits, excised axillary buds, carpels, mature zygotic embryos, cotyledons, root segments, epicotyls, leaves, petioles, rachis.

For immature fruits, nine different stages of development were used based on fruit length. These varied in length from 0.5 to 1.4 cm (Plate 1**)**, representing the early stages of fruit development, considering that mature fruits are usually 4cm long.

Axillary buds were excised from green, semi-hard branches of mature trees. Carpels were detached from field-collected, freshly opened flowers (Plate 2). Mature zygotic embryo and cotyledon explants were obtained from seeds extracted from the stored endocarps. Root segments, epicotyls, leaves, petioles and rachis were obtained from *in vitro*–germinated seedlings.

Plate 1: The stages of immature fruits Melia volkensii used as explants. Stage 1 is the initial stage of fruit setting as evidenced by presence of the style and stigma and initial swelling of the ovary. (Stages 1 to 5, ≤ 0.7 cm long and stages 6 to 9, ≤ 1.3 cm long; Mature fruits = 4 cm length).

Plate 2: Flowers of M. volkensii. The opened flowers (arrows) were used as sources of carpel explants

For root segments, three parts of the roots were used; root tips, midsections and upper sections in order to determine effect of position on the callogenesis and/or regeneration potential.

3.1.3.2 Aseptic Techniques for Explant Disinfection

Explants required surface-disinfection to remove epiphytic bacteria and fungi so as to prevent contamination of the plant tissue cultures.For explants obtained from the field, the effectiveness of two types of surface-disinfection agents was tested; 0.1% (m/v) mercuric chloride solution and 10 or 20 % of commercial bleach (Jik®) with sodium hypochlorite concentrations of0.39% and 0.77 % (m/v), respectively. Before disinfection, field-collected explants were washed with sterile water containing 0.5 % (v/v) Teepol® detergent for removal of dust and soil. This was followed by a 15-minute gentle shaking in either 10 or 20% (v/v) commercial bleach. Two drops of the commercial detergent Teepol® were added to each 100 ml of disinfectant solution as a wetting agent and surfactant. After sterilization, the explants were rinsed three times with sterile (autoclaved) water for removal of disinfectant. Based on the immediate effects on the explant and the responses of the explants during culture, a 15-minute treatment with 10% Jik was adopted as the explant disinfectant method for the entire study. No surface-disinfection treatment was required for explants obtained from seedlings grown aseptically *in vitro*.

3.1.3.3 Media Preparation and Sterilization

Two types of culture media were used in this study, Murashige and Skoog (1962) medium, generally referred to as MS medium and Gamborg *et al.* (1968) medium, also known as B5. Some stages of the study used ¼, ½ and ¾ -strength variants of MS medium (Appendix 2). Preliminary studies were done using media constituted in the lab by mixing of MS or B5 macro and micronutrients, iron source, vitamins (Appendix 2) supplemented with 100 mg/L myo inositol, 15, 20 or 30 g/L sucrose. pH was adjusted to 5.8 ± 0.1 using 1M HCl and 1M NaOH and 8g/L added to solidify the medium. The initial experiments were done using MS and B5 media prepared in

the lab (Appendix 2). Final tissue culture experiments were carried out using plant tissue-culture-tested MS and B5 media formulations sourced from Duchefa Biochemie B.V., Netherlands.

Both the media prepared in the lab and those obtained from Duchefa Biochemie were subjected to further manipulation through variation of the strength of the media, types and concentrations of plant growth regulators (PGRs). This also included the explorative testing of the efficacy of a low-cost agrochemical cytokinin Thidiazuron (TDZ) as a substitute for the exhorbitant plant-cell-culture tested TDZ. The agrochemical TDZ used in this study was obtained from Kingtai chemicals Ltd., China in December 2011 at the cost of US$ 160 per 700g, while the price of Sigma-Aldrich plant-tissue-culture-tested TDZ at that time was € 2,025 for 0.5g (Pers. Comm.).

Culture media were used with and without supplementation. Media supplements included sucrose (10, 20 or 30 g/l), amino acids L-glutamine, L-tryptophan, L-arginine, L-proline and L-ornithine (50 or 100 mg/l), auxins (IAA, IBA or NAA) and cytokinins (Kinetin, BAP or TDZ). Actual concentrations of plant growth regulators varied from one study to another and are detailed in the respective subsections of materials and methods. Gamborg's B5 medium was not varied in strength as it is already reported to be low in nutrient content (Dodds and Roberts, 1985).

Media were gelled by addition of agar powder at the concentration of either 8 g/l agar (Thomas Baker®) or 12 g/l agar (Oxoid®), as recommended by the manufacturers. After addition of agar, the media were topped up to the desired volume (½ or 1 litre) with distilled water and homogenized by shaking. The pH was

then adjusted to 5.8 ± 0.1 using 1M HCl or 1M NaOH before autoclaving. Measurements of pH were done using Exstick® digital pH meter.

For sterilization of media, 50 ml of dissolved respective culture media were dispensed into 400 ml honey jars and autoclaved for 15 minutes at 121^{o}C (1.06 kg cm^{-2} steam pressure). Media for cultures used in the induction stages were dispensed in foil-covered jars so that the explants could receive diffuse light. Those for shoot elongation, rooting and acclimatization stages were dispensed in jars covered with transparent tops to allow both diffuse and overhead illumination.

3.1.3.4 Inoculation and Culture of Explants

Inoculation of media was carried out in a laminar air-flow cabinet. Work surfaces were disinfected with 70% ethanol. Forceps and scalpels were immersed in 70% ethanol and sterilized by flaming till red-hot before and after each single use. Flamed forceps and scalpels were cooled by dipping the red-hot end in autoclaved water at ambient temperature.

Inoculation was done in a laminar air-flow cabinet by simply placing the aseptic explants onto the surface of the sterilised semi solid medium. The effects of abaxial or adaxial contact with the medium were investigated for cotyledons and leaves. Positional effects of explants were tested in root explants using root-tip segments, mid-root segments and upper-root segments.

Cultures were observed at two day intervals unless otherwise specified and all relevant callogenic and morphogenic events recorded. Macroscopic growth and developmental stages were imaged using Sony digital camera (Model DSC-W390). However, detailed imaging of microscopic morphogenic events was recorded at the

Kenya National Museums, using a Keyence® (Z35) VHX Digital Scanning Microscope.

3.1.3.5 Culture Conditions and Experimental Design

The cultures were then incubated in at 28 ± 2 ^{o}C, with cool white fluorescent light of approximately 60 µmol photons m^{-2} s^{-1}, which is intermediate between the 70 µmol m^{-2} s^{-1} used for *Melia volkensii* (Indieka *et al.,* 2007) and 50 µmol m^{-2} s^{-1} used for *Melia azedarach* (Sharry and Silva, 2006; Husain and Anis, 2009). A 16:8 hour light: dark cycle was used as it has been successfully used in tissue culture of *M. volkensii* (Indieka *et al*., 2007) and *M. azedarach* (Sharry and Silva, 2006; Husain and Anis, 2009). Light intensity was measured in lux using Phillip Harris digital light sensometer and the values converted into µmol photons m^{-2} s^{-1} using the formula 56 lux = 1µmol (photons) m^{-2} s^{-1}.

Culture bottles were arranged in a completely randomized design. In each experiment, three replicate bottles were inoculated with a minimum of five explants each giving 15 explants per experiment (n). Experiments were repeated three or four times, giving the total number of explants per treatment (N) as 45 or 60.

Cultures were maintained by sub-culturing to fresh medium of same or different composition at three week intervals. This interval was based on initial observations made during the trial stages which showed that senescence and necrosis begin to occur in most explants after 21 days on the same medium.

3.1.4 Callus Induction and Regeneration from Zygotic Embryo Explants

Surface-disinfected zygotic embryos extracted from mature seeds belonging to Mbeere, Kitui and Mwingi ecotypes were inoculated onto Gamborg *et al.'s* (1968) B5

medium and Murashige and Skoog's (1962) MS medium (Duchefa Biochemie B.V., Netherlands), supplemented with 20 g/l sucrose and 12 g/l Oxoid®agar. The callus induction media contained low-cost agrochemical TDZ from Kingtai Chemicals Ltd China, at the following concentrations: 0, 0.05, 0.125, 0.25, 0.5, 1, 2 and 4 mg/l. Each TDZ treatment had 3 replicate bottles per ecotype, with each bottle having 5 zygotic embryos. The experiment was repeated three times.

Cultures were observed at 3 day intervals and relevant observations recorded till day 21 when the callus induction score and callus fresh mass were determined. The callus masses were then subcultured to plant growth regulator-free (PGR-free) B5 or ½ MS media for induction and growth of somatic embryos. After 21 days on induction medium callus masses with well-developed green and leafy somatic embryos were transferred to ½ MS medium supplemented with 0.1 mg/l BAP alone or in combination with 5 or 10% coconut water for conversion of the somatic embryos into micro shoots. Well defined micro shoots were separated from the callus masses and subcultured to ½ MS medium with 0.1 or 1 mg/l BAP with or without 10% (v/v) coconut water for elongation. Shoots of ≥ 2.5 cm height were transferred to MS or ½ MS medium with 0.05, 0.1, 0.2, 0.5, 1 or 2 mg/l Indole-3-butyric acid (IBA) for rooting. Callus induction and shoot elongation medium contained 20 g/l sucrose but rooting media contained 15g/l sucrose.

3.1.5 Regeneration from Cotyledon Explants

Surface-disinfected **c**otyledon explants from Mbeere, Kitui and Mwingi ecotypes were inoculated on to callus induction medium which consistedof half-strength MS (Duchefa Biochemie B.V., Netherlands) supplemented with 0, 0.05, 0.125, 0.25, 0.5, 1.0, 2.0 and 4.0 mg/l of low-cost agrochemical Thidiazuron from Kingtai Chemicals

Ltd., China. Four PGR combinations were tested for shoot elongation: 0 PGR, 0.1 mg/l BAP alone and 0.1 mg/l BAP plus 0.01 mg/l IAA or 0.1 mg/l BAP plus 10% CW. Eight PGR combinations were tested for rooting: 0 PGR, 0.05, 0.1 or 0.2 mg/l IBA only, 0.05, 0.1 or 0.2 mg/l NAA only and 0.1 mg/l IBA + 0.1 mg/l NAA combined. Embryo induction and shoot elongation medium contained 20 g/l sucrose but rooting media contained 15g/l sucrose.

3.1.6 Hardening of Plantlets and Transfer to Pots

A four-step hardening and acclimatization method was used:

(i) Switching the plantlets from heterotrophic to autotrophic nutrition to become independent plants by transferring them from agar medium to sterile vermiculite irrigated with basal ½ MS. Plantlets with visible roots were carefully removed from the agar medium and the roots gently washed with sterile water to remove the agar medium. The plantlets were then planted in 50 ml of sterile vermiculite in 500ml capacity honey jars.

(ii) Progressive exposure to desiccation for hardening through development of leaf cuticles was done through a 2-week gradual opening of lids of the culture bottles after shoots had established in vermiculite.

(iii) Transfer to soil in pots and further acclimatization to the open atmosphere in the culture room.

(iv) Fully acclimatized plants were transferred to the greenhouse at the School of Biological Sciences, Chiromo Campus. Plants were grown individually in nursery pots filled with homogenized red soil, obtained from the botanical garden.

3.1.7 Data Analysis for *in vitro* Regeneration Studies

Data were analyzed by either one-way or two-way analysis of variance depending on the nature of the data, using GenStat 15th edition (32-bit) software. Percentage scores from count data were arcsine transformed before application of ANOVA, using the equation:

$Y = arcsine \sqrt{p}$, ……Equation 1

Where p = the proportion obtained by dividing the respective percentage value by 100, as suggested by Rangaswamy (2010). Separation of means was done using Tukey's HSD test at $p \leq 0.05$.

3.1.8 Optimized Protocol for Somatic Embryogenesis and Shoot Regeneration from Zygotic Embryos

Optimal callus induction was obtained by culturing the zygotic embryos for 21 to 25 days on ½ MS supplemented with 0.05 mg/l Thidiazuron, with a 16-hour photoperiod and 28 ± 2 ^{o}C. Callus masses were transferred to PGR-free ½ MS for another 21 to 25 days for optimal somatic embryogenesis. Callus masses having somatic embryos were then transferred to ½ MS supplemented with either 0.1 mg/l BAP alone or 0.1 mg/l BAP plus 0.01 mg/l IAA for 25 to 30 days for conversion of somatic embryos into microshoots.

After 25 to 30 days, microshoots of ≥ 1.0 cm length were cut off from the callus and the cut ends planted into fresh ½ MS supplemented with 0.1 mg/l BAP and 0.01 mg/l IAA for shoot elongation. Shoots of ≥2.5 cm length were transferred to

rooting media consisting of ½ MS with 0.1 mg/l NAA and 0.1 mg/l IAA, for 14 to 20 days. Rooted plantlets were switched from heterotrophic to autotrophic mode of nutrition by transferring them to honey jars containing sterile (autoclaved) vermiculite watered with ½ MS basal salts. This was after gentle washing of agar medium from the roots using sterile water. After two weeks in vermiculite, the plantlets were watered with ½ MS basal salts mixed with 0.2 % (m/v) urea solution.

After 30 days in vermiculite, shoots were progressively hardened to allow for development of dessication resistance through dessication-induced formation of waxy cuticles. This was achieved by partial but incremental opening of the honey jar tops at 2-day intervals followed by complete removal of the tops after 14 days. Hardened plants were then transferred to pots containing a mixture of sterile (autoclaved) soil and vermiculite in 1:1 ratio, watered with ½ MS. Potted plants were further acclimatized to the culture room conditions for 14 to 20 days before transfer to the greenhouse. The minimum duration of this protocol was 164 days, approximately 5.5 months.

3.1.9 Optimized Protocol for Direct Somatic Embryogenesis and Shoot Regeneration from Cotyledons

Optimal direct somatic embryogenesis was obtained by culturing cotyledons for 25 to 30 days on ½ MS medium supplemented with 0.25 mg/l Thidiazuron with a 16-hour photoperiod and 28 ± 2 ^{o}C. Somatic embryos formed within 10 to 14 days. Whole and half-segments of cotyledon explants having somatic embryos were transferred to ½ MS supplemented with either 0.1 mg/l BAP alone or in combination with 0.01 mg/l IAA for conversion of somatic embryos into microshoots for 25 to 30 days. Optimal elongation of microshoots was achieved by cutting the shoots from the cotyledons and

planting the cut ends in fresh medium containing ½ MS with 0.1 mg/l BAP and 0.01 mg/l IAA.

Shoots of ≥ 2.5 cm length were transferred to rooting media consisting of ½ MS with 0.1 mg/l NAA and 0.1 mg/l IAA. After 14 to 20 days, rooted plantlets were removed from agar medium and the agar washed from the roots using strile water. The plantlets were then transferred to honey jars containing sterile vermiculite watered with ½ MS basal salts forswitching from heterotrophic to autotrophic mode of nutrition. After two weeks in vermiculite, the plantlets were watered with ½ MS basal salts mixed with 0.2 % (m/v) urea solution. Urea was added to promote leaf growth and greening which are essential for photoautotrophic nutrition.

After 30 days in vermiculite, shoots were progressively hardened by partial but incremental opening of the honey jar tops at 2-day intervals followed by complete removal of the tops after 14 days. Hardened plants were then transferred to pots containing a mixture of sterile (autoclaved) soil and vermiculite in 1:1 ratio, watered with ½ MS. Potted plants were further acclimatized to the culture room conditions for 14 to 20 days before transfer to the greenhouse. The minimum duration of this protocol was 147 days, approximately 4.9 months.

3.2 Histological Study

The mode of regeneration was ascertained by histological examination. Tissues were prepared for histology using the method of Johansen (1940). Cotyledon and zygotic embryo explants at different stages of regeneration were fixed for 24 hours in Formalin-Acetic acid-Alcohol (FAA) fixative (95% ethyl alcohol:glacial acetic acid: 37% formaldehyde:water, 10:1:2:7 v/v). Each 200 ml of the fixative contained 100 ml of 95% ethanol, 70 ml distilled water, 20 ml of 37% formaldehyde and 10 ml of

concentrated glacial acetic acid. Fixed tissues were dehydrated for three hours in an alcohol series consisting of 30 minute immersion in each of 6 ethanol solutions consisting of : 80, 90, 95 and three changes of 100%.This was followed by another three hours of dehydration in absolute xylene, consisting of 1 hour each in 3 changes of absolute xylene. The dehydrated tissues were then subjected to an 8-hour wax infiltration using molten McCormick®'s paraplast tissue embedding wax maintained at 56 ^{o}C. The tissues were passed through two changes of molten wax lasting 2 hours each with a final change of 4 hours. The entire dehydration and wax infiltration process was done in an automatic tissue processor (Shandon Elliot®).

Processed tissues were immediately embedded in molten paraplast at 56 ^{o}C. The wax moulds were left overnight for cooling and setting of the wax. Set blocks were trimmed and sectioned at 5 – 8 µm thickness using a rotary microtome (R. Jung AG Heildelberg®). Ribbons with good tissue sections were stretched by placing on warm water at 30 ^{o}C and then mounted onto clean microscope slides. Surfaces of slides were pre-smeared with 1:1 mixture of egg albumin and glycerine prior to mounting of sections.

Mounted sections were de-waxed and stained using Johansen's (1940) Safranin and Fast-green staining method. Safranin stain was constituted as follows; 4g safranin dissolved in 200ml methyl cellusolve (2-methyoxyethanol), 100 ml 100% ethanol, 100ml distilled water, 4g sodium acetate and 8ml formalin. Fast-green stain was prepared as follows; 0.23g fast-green (dissolved in 150 ml methyl cellusolve), 150ml 100% ethanol and 150ml methyl salicylate. Clearing solution was 200ml methyl salicylate, 100ml 100% ethanol and 100ml xylene.

Deparaffinization was done using two 5-minute changes in xylene followed by 1-minute passage through each of 100%, 95%, 90%, 70% ethanol. This was followed by staining for 1 hour in Safranin solution followed by 5 minutes in running water, 10 seconds in 95% ethanol plus 0.5% picric acid, 10 seconds in 95% ethanol plus 4 drops of ammonium hydroxide and 10 seconds in 100% ethanol. This was followed by 10 seconds counter staining with Fast-green solution, 10 seconds in clearing solution, 10 seconds in xylene plus 4 drops of 100% ethanol and 1 minute in xylene. Histological sections were observed using a Leica ICC 50 photomicroscope and images taken at low and high power using its integrated LAS EZ digital imaging system.

3.3 Morphological Characterization of Tissue-cultured and Seed Derived Plants

3.3.1 Morphometric and Meristic Characters Used

A total of eleven morphological characters were used to characterize the extent of similarity or variance of the *in vitro* plants (regenerants) obtained using the two modes of regeneration. Comparison was also made between the morphological characteristics of the *in vitro* plants and seed-derived seedlings. The morphological characters were placed into two groups consisting of six morphometric and five meristic parameters (Table 1).

Morphometric variables were measured to the nearest mm using a ruler. To minimize on the effects of size-related differences and variations caused by the allometric nature of plant growth only shoots at the 8 – 11 leaf stage of development were used. Morphometric data was also subjected to log transformation to minimize effects of size related differences as suggested by Palmer and Strobeck (2003). Since meristic characters are usually independent of size of the organism, and tend to be fixed in early stages of growth, the raw meristic data were used in analysis.

Table 1: Traits used for morphological characterization of Melia volkensii

Morphometric Measurements		
Character	Description	Acronym
1. Shoot Height	From shoot apex to base of the stem	SH
2. Internode length	Mean length of internodes in lower half of the shoot	IL
3. Leaf Rachis Length	Mean length of rachis of three lowermost compound leaves	LRL
4. Leaflet Mid-width	Mean mid-section widths of leaflets of three lowermost leaves.	LMW
5. Leaflet Length	Mean length of leaflets of three lowermost leaves	LFL
6. Length of Taproot	From tip of taproot to base of stem	LTR
Meristic Counts		
Character	Description	Acronym
1. Number of leaf nodes per shoot	Total number of leaf nodes present on the shoot	NL
2. Number of leaflets per rachis	Mean number of leaflets per rachis, for three lowermost leaves	NLT
3. Number of lobes per leaflet	Mean number of leaf lobes or pinnules on leaflets of three lowermost leaves	NLB
4. Number of axillary bud sprouts	Mean number of axillary bud sprouts per shoot	NAB
5. Number of lateral Roots	Mean number of lateral roots per plant	NLR

3.3.2 Morphometric and Meristic Data Analysis

Morphological data was analyzed using the PAleontological STatistics (PAST) software (Hammer *et al.*, 2001). Log-transformed morphometric data and the raw meristic data were subjected to separate multivariate analysis using principal

coordinate (PCOORDA) analysis using the Gower measure of distance and 95% confidence ellipses used to indicate the extent of overlap or separation. Cluster analysis was used to generate paired-group dendrograms to show the extent of separation based on morphometric and meristic traits. Analysis of similarities (ANOSIM) was used to determine the significance of similarity or dissimilarity at $p \leq 0.05$. The Similarity Percentages (SIMPER) test was used to determine individual and cumulative contributions of each trait to the overall percentage dissimilarity, so as to reveal the most important sources of dissimilarity.

3.4 Molecular Characterization of Tissue-cultured and Seed Derived Plants

3.4.1 Optimized DNA Extraction Method

The terminal portions of shoots of *in vitro* plants and normal seedlings were cut and 0.3 g fresh mass was used for DNA extraction. The tips were ground to a fine white powder in liquid nitrogen and 2ml of extraction buffer added. The composition of the extraction buffer was as follows: 5 ml 1M Tris.Cl at pH 8, 10 ml 1M EDTA, 2.5 ml 5M NaCl, 250 µl Proteinase K (packed concentration:20 mg/ml), topped up to 50 ml using double-distilled water.

The mixtures of homogenate in extraction buffer were stored overnight at -20 ^{o}C for maximum extraction of the DNA. Further processing was initiated by thawing the frozen samples at room temperature. Genomic DNA (g-DNA) was then extracted from the homogenates using the Cetyl trimethyl ammonium bromide (CTAB) method of Doyle and Doyle (1987), with slight modifications. Thawed samples were vortexed using the Maxi-Mix1 (Type 16700) Mixer and from each vortexed sample, three replicate lots of 540 µl were removed and pipetted into separate 1.5 ml Eppendorf tubes. Then 60 µl of 10% sodium dodecyl sulphate (SDS) was added to each tube, to

give a final concentration of 1% SDS in the mixture. The mixtures were then incubated for 30 minutes at 60 ^{o}C (c.a. optimum temperature for proteinase k activity). After this, 100 µl of 5M NaCl was added and the contents mixed thoroughly to ensure a final salt concentration of 0.7M in final volume of 700 µl. This concentration of NaCl allows precipitation of polysaccharides by CTAB and avoids precipitation of nucleic acids by same agent. The precipitation of polysaccharides was achieved by adding 80 µl of CTAB/NaCl solution (2% CTAB and 0.7M Nacl) per sample followed by vortexing for thorough mixing of the contents. The vortexed mixtures were incubated for 10 minutes at 60^{o}C and left to cool to room temperature.

An equal volume (700 µl) of Chloroform : isoamyl alcohol (24: 1 v/v ratio) was added to the cooled mixtures, followed by 20 minutes centrifugation at 10,000g , 4^{o}C using the Eppendorf Centrifuge® (model 5415R). Then 700 µl of the aqueous phase was transferred into sterile Eppendorf tubes and the procedures repeated once more, from the addition of 700 µl chloroform: isoamyl alcohol to centrifugation, in order to get rid of contaminating macromolecules, which usually form a white interphase between the two layers.

Then 600 µl of the aqueous portion was pipetted from each tube and transferred to a fresh Eppendorf tube. DNA was precipitated by addition of 0.6 volume (420 µl) of ice-cold isopropanol (ca. -20 ^{o}C) and the mixtures incubated overnight at -20 ^{o}C. The DNA precipitates were pelleted by centrifuging for 20 minutes at 10,000g, at 4^{o}C. The pellets were recovered by carefully decanting the supernatant in a biosafety cabinet for air-drying. The tubes were then inverted over a paper towels for complete draining of the isopropanol.

The DNA pellets were washed with 400 µl of 70 % ethanol to remove residual CTAB and unwanted ions. The washed DNA was re-pelleted by another round of centrifugation for 20 min at 10,000 g, 4 ^{o}C. The ethanol was then discarded through careful decantation and the tubes inverted over paper towels for air-drying in the biosafety cabinet. The dried pellets were finally re-dissolved in 40 µl of Tris-EDTA (TE) buffer at pH 8 and stored at -20 ^{o}C until required.

3.4.2 Genomic DNA Confirmation and Quantification

Presence of genomic DNA was confirmed by carrying out electrophoresis of the samples for 45 minutes at 80 V (at 5.5 V/cm) in 0.8% (w/v) agarose gel, with 3 µl of 10 mM ethidium bromide as visualization stain. A 1 kilobase ladder (Bioline Hypperladder®) was used as molecular size marker. The negative samples consisted only of TE buffer with bromophenol blue loading dye. Wells were loaded with 3 µl of sample plus 3 µl of loading dye. The gels were visualized at 302 nm using a UV Transluminator and images captured using the integrated Multi Doc-it Digital Imaging System.

RNA was removed from the samples by adding 2 µl of RNAase cocktail (consisting of 500 Units/ml of RNAase A and 20,000 Units/ml of RNAase T1) to 20 µl of total nucleic acid dissolved in TE buffer (Tris-EDTA, pH 8.0). The mixtures were incubated for 30 minutes at 37 ^{o}C followed by 15 minutes at 65 ^{o}C for inactivation of the RNAase. RNA removal was confirmed by 45 minutes of electrophoresis at 80 volts (at 5.5 V/cm) using 3 µl of sample.

The RNA-free DNA was quantified in ng/µl at absorption of 260 nm (A_{260}) using a Thermoscientific Nanodrop 2000 Spectrophotometer. Base correction was done using 1 µl of TE buffer. Sample wells were loaded with 1 µl of RNA-free

genomic DNA. Readings of DNA concentration in ng/µl, together with the A_{260}/ A_{280} and A_{260}/A_{230} absorbance ratios were obtained for assessment of purity as suggested by ThermoScientific (2014).

3.4.3 Selection of RAPD Primers

RAPD markers were selected for this study for three reasons. First, results of this study could be related to the RAPD-based study of population genetic structure of natural populations of *M. volkensii* by Runo *et al* (2004). Secondly, the scarcity of genetic information on *M. volkensii* at the time of the study made it difficult to use other markers such as SSR, unlike RAPD markers which do not require any previous knowledge of the genome. Also, RAPDs can provide useful information if gene sequencing is done.

Initial screening of primers for amplification was done using eight random decamer primers, synthesized by Inqaba Biotechnical Industries (Pty) Ltd., South Africa. Each primer was tested on 10 samples of *M. volkensii* genomic DNA (gDNA) from 5 seed derived seedlings (wild type) and 5 tissue-culture generated *M. volkensii* plants. The selection of the nucleotide sequences of the primers was based on other RAPD-based studies on genetic relatedness and diversity studies in tree species belonging to three genera of the meliaceae family; *Azadirachta, Lansium* and *Xylocarpus,* (Table 2). Seven of the primers were successfully used in these other studies while the eighth primer (Mel-8) is an arbitrary sequence derived in this study.

3.4.4 Optimized PCR Amplification Method

The PCR amplification reaction was performed using the standard PCR-RAPD procedure, with some modifications. PCR reaction mixture and thermal cycler conditions were optimized by varying the type of DNA polymerase (Dream Taq® and

Taq® polymerase from ThermoScientific and MyTaq® polymerase from Bioline) concentration of Taq® polymerase (0.75, 1.0, 1.25 and 2.5 units per 25 µl reaction), strength of PCR buffer (x1 or x1.5), concentration of primer (25 or 50 pmoles per 25 µl reaction), annealing temperature (34, 35, 36, 37 and 38 ^{o}C) and extension time (1 or 2 minutes).

Table 2: Key features of the primers used in the RAPD study

Primer code name	Nucleotide sequence 5' 3'	GC %	Related species where similar primer was used
1. MEL-1	GGC ACT GAG G	70	Neem (*Azadirachta indica* A. Juss), meliaceae (da Silva *et al*., 2013)
2. MEL-2	GTA GAC CCG T	60	
3. MEL-3	CAA TCG CCG T	60	
4. MEL-4	CCT TGA CGC A	60	*Lansium domesticum* Corr., meliaceae (Song *et al*., 2000)
5. MEL-5	TGC CGA GCT G	70	
6. MEL-6	GGC ATG ACC T	60	Genus *Xylocarpus:X. granatum* Koen.,*X. moluccensis* Lamk. and *X. mekongensis* Pierre, meliaceae (Pawar *et al*., 2013)
7. MEL-7	TGG CGC AGT G	70	
8. MEL-8	GGC TAT CCG A	60	Arbitrary sequence (present author)

The optimum amplification reaction mixture per 25 µl reaction was as follows: 5 µl of 5X MyTaq® reaction buffer (Bioline), 0.5 µl MyTaq® DNA polymerase (Bioline), 5 µl of 10 mM primer, 3.0 µl of gDNA in TE buffer (after diluting the gDNA-TE mixture to half-strength using equal volume of PCR water) and 11.5 µl of nuclease-free PCR water (Sigma-Aldrich). In terms of final concentration, each of the 25 µl reactions had 1 mM dNTPs and 3 mM $MgCl_2$ (contained in the 1X final

concentration of MyTaq® buffer), 50 pmoles of primer and 2.5 units of MyTaq® DNA polymerase.

The polymerase chain reaction was carried out in a PTC-100 thermal cycler (Programmable Thermal Controller- MJ Research Inc., USA). Optimum PCR for all the eight primers used was attained with the following conditions: 5 minutes initial denaturation at 95 ^{o}C, followed by 35 cycles consisting of, 30 seconds denaturation at 94 ^{o}C, 45 seconds annealing at 35 ^{o}C, 60 seconds extension at 72 ^{o}C, and a single final extension of 5 minutes at 72 ^{o}C.

3.4.5 Gel Electrophoresis and Imaging

Electrophoresis of genomic DNA was done for 45 minutes in 0.8% (w/v) agarose gel (dissolved in 1x TAE buffer) at 80 V (at 5.5 V/cm). Visualization stain was 3 µl of 10 mM ethidium bromide added to the gel before setting. A 1 kb ladder (Bioline Hyperladder®), with size markers from 200 to 10,037 base pairs, was used as molecular size marker. Wells were loaded with 3 µl of sample + 3 µl of 6x bromophenol blue loading dye. Negative samples for genomic DNA gel runs consisted only of TE buffer with loading dye.

PCR products were also electrophoresed for 45 minutes at 80 V (5.5 volts/cm) but in 1.5 % (m/v) agarose gel (dissolved in 1x TAE buffer). The gels were also visualized using ethidium bromide as described for genomic DNA. Each well was loaded with a mixture of 7 µl of PCR product and 3 µl of 6x bromophenol loading dye containing a tracking dye. All gels were visualized at 302nm using a UV Transilluminator and images captured using the integrated Multi Doc-it Digital Imaging System.

3.4.6 RAPD Data Scoring and Analysis

Only clearly resolved bands were scored. The RAPD bands/markers were scored for each lane using 1 for presence and 0 for absence of a band. Lanes represented the DNA samples of different individuals. The resultant binary matrix of 1 and 0 scores was analyzed using the Popgene Version 1.32 Population Genetic Analysis software (Yeh *et al.*, 2000). Six summary genic variation statistics were obtained from this analysis: observed number of alleles (N_a); effective number of alleles (N_e); Nei's (1973) gene diversity index (H); Shannon's information index (I); number of polymorphic loci and percentage of polymorphic loci.

These Popgene-generated genic variation values were subjected to four different multivariate analyses using PAST software version 2.17c; Principal coordinates analysis (PCOORDA), cluster analysis, one-way Analysis of Similarity (Anosim) and Similarity Percentage (SIMPER) analysis (Hammer *et al.*, 2001). PCOORDA scatterplots and 95% confidence ellipses were drawn using the Gower measure of distance, which is applicable to mixed type data (continuous and ordinal)(Hammer *et al.*, 2001). Clustering was performed using the paired-group algorithm and Gower measure of distance, with 1000 bootstrap replications.

Anosim and SIMPER were done using Bray-Curtis measure of similarity, which is the default measure. Anosim produced the R statistic and pair-wise percentages of dissimilarity between groups based on 10,000 permutations. The R statistic measures the overall dissimilarity between groups. R values closer to zero indicate no dissimilarity (significant similarity) while those closer to 1 indicate significant dissimilarity between the groups. The SIMPER test gave individual and

cumulative contributions of the characters to the overall average percentage dissimilarity.

CHAPTER FOUR: RESULTS

4.1. *In Vitro* Regeneration

4.1.1 Responses of Tissues and Organs to *in vitro* Culture for Explant Selection

On the basis of their responses to the plant culture media tested, explants were categorized into three distinct groups: - non-responsive, callogenic and non-callogenic explants.

4.1.1.1 Non-responsive Explants

Two types of explants were non-responsive. These were axillary buds excised from semi-hard stem cuttings and longitudinal sections of nine stages of immature fruits. Both types of explants showed 100% necrosis of explants after 15 days on MS media supplemented with 0, 0.5, 1.0, 2.0, 4.0, 8.0 mg/l BAP + 0.4 mg/l 2,4-D. Total explants used were 98 for axillary buds and 137 for immature fruits.

There was no swelling or callusing of fruit explants. Instead, the pericarps and mesocarps of the explants turned from green to pale green and then yellow within 10 days of culture. Endocarps turned light brown (Plate 3). No morphogenic events were

Plate 3:Non-responsive Melia volkensii immature fruit segment explants after 15 days on MS medium with 1 mg/l BAP and 0.4 mg/l 2,4-D.

observed and the cultures were discarded after 30 days. Similar observations were made in excised axillary buds.

4.1.1.2 Responsive Explants

Out of the 10 explant types tested 6 types, namely carpels, leaves, petioles/rachis, mature zygotic embryos, root segments and seedling epicotyls formed callus and were thus categorized as callogenic (Table 3). These explants callused easily on a variety of test media including MS medium supplemented with 0 – 8 mg/l BAP combined with 0.05 - 0.4 mg/l of either 2,4-D or NAA; MS medium supplemented with 0 – 1 mg/l TDZ with or without 0.2 mg/l 2,4-D, and B5 medium supplemented with 2 mg/l Kinetin. Table 3 summarizes the media and PGR combinations that elicited good callus induction responses in each type of explant.

The other two types of explants, which are the green cotyledons detached from seedlings and the cream cotyledons obtained from mature seeds, were also responsive to *in vitro* culture. However, they did not callus at all. Instead, they exhibited direct morphogenesis which was preceded by marked greening and expansion of the explants.

4.1.1.2.1 Callus Induction in Carpels

In the experiment involving use of carpels, swelling of ovaries was evident within 4-5 days in culture on MS medium supplemented with 1 mg/l BAP and 0.4 mg/l 2, 4-D. The swollen ovaries then ruptured within 8 to 10 days, forming a compact white or cream callus with an adjoining translucent callus on the peduncle side of the ovaries (Plate 4A, B). In few of the carpel explants, the callusing spread to the style (Plate 4C).

Table 3: Melia volkensii explants, test media and plant growth regulators that supported callogenesis

Explant& (Media)	**Callus induction medium PGR/mg/l**					**Total explants**	**% of explants with callus (Mean ± S.E)**
	BAP	**NAA**	**2,4-D**	**KN**	**TDZ**		
1.Carpels (MS)	1.0	-	0.4	-	-	66	68.78 ± 5.74[a]
2.Leaves (B5)	- or 0.5	- 0.05	- -	2.0 -	- -	47 76	66.67 ± 3.85[a] 81.80 ± 6.85[b]
3.Petioles/ Rachis. (MS)	-	-	0.2	-	1.0	138	70.63 ± 8.64[b]
4.Zygotic Embryos (B5)	-	-	-	-	0.05	60	100[c]
5.Root Segments (MS)	-	-	-	-	0.05	60	100[c]
6.Epicotyls (MS)	-	-	-	-	-	50	100[c]

Values with same superscript in the last column are not significantly different using Tukey's HSD test at $p \leq 0.05$.

Plate 4:Callogenesis in Melia volkensii carpel explants after 10 days on MS medium + 1 mg/l BAP + 0.4 mg/l 2, 4-D + 20 g/l sucrose + 2 g/l yeast extract;(A) Swelling of ovary (B and C) Callus formed Scale bars: (a)= 1.53 mm (b) = 3.86 mm, (c) = 2.00 mm.

4.1.1.2.2 Callus Induction in Leaves

Leaf explants cultured on B5 medium supplemented with either 2 mg/l Kinetin or with 0.5 mg/l BAP plus 0.05 mg/l NAA started to callus from day 7. Callus induction started as green and pale yellow nodular growths on the leaf lamina, mid-ribs and veins (Plate 5). The whole leaf was callused after 20 days of culture. Leaf callus was generally compact with mixtures of green and pale yellow calluses.

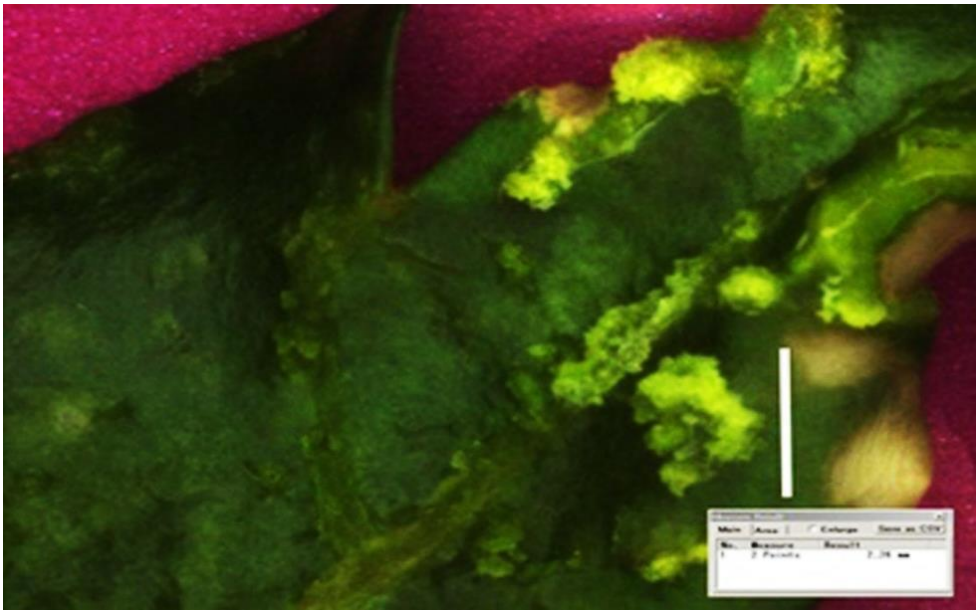


Plate 5: Callusing of Melia volkensii leaves after 10 days on B5 medium + 2mg/l kinetin. Callus formed on the blades and veins.Vertical scale bar = 2.26 mm.

4.1.1.2.3 Callus Induction in Petioles and Rachis

Petiole and rachis explants responded within 5 – 8 days of culture on medium with MS with 1.0 mg/l TDZ plus 0.2 mg/l 2,4-D and MS with 0.5 – 8 mg/l BAP plus 0.4 mg/l 2,4-D. Explants formed slight swellings at the petiole-rachis nodes (Plate 6), with very low vigour of callusing. They turned light brown within 14 days of culture.

Plate 6:Callus induction in Melia volkensii leaf rachis after 10 days on MS + 1mg/l TDZ + 0.2 mg/l 2,4-D. Callusing occurred at the petiole junction. Scale bar = 1mm.

4.1.1.2.4 Callus Induction in Epicotyl Explants

Epicotyl segments callused readily on PGR-free MS and ½ MS medium but failed to callus and turned brown (necrotic) in presence of low (0.05 mg/l) to moderate (2.0 mg/l) concentrations of TDZ (Plate 7). Callusing started within 4-5 days of culture on PGR-free media. Some of the calluses formed roots within 15 – 20 days. The frequency of rooting recorded on day 20 in the callused epicotyl explants was 56.67 ± 3.33 %.

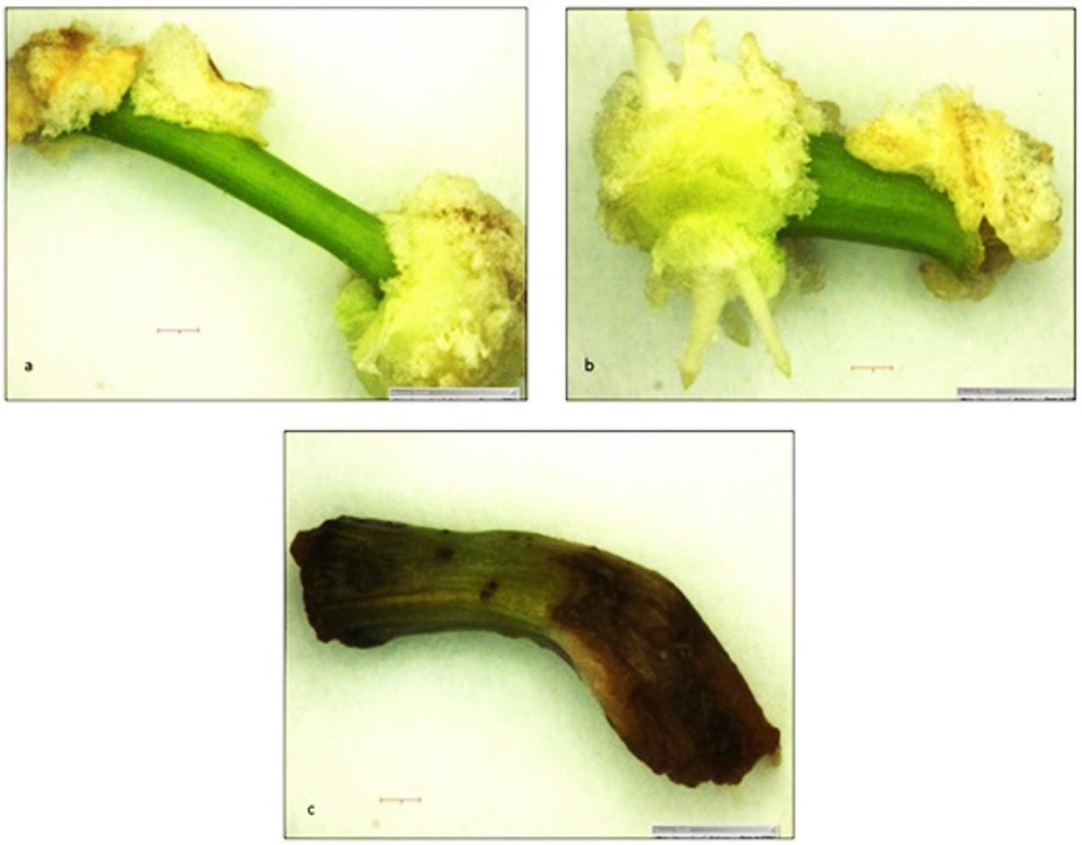


Plate 7: Callus and root induction in Melia volkensii epicotyl explants showing (a): callusing at the ends after 15 dayson ½ MS + 0 PGR medium, (b): root regeneration from callused basal end of explant after 20 days on ½ MS + 0 PGR medium, and (c): failure of callus induction and necrosis after 20 days on ½ MS + 0.05 mg/l TDZ induction medium ((Scale bars = 1mm).

4.1.1.2.5 Callus Induction in Zygotic Embryos

Zygotic embryos started to callus after days 3 to 4 of culture, exhibiting fast growth on ½ MS and B5 medium with or without plant growth regulators. Calluses of zygotic embryos could be maintained on induction media for up to 35 days without browning and onset of necrosis. In presence of low levels of TDZ (0.05 mg/l), some of the zygotic embryo calluses recorded some regeneration by developing tiny green globular structures which gave rise to microshoots (Plate 8).

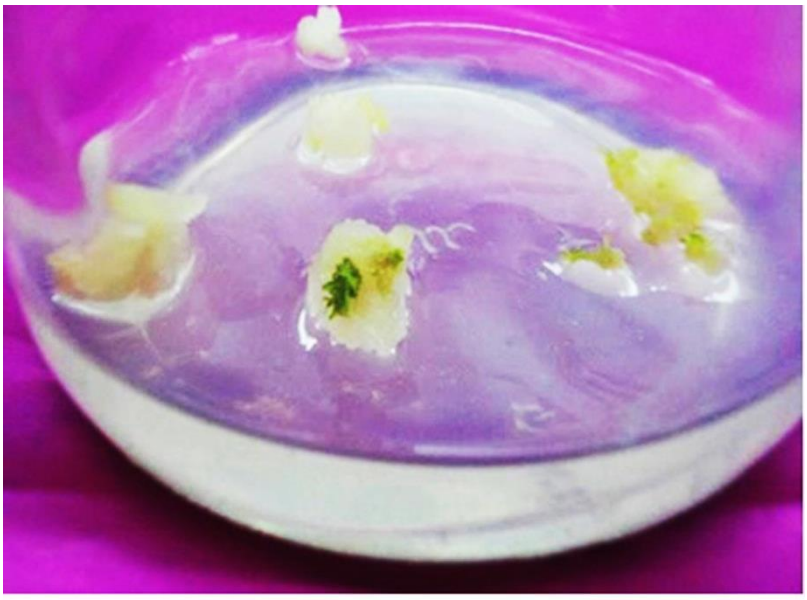


Plate 8: Callogenesis and initial regeneration in Melia volkensii zygotic embryos after 18 days on ½ MS + 0.05 mg/l TDZ.

4.1.1.2.6 Callus Induction in Root Segments

In root segment explants, callusing started to occur within 5 to 8 days of culture in MS and ½ MS with 0 or 0.05mg/l TDZ PGR-free media (Plate 9) and in media containing 0.05 – 2 mg/l TDZ, BAP or Kinetin. However, the explants on media with TDZ turned brown at the cut ends of the root segments before the browning spread to the rest of the explant and to the callus itself within 15 days in culture. Calli of root segments showed browning in three-quarters to whole of explant by day 20 of culture.

Plate 9: Callogenesis in Melia volkensii root segments after 12 days on ½ MS + 0.05 mg/l TDZ

In roots, callogenic potential varied along the root both in PGR-free and medium containing TDZ. Segments obtained from the middle part of the root were the most responsive followed by those from the upper part of the root (Plate 10). Root tips showed the least callogenic potential. Some morphogenesis, in form of numerous nodular growths, was observed on surfaces of the callus in contact with the TDZ-containing media (Plate 10). There were no similar morphogenic events in the control media.

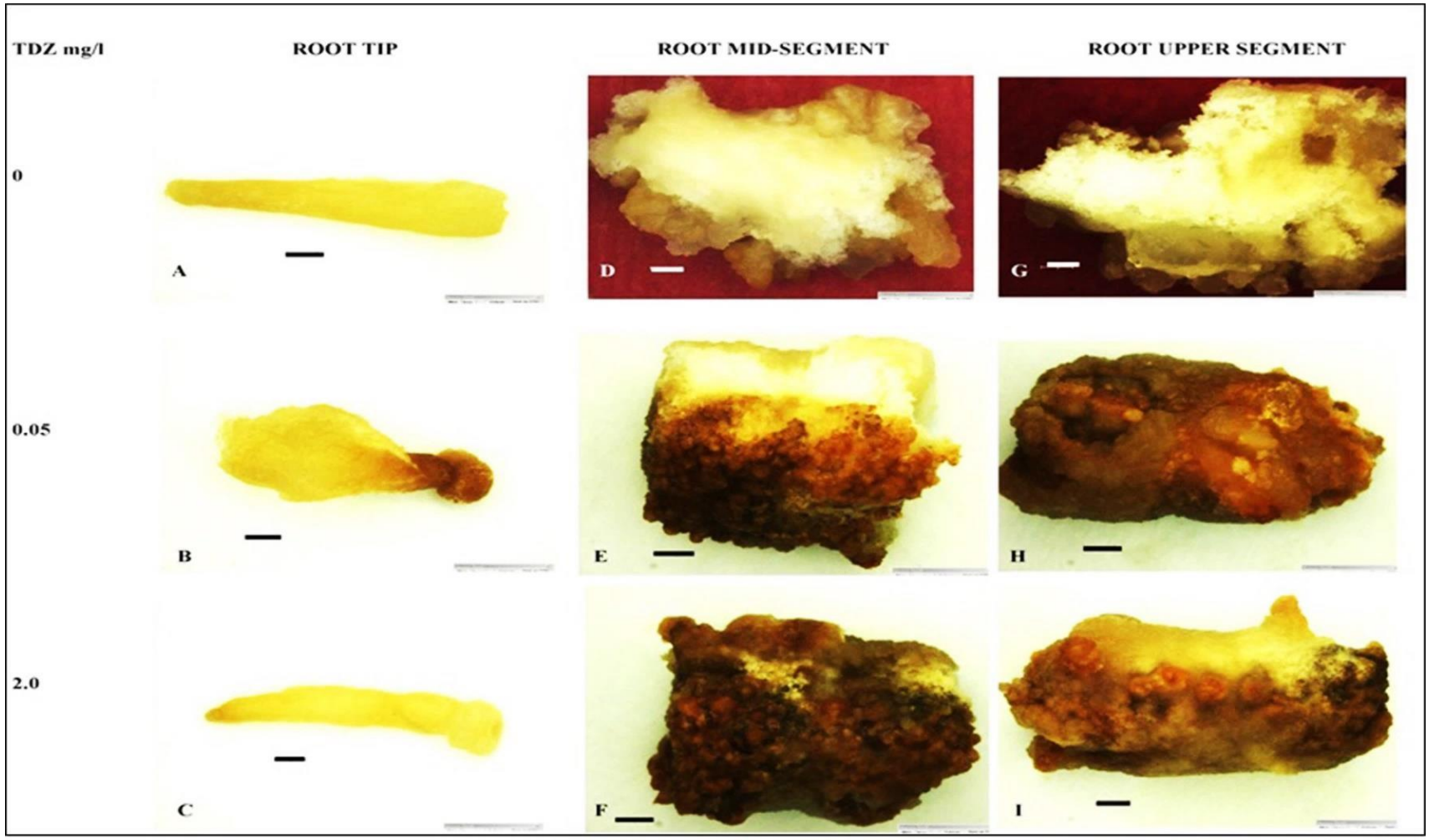


Plate 10: Callogenic and morphogenic effects of TDZ in different parts of Melia volkensii root after 20 days on ½ MS + varying TDZ. (Scale = 1mm). Root tip segments (A,B,C),; Mid-root segments (D,E,F) and Upper root segments (G,H,I) on 0, 0.05 and 2.0 mg/l TDZ respectively.

4.1.1.2.7 Non-callogenic Explants

Responses of cotyledon explants differed completely from those of the other explant types. The two types of cotyledons used, green cotyledons excised from young seedlings and the cream cotyledons of mature seeds, exhibited direct regeneration without an intervening callus phase. They were non-callogenic but highly morphogenic through direct regeneration of shoots. For both types of cotyledons, 100% regeneration was attained in ½ MS, MS and B5 media supplemented with 0.05 – 4 mg/l of the cytokinins BAP, Kinetin or TDZ (Table 4).

Table 4: Explants, test media and PGR combinations that supported direct regeneration of Melia volkensii

Explant	**Induction medium**	**Total explants**	**% of explants with direct regeneration (Mean ± S.E)**
Green seedling cotyledons	½ MS + 2 - 4mg/l TDZ	90	100
Cream seed cotyledons	½ MS + 1 – 4 mg/l TDZ	90	100

Morphogenesis was preceded by deepening of the green colour in the case of the green seedling cotyledons and change from cream to green colour for the mature seed cotyledons. These colour changes occurred within 3 – 7 days of culture and were followed by the formation of numerous tiny green nodular growths from which tiny shoots regenerated (Plate 11). Green cotyledons detached from seedlings showed regeneration mostly at the proximal ends of the explants (Plate $11A_3$). On their part, mature seed cotyledons had profuse shoot regeneration over the whole explant was observed in the mature cotyledons obtained from seeds (Plate $11B_2$ & B_3).

Plate 11: Comparison of patterns of shoot regeneration in Melia volkensii seedling cotyledons and seed cotyledons; A1-A3: Shoot regeneration in seedling cotyledons; ***Arrow*** *in A_3 shows regeneration at proximal end of cotyledon after 11 days on ½ MS + 4mg/l TDZ medium. B1 - B3: Shoot regeneration in seed cotyledons after 3 (B_1), 10(B_2) and 21(B_3) days on ½ MS + 2mg/l TDZ.*

4.1.2 Responses of explants to BAP, Kinetin and Thidiazuron

4.1.2.1 Zygotic Embryos

Mature zygotic embryos callused in all four concentrations (0.05, 0.25, 1 and 4 mg/l) of the three cytokinins used, namely BAP, Kinetin and TDZ (Figure 3A). Explants started to callus within 4-6 days of culture. Calluses produced by TDZ were generally larger than those obtained using either BAP or Kinetin. However, callus fresh mass did not differ significantly between the three cytokinins or cytokinin concentrations 0.05 to 1 mg/l. Fresh mass was only significantly different ($p < 0.001$) only at 4mg/l BAP, Kinetin or TDZ (Figure 3A).

Cytokinin type and concentration had significant effects on the percentage of zygotic embryo calli forming somatic embryos while still on the callus induction medium (Figure 3B). In general, TDZ elicited the highest percentage induction of somatic embryos, concurrent with callus formation, at all concentrations used. However, at 4mg/l all three cytokinins recorded drastic inhibition of somatic embryo induction. Kinetin failed to elicit any somatic embryos at 0.05 and 4 mg/l concentration.

Interaction between cytokinins type and concentration was insignificant for both fresh mass per callus ($p = 0.323$) and percentage of callus forming somatic embryos ($p = 0.495$) on the callus induction medium (Table 5). Lower concentrations of the three cytokinins (0.05 and 0.25 mg/l) induced some root morphogenesis while higher concentrations (1 and 4 mg/l) completely inhibited rooting. At 0.05mg/l, Kinetin caused more rooting of calluses than either BAP or Kinetin. However, interaction between cytokinins type and concentration was insignificant ($p = 0.225$) [Table 5].

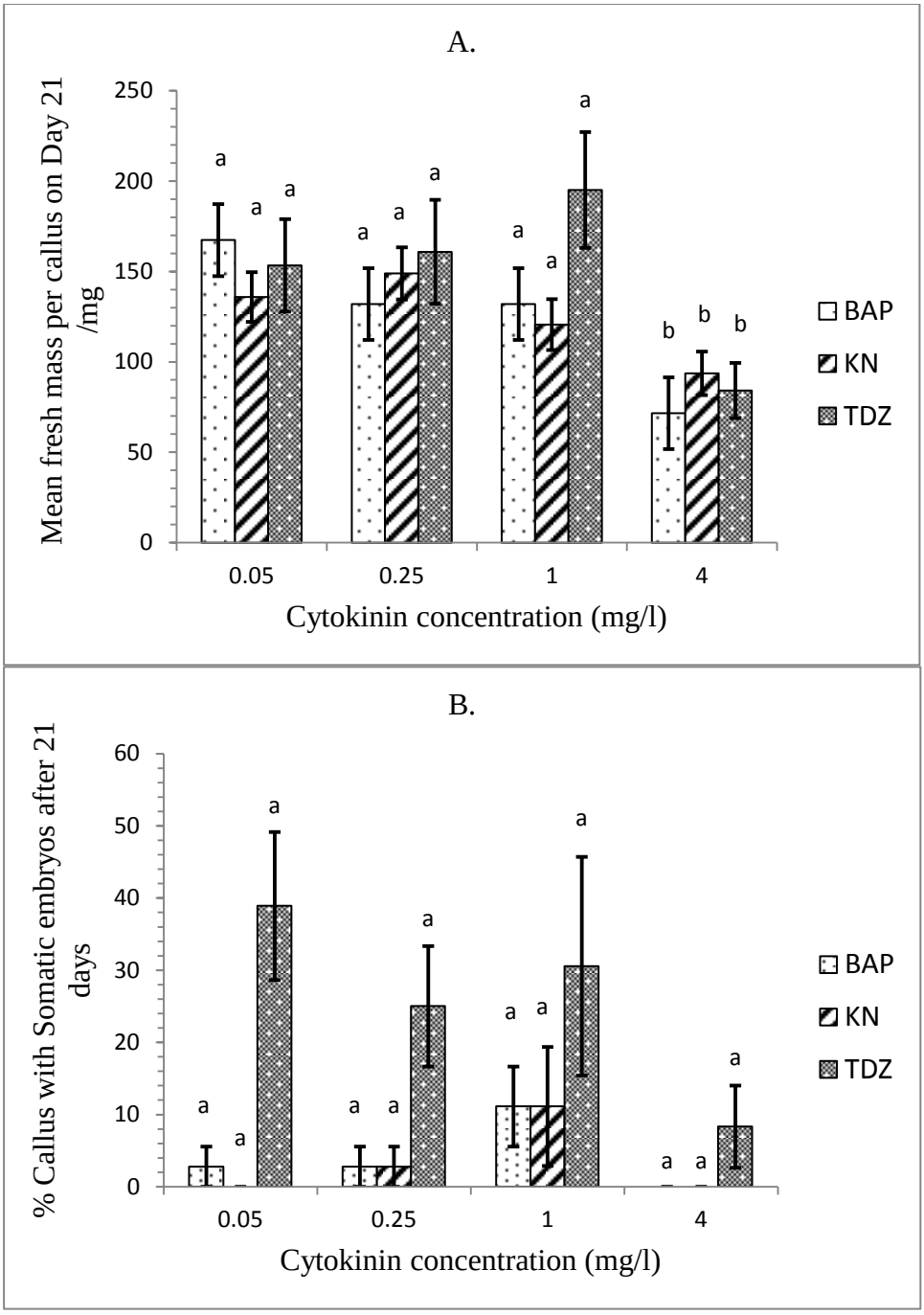


Figure 3: Effects of cytokinin type and concentration on callus fresh mass and somatic embryo induction in Melia volkensii zygotic embryos; A: callus fresh mass, B: percentage of callus having somatic embryos after 21 days on induction medium. Bars with same letters are not significantly different using Tukey's HSD test at $p \leq 0.05$.

Table 5:Two-way Anova output for effects of type and concentration of cytokinin on callus induction, somatic embryogenesis and rooting in Melia volkensii zygotic embryos

Fresh mass per zygotic embryo callus/mg				
Source of variation	d.f	F-value	Significance (*p* value)	%CV
Cytokinin concentration	(3,57)	8.31	<0.001	33.73
Cytokinin type	(2,57)	3.08	0.054 ns	
Concentration x type	(6,57)	1.19	0.323 ns	
% of Zygotic embryo callus with somatic embryos				
Source of variation	d.f.	F-value	Significance (*p* value)	%CV
Cytokinin concentration	(3,57)	1.01	0.395 ns	4.54
Cytokinin type	(2,57)	1.06	0.354 ns	
Concentration x type	(6,57)	0.91	0.495 ns	
% of zygotic embryo callus with root induction				
Source of variation	d.f	F-value	Significance (*p*value)	%CV
Cytokinin concentration	(3,57)	4.73	0.005	202.67
Cytokinin type	(2,57)	1.92	0.005	
Concentration x type	(6,57)	1.41	0.225	

Data taken after 21 days on induction medium containing 0.05mg/l of BAP, KN or TDZ (ns =not significant).

4.1.2.2 Cotyledons

In cotyledon explants, TDZ outperformed BAP and Kinetin both in fresh mass per explant (Figure 4A) and the percentage of explants forming somatic embryos (Figure 4B, Plates 12 & 13). The highest fresh mass produced by TDZ (956.4 mg per explant) was achieved at 0.25 mg/l concentration whilst those for the other two

cytokinins were attained at 4 mg/l concentration, with the respective fresh mass per explant being 837 mg for BAP and 757.6 mg for Kinetin (Figure 4A).

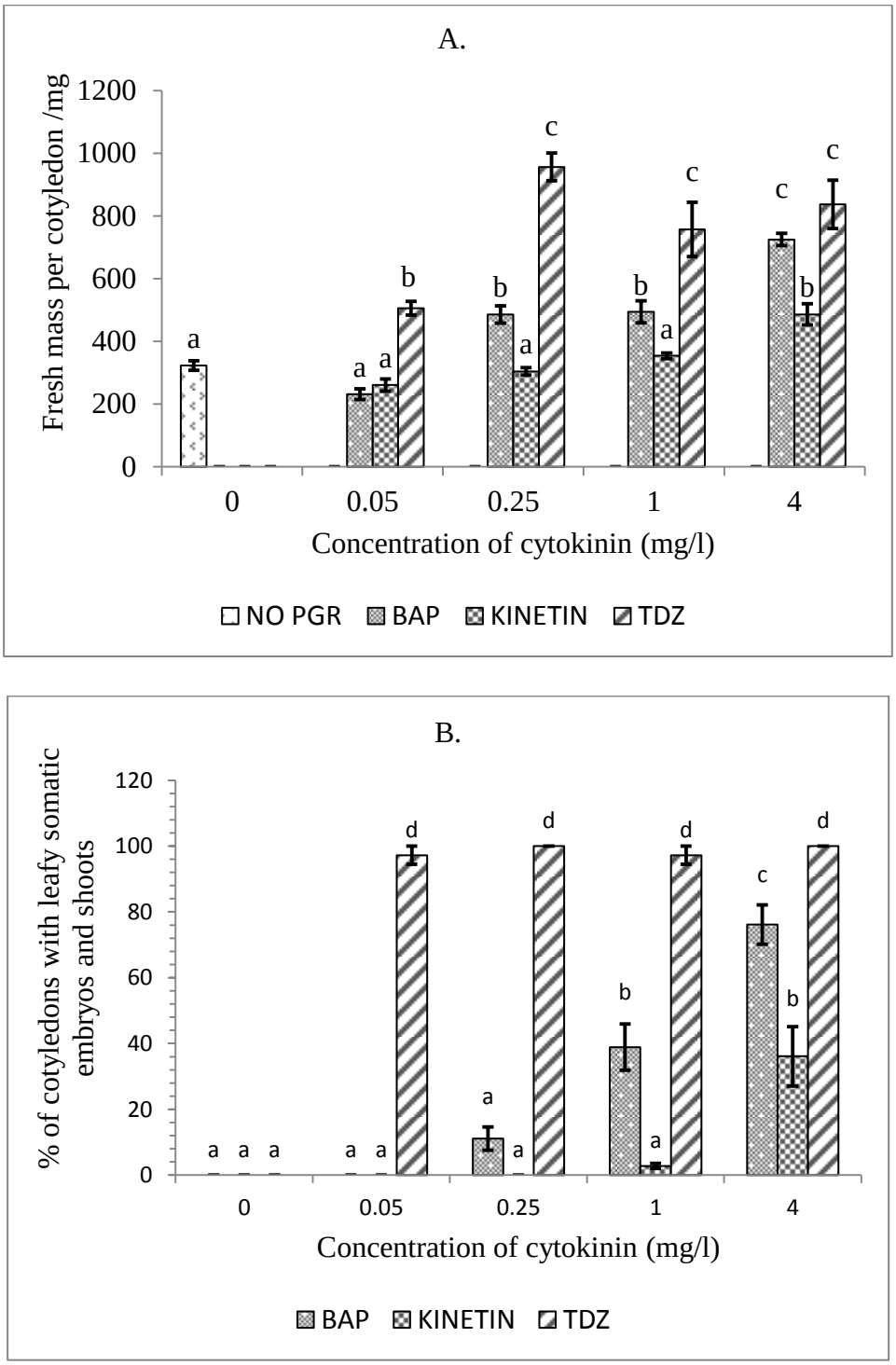


Figure 4: Effects of cytokinin type and concentration (A) on cotyledon fresh mass and (B) percentage of M. volkensii cotyledon explants having somatic embryos after 21 days in induction medium. Bars with same letter do not differ significantly using Tukey's HSD test at $p \leq 0.05$.

Over the range of cytokinin concentrations used, TDZ had significantly higher ($p < 0.05$) percentages (97.22 - 100 %) of cotyledon explants with somatic embryo induction and shoot regeneration than BAP (0 - 76.11%) or Kinetin (0 – 36.11%) (Figure 4B). The responses of the cotyledons to BAP and Kinetin appeared to be dose-dependent unlike their response to TDZ. At 0.05mg/l, only cotyledons grown in TDZ-medium produced somatic embryos and shoots, with an induction frequency of 97.22 % of the explants (Figure 4B). At 0.25 mg/l, embryo induction and shoot regeneration was attained with BAP (11.11%) and TDZ (100%), but not with Kinetin. Kinetin only started to show response at 1 mg/l with a very low frequency (2.78%) of regeneration, in comparison with 38.89 % attained with BAP and 97.22 % with TDZ. At 4mg/l, the regeneration response in BAP (76.11%) was closer to that of TDZ (100%). However, regeneration by Kinetin (36.11%) was still significantly lower ($p < 0.05$) than that achieved using TDZ or Kinetin (Figure 4B).

The patterns of regeneration also differed according to the cytokinin used (Plate 12). Cotyledons cultured on media containing TDZ regenerated with the highest frequency and vigour with regeneration observed at the distal and proximal ends and along the margins (Plates 12 & 13). Those on media with BAP regenerated with low frequency and vigour at the distal and proximal ends of the cotyledons. Cotyledons on media with Kinetin responded with the lowest frequency and vigour of regeneration which was observed only at proximal ends of explants with Kinetin concentrations of 1 or 4 mg/l (Plate 12, Figure 4B).

Increasing the concentration of TDZ from 0.05 to 4 mg/l had little effect on somatic embryo induction, with percentage of explants with somatic embryos ranging from 97.22 to 100%. However, increasing the concentration of either BAP or Kinetin

over the same range significantly increased ($p < 0.05$) the percentage of explants with somatic embryo regeneration (Figure 4B).

When the effects of the three cytokinins on fresh mass and somatic embryo induction were subjected to two-way Anova, both cytokinin type and concentration had significant effects ($p < 0.001$) on cotyledon fresh mass and percentage induction of somatic embryos. Interaction between cytokinin type and concentration was also significant at $p < 0.001$ (Table 6).

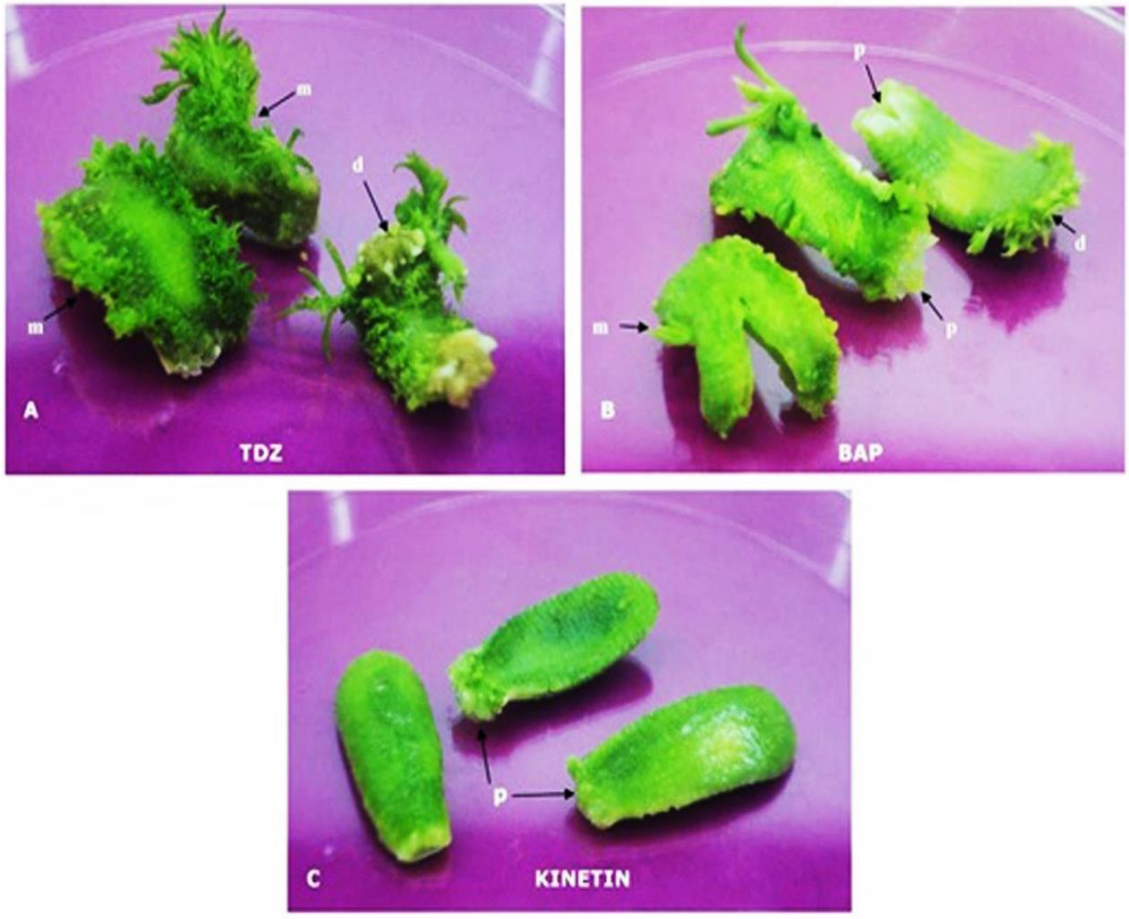


*Plate 12: Comparison of the effects of TDZ, BAP and Kinetin on shoot regeneration in Melia volkensii cotyledons after 20 days in ½ MS supplemented with; A: 0.25mg/l TDZ- with high-vigor multiple microshoot regeneration at the margins (**m**), distal ends (**d**) and some proximal ends (**p**) of explants; B: 0.25 mg/l BAP- with low-vigor shoot regeneration, at distal and proximal ends; and C: 0.25 mg/l Kinetin- with complete absence of regeneration.*

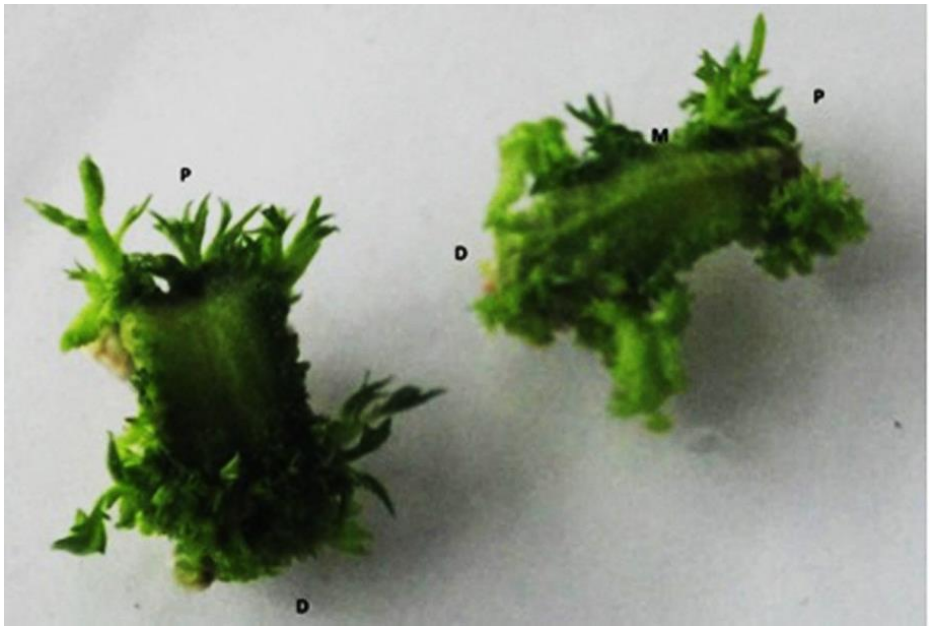


*Plate 13: Close up of microshoots regenerated from Melia volkensii cotyledons, after 21 days on ½ MS + 1 mg/l TDZ. Shoot regeneration occurred at the margins (**m**), distal ends (**d**) and proximal ends (**p**) of the explants.*

Table 6:Two-way Anova output for the effects of type and concentration of cytokinin on somatic embryo induction in Melia volkensii cotyledons.

Source	% of cotyledon explants with somatic embryos			
	d.f.	F-value	Significance (p value)	%CV
Cytokinin concentration	(3,52)	74.35	<0.001	22.5
Cytokinin type	(2,52)	367.33	<0.001	
Concentration x type	(6,52)	22.68	<0.001	
	Fresh mass per cotyledon explant/mg			
Cytokinin concentration	(3,52)	38.43	<0.001	18.18
Cytokinin type	(2,52)	87.35	<0.001	
Concentration x type	(6,52)	7.32	<0.001	

Data taken after 21 days on induction medium containing 0.05mg/l of BAP, KN or TDZ.

4.1.2.3 Developmental Aberrations Associated with Cytokinins

4.1.2.3.1 Shoot Fasciation

Use of the cytokinins BAP and Kinetin at concentrations of ≥ 1mg/l was associated with shoot fasciation. Symptoms included flattening of stems, precocious sprouting of axillary buds and fusion of the lateral branches with the main stem, giving the stem a broader than usual appearance (Plate 14). This type of aberration occurred in shoots irrespective of whether they were regenerated from zygotic embryos or cotyledon explants.

*Plate 14:BAP-induced shoot fasciation in Melia volkensii shoots (shown by **arrows**), arising from precocious sprouting of axillary buds and fusion of the resulting lateral branches, after 30 days on ½ MS + 4mg/l BAP followed by 7 days on ½ MS + 0.1 mg/l Kinetin.*

4.1.2.3.2 Hyperhydric shoots

Use of BAP at concentrations of ≥ 0.25 mg/l also resulted in hyperhydricity of shoots, both in zygotic embryos and cotyledon explants. Symptoms of this disorder included a glassy and transluscent appearance of shoots, pale green colour and swelling of the organs due to waterlogging (Plate 15). The swollen stems and leaves were also easy to break.

Plate 15: BAP-induced hyperhydricity in Melia volkensii shoots; ***Arrows*** *show two typical symptoms of hyperhydricity, swollen stems with glassy appearance, in shoots regenerated from cotyledons after 30 days on 0.25 mg/l BAP.*

4.1.3 Responses of Explants to Media Type and Strength

4.1.3.1 Zygotic Embryos

Full-strength MS medium, its reduced-strength variants (¾, ½ and ¼) and B5 medium all induced 100% callusing of zygotic embryos (Table 7). However, responses differed significantly ($p < 0.05$) in callus fresh mass and the percentage of callus forming somatic embryos. Half strength MS and B5 were superior to the rest of the media tested in formation of fresh mass. The effect of B5 medium on fresh mass yield was closer only to that of ½ MS. Quarter-strength MS induced tiny calluses, whose average fresh mass was about half of the fresh masses obtained with the rest of the media types.

All media tested (¼ MS, ½ MS, ¾ MS, MS and B5) supported induction of somatic embryos (Table 7). Callusing and somatic embryogenesis occurred simultaneously on the callus induction medium which consisted of the respective type and strength of medium plus 0.05 mg/l TDZ as the sole plant growth regulator. The highest percentage of concurrent callusing and somatic embryo induction (52.78%) was

observed in MS + 0.05 mg/l TDZ medium. Reduction of MS nutrients generally reduced the frequency of callus forming somatic embryos (Table 7). Consequently regeneration frequency in ¼ MS was just one-third that in full-strength MS. B5 medium produced callus induction and somatic embryogenesis frequencies similar to those of ¾ MS.

Table 7: Effect of media type and strength on callus induction and somatic embryogenesis in M. volkensii zygotic embryo explants.

Media strength and type	Means ± s.e.m			
	% of explants with callus	Fresh mass per explant/mg	% of callus with somatic embryos†	Appearance of somatic embryos
¼ MS	100[a]	116.00±10.36[a]	16.67±5.27[a]	Tiny green spots
½ MS	100[a]	260.28±22.61[b]	41.67±7.17[ab]	Tiny green spots
¾ MS	100[a]	193.88±28.81[ab]	47.22±5.12[ab]	Tiny green spots
MS	100[a]	185.83±16.26[ab]	52.78±9.04[b]	Tiny green spots
B5	100[a]	246.94±54.94[ab]	47.22±9.04[ab]	Tiny green spots
F and *p* values	-	$F_{(4,24)} = 3.04$ *p= 0.037*	$F_{(4, 24)} = 3.27$ *p= 0.028*	-

Data recorded after 30 days in callus induction medium. All media supplemented with 0.05 mg/l TDZ. Ecotype = Mbeere. Means denoted with same superscript were not significantly different using Tukey's HSD test.

4.1.3.2. Cotyledon Explants

The percentage of cotyledon explants forming somatic embryos in the four variants of MS media used did not differ significantly from each other or from those on B5 medium in (Table 8). All types of media supported high frequency of somatic embryogenesis (88-97.22%). However, lower strengths of MS (¼ and ½ MS) induced higher frequencies of direct regeneration than higher strengths of MS and full-strength B5. The regeneration response in B5 medium was similar to that of ¾ MS as the two media had the same percentages of cotyledon explants with somatic embryos (88.87 %) and similar number of shoots per explant (5.36 for ¾ MS and 5.92 for B5)[Table 8].

With regard to yield of fresh biomass in cotyledons, MS and ½ MS outperformed the rest of the media with average mass per explant of 1362 mg and 1248 mg respectively (Table 8). Biomass yields in ¼ MS, ¾ MS and B5 medium were significantly lower than those of MS and ½ MS. However, no definite pattern of change in fresh biomass was seen in relation to reduction in strength of MS medium. In terms of the number of shoots per explant, MS medium was superior to the lower strengths of MS and to B5 medium as it produced an average of 10.37 shoots per explant (Table 8).

Table 8: Effect of media type and strength somatic embryogenesis and shoot formation in Melia volkensii cotyledon explants.

Media type and strength	% of explants with somatic embryos (Mean±s.e.m)	Fresh mass per explant/ mg (Mean±s.e.m)	Shoots per explant (Mean±s.e.m)	Frequency of *in vitro* flowering (% of total shoots).
¼ MS	97.22 ± 2.78^{a}	1010.70±25.65^{ab}	7.67 ± 0.91^{ab}	0.44
½ MS	97.22 ± 2.78^{a}	1248.00±115.42^{b}	8.90 ± 1.92^{ab}	0.00
¾ MS	88.87 ± 3.52^{a}	710.80±72.38^{a}	5.36 ± 0.59^{a}	3.63
MS	91.65 ± 4.82^{a}	1362.50±73.50^{b}	10.37 ± 1.31^{b}	0.80
B5	88.87 ± 3.52^{a}	1023.10±85.25^{ab}	5.92 ± 0.71^{a}	0.00
F and *p* values	$F_{(4,23)} = 1.64$ *p= 0.198*	$F_{(4, 21)} = 9.40$ *p=0.001*	$F_{(4, 21)} = 4.31$ *p= 0.011*	-
% CV	8.7	17.7	29.9	-

Data scored after 30 days on induction medium. All media supplemented with 0.05 mg/l TDZ. Only shoots ≥ 5 mm in height counted. Means denoted with same superscript were not significantly different using Tukey's HSD test.

One of the developmental aberrations was the occasional precocious *in vitro* flowering of shoots that was observed in some of the media used (Plate 16). The highest frequency of *in vitro* flowering (3.63%) was recorded in ¾ MS plus 0.05mg/l TDZ with full-strength MS medium eliciting the second highest frequency (0.8%) of precocious *in vitro* flowering among the media tested (Tables 8 &9). In addition, shoots formed on the full-strength MS and ¾ MS plus 0.05 mg/l TDZ media exhibited thickening and stunting with more pronounced leaf megalies (Table 9). Reduction of

MS media nutrients generally resulted in reduced abnormalities, higher frequencies of normal shoots and lower frequencies of *in vitro* flowering of shoots.

Plate 16:Effect of media type on precocious in vitro flowering in Melia volkensii shoots regenerated from cotyledons, after 30 days in culture; A: Low frequency flower induction in full MS Medium + 0.05 mg/l TDZ. B: Increased frequency of flower induction in ¾ MS + 0.05 TDZ. C: Single shoot with flower induction in MS + 0.05 mg/l BAP.

Table 9: Significant developmental features observed in regenerated Melia volkensii shoots in different media types and strengths.

Media type & strength	Significant observations after 30 days in the medium
¼ MS	Best elongation of shoots, shoot height reaching 2.5 cm in 30 days, Shoots generally normal, very low frequency of *in vitro* flowering.
½ MS	Some stunting of shoots, secondary shoot development on primary regenerants, increased number of normal shoots, no *in vitro* flowering.
¾ MS	Leaf megaly but less than in full MS, stunting of shoots and highest frequency of precocious flowering of shoots.
B5	Leaf megaly, stunting of shoots, no *in vitro* flowering
MS	Leaf megaly, stunting of shoots, occasional thick and spongy roots at proximal ends of cotyledons, low frequency of precocious flowering of shoots, all flowers at shoot apex, flowering terminates apical growth.

4.1.4 TDZ-induced Indirect Somatic Embryogenesis and Recovery of Shoots from Mature Zygotic Embryos

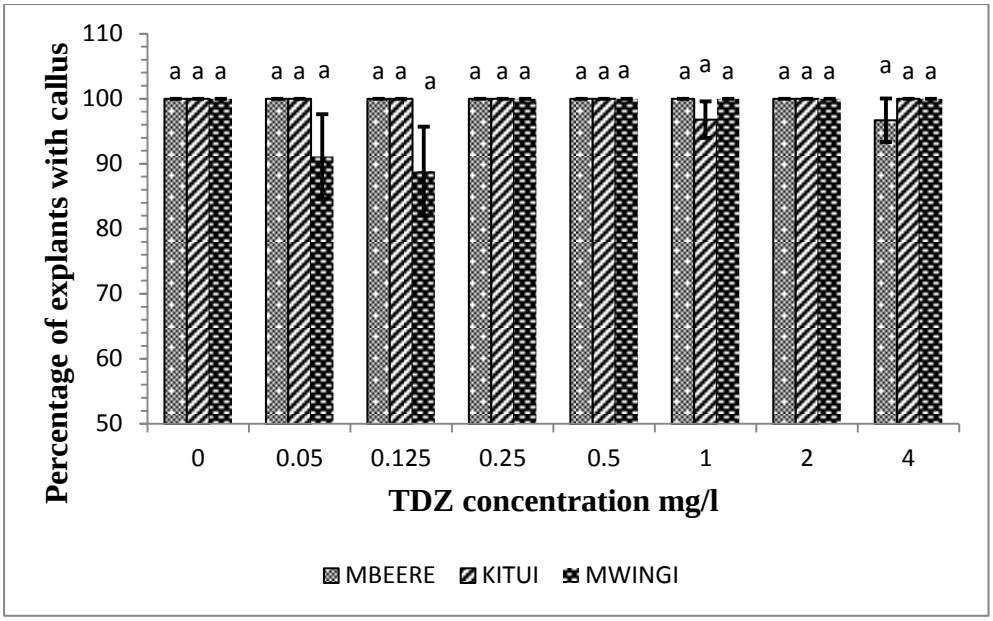
4.1.4.1 Effects of Thidiazuron Concentration and Ecotype on Callus Induction

Swelling of zygotic embryos was observed after 3 to 4 days in culture. Explants started to callus after 4 days of culture forming a compact, nodular, cream callus. Callus induction occurred at a very high frequency (88.88 to 100%) in all three ecotypes; Kitui, Mbeere and Mwingi. The zygotic embryos callused in all TDZ treatments as well as in the control media (Figure 5A). TDZ concentration had no significant effect (F test, $p > 0.05$) on percent of zygotic embryos with callus induction but its effect on callus fresh mass was significant (F test, $p < 0.001$) (Figure 5B; Table 10).

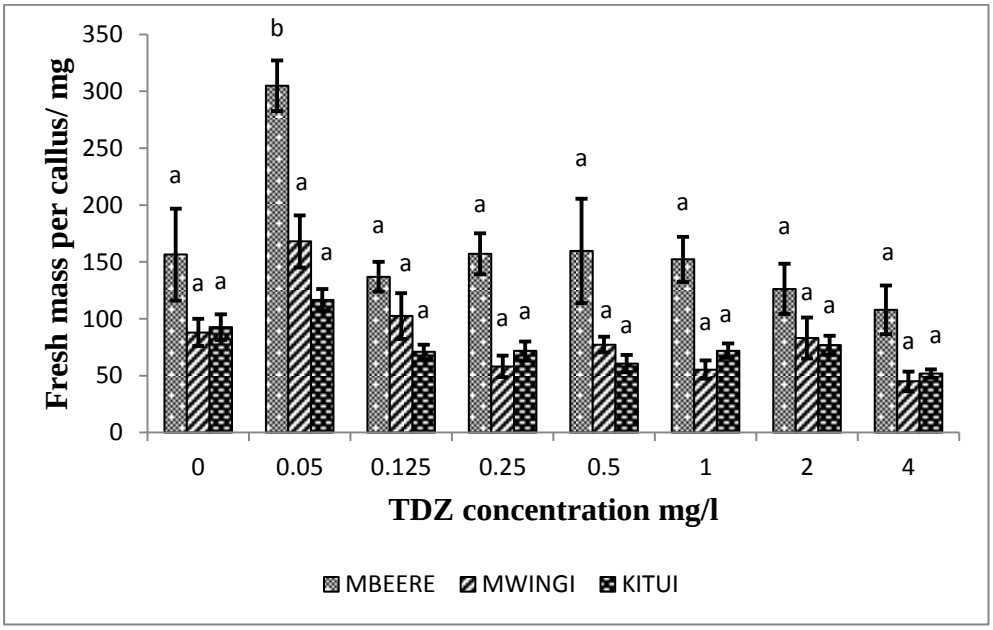
In all three ecotypes the largest mean fresh mass of zygotic embryo callus were obtained at 0.05mg/l TDZ (304.7 mg for Mbeere, 168 mg for Mwingi and 116.7 mg for Kitui) (Figure 5B). However, despite zygotic embryo explants from Mbeere clearly outperforming those from Mwingi and Kitui in formation of callus fresh mass, interaction between TDZ concentration and ecotype in production of callus fresh mass was not significant (F test, $p > 0.05$) (Table 10).

4.1.4.2. Somatic Embryogenesis and Shoot Regeneration from Callus

Sixty to 80% of the callus formed by zygotic embryos (Mulanda, Unpublished Data) were non-morphogenic when maintained on TDZ medium and only developed somatic embryos when transferred to growth regulator-free B5 or ½ MS medium. However, calli formed on control media in the absence of TDZ failed to form somatic embryos even after a similar transfer to growth regulator-free medium (Figure 5). All the TDZ concentrations used (0.05 to 4 mg/l) had a significant differences on the percentage of callus forming somatic embryos (F test, $p < 0.001$), with the optimal response occurring at 0.05 mg/l in the three ecotypes studied (Mbeere (100%), Mwingi (100%) and Kitui 96.67%).



(A)



(B)

Figure 5: Effects of Thidiazuron concentration and ecotype on embryogenic callus induction in Melia volkensii zygotic embryos; (A) Percentage of explants with callus and (B) Callus fresh mass. (N = 45 explants per ecotype per TDZ treatment). Bars with same letter do not differ significantly using Tukey's HSD test at $p \leq 0.05$.

Table 10:Two-way anova output for the effects of TDZ concentration x ecotype on callus fresh mass in Melia volkensii zygotic embryos.

Source	d.f.	F-value	Significance p-value	% CV
TDZ concentration	(7,179)	11.83	< 0.001	48.57
Ecotype	(2, 179)	55.53	< 0.001	
TDZ conc. X Ecotype	(14, 179)	1.70	0.059 (NS)	

The formation of somatic embryos in calluses of mature zygotic embryos was preceded by the greening of the callus at one, two or three focal points before spreading to the rest of the callus. These green foci developed multiple green globular somatic embryos (Plate 17). These embryos developed into well-defined micro shoots (Plates 17 & 18) when transferred to hormone-free B5 or ½ MS medium. The mean regeneration frequency was 24.25 ± 9.34 micro shoots per callus.

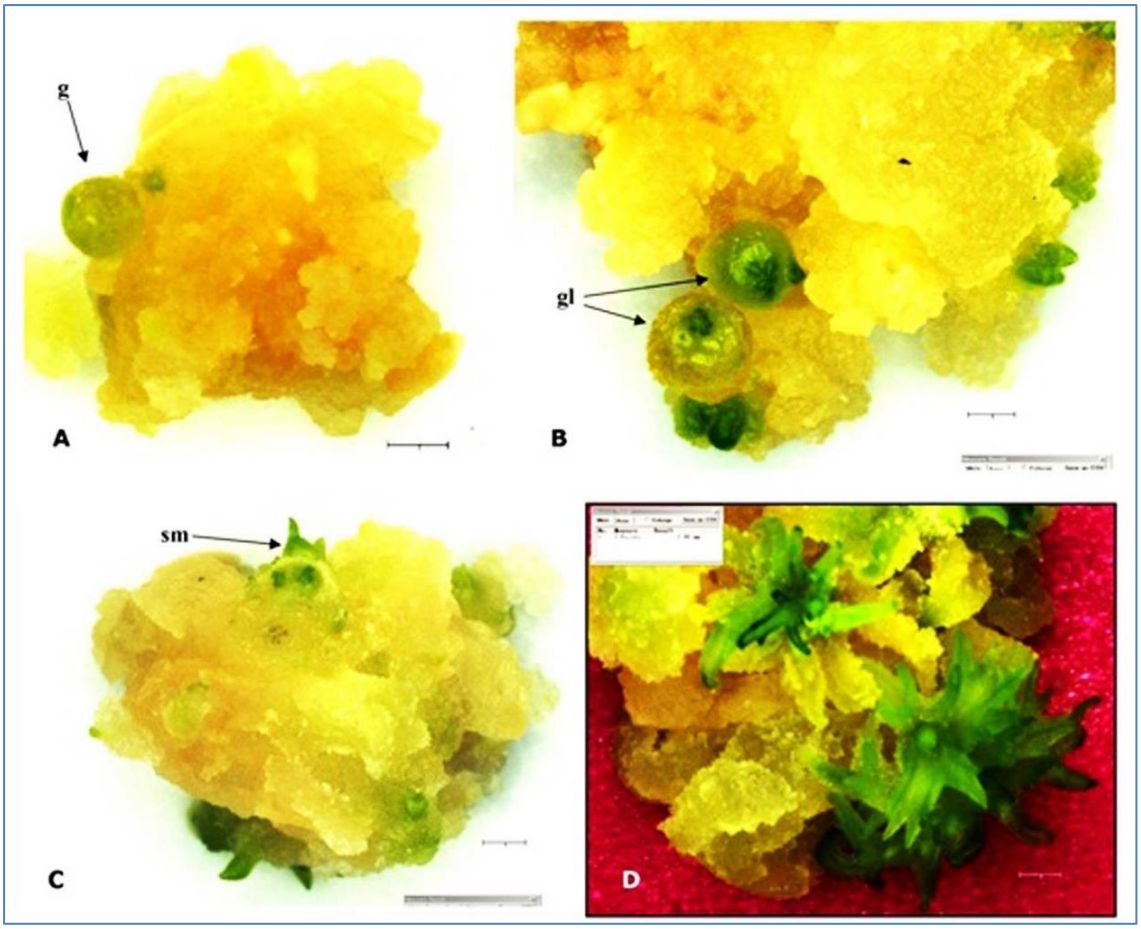


*Plate 17: Somatic embryogenesis in Melia volkensii zygotic embryo callus after subculture to PGR-free B5 medium, with different stages of embryogenesis indicated by labelled arrows;A: A globular stage somatic embryo (**g**) forming from callus after 14 days, cumulative age = 35 days; B: globular somatic embryos forming leaf primordia (**gl**) during initial stages of shoot morphogenesis (**sm**) after 15 days; cumulative age = 36 days; C: Callus with further shoot regeneration after 17 days, cumulative age = 38 days. D: Callus with multiple microshoots after 20 days, Cumulative age = 41 days [Scale bars = 1mm].*

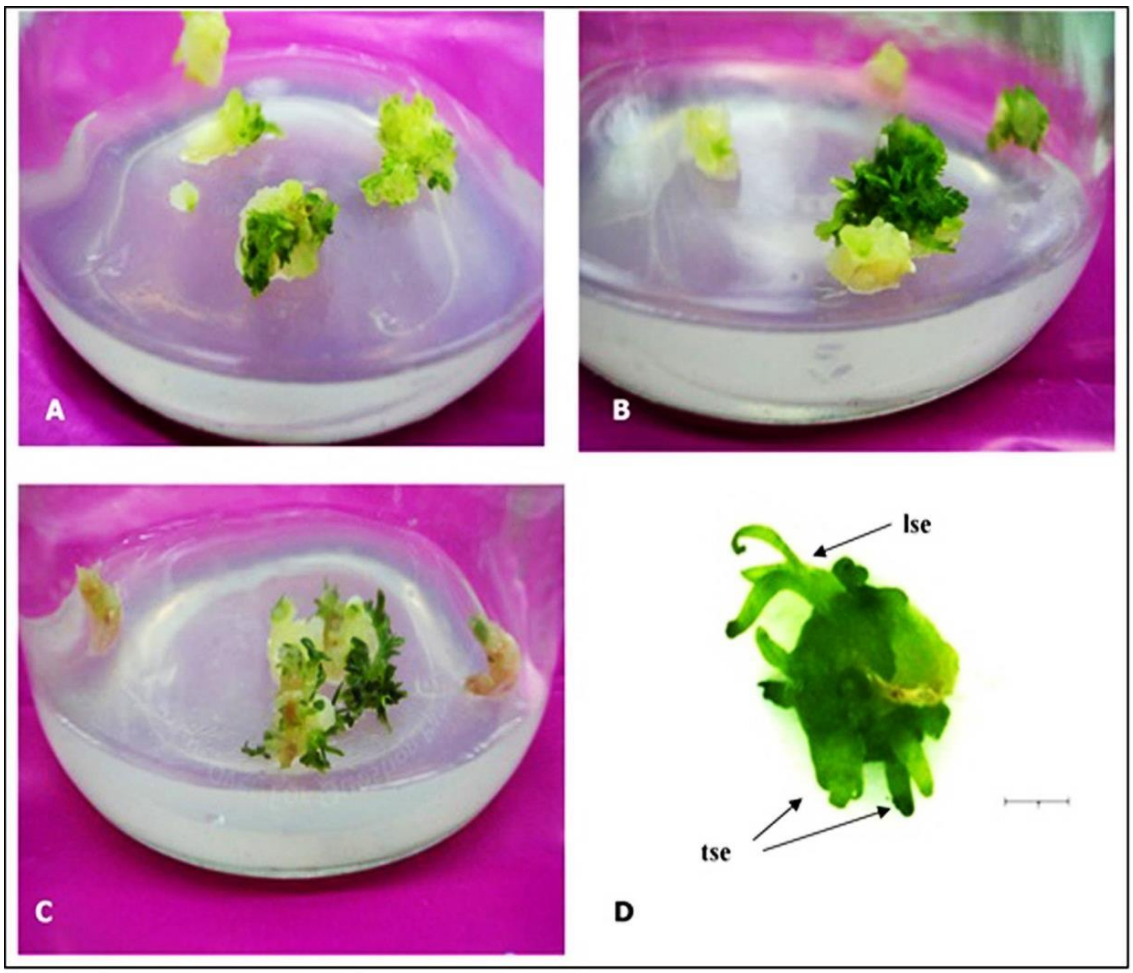


*Plate 18: Conversion of somatic embryos into microshoots in Melia volkensii zygotic embryo calli, with developmental stages shown by arrows**;** A to C: Macroscopic images showing callus induction and development of somatic embryos (green dots and leafy structures) on day 21 (A), day 26 (B) and day 36 (C) after subculture to growth regulator-free ½ MS medium. D: Photomicrograph showing a leafy somatic embryo (lse) and torpedo somatic embryo (tse) stages after 20 days on growth regulator-free B5 medium; Scale = 1mm.*

4.1.4.3 Elongation of Microshoots Regenerated from Zygotic Embyros

Shoots regenerated from zygotic embryoexplants using TDZ failed to elongate when subcultured on to either B5 or ½ MS with no PGRs. They only elongated when transferred to media containing either 0.1 mg/l BAP alone or in combination with 10 % coconut water (Table 11). Gibberellic acid (GA_3) was unsuitable for elongation of the micro shoots as it resulted in fasciated shoots with morphological and physiological defects such as twisted, unusually thick and hyperhydric stems, failure of leaf

expansion, and creeping of some stems along the surface of the media in what appeared as inability to show the normal phototropic or geotropic responses.

Table 11: Some observations on the effects of BAP, gibberellic acid and coconut water on Melia volkensii microshoots

BAP mg/l	G.A mg/l	Coconut water (%)	% of explants with shoots ≥ 2.0 cm	Days taken	Phenotypic appearance
0	0	0	0[a]	35	Shoots stunted, with callusing of stem bases
0	1.0	0	52.08 ± 6.61[b]	35	Shoots fasciated, with thick stems
0.1	0	0	52.00 ± 8.33[b]	35	Shoots normal but elongation slow
0.1	0.1	0	70.17 ± 10.67[b]	31	Shoots fasciated, with thick and twisted stems
0.1	0	10	81.25 ± 5.54[b]	35	Shoots normal, with multiple shoot induction from the bases of parent shoots

Values with the same superscript do not differ significantly using Tukey's HSD test at $p \leq 0.05$ (n=20).

Elongation media consisting of ½ MS supplemented with either 0.1 mg/l BAP alone or in combination with 10% coconut water supported normal shoot development with good elongation of the microshoots. The percentage of explants with shoots of ≥ 2 cm height after 35 days of culture was 52 ± 8.33 % on ½ MS media with 0.1 mg/l BAP alone and 81.25 ± 5.54 % on ½ MS media with 0.1 mg/l BAP combined with 10%

coconut water (Table 11 & Plate 19). Media with 0.1 mg/l BAP and 10% coconut water also supported some secondary callusing and formation of multiple shoots from the bases of the original shoots (Plate 19).

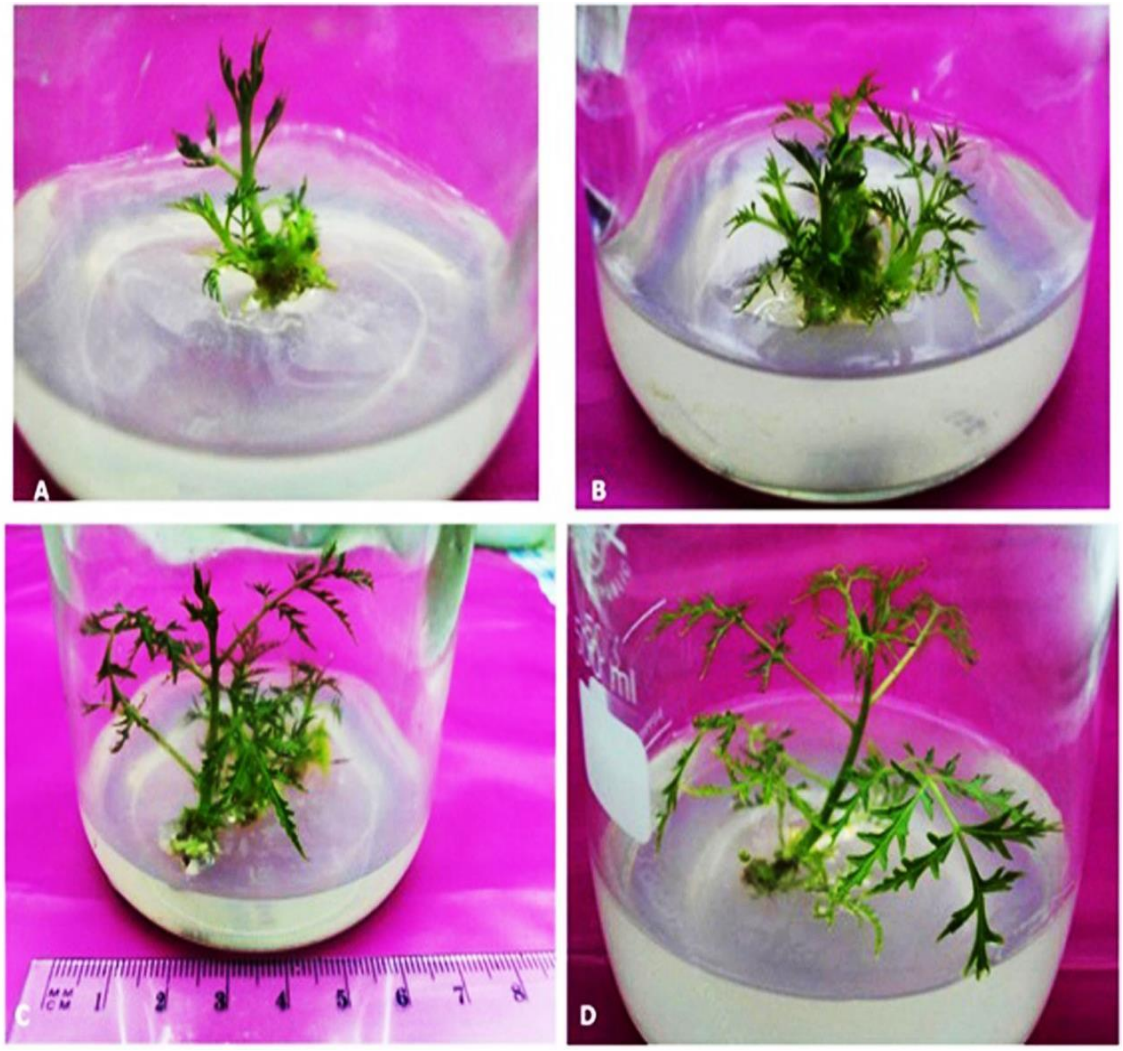


Plate 19: Elongation of Melia volkensii microshoots formed via indirect regeneration. A/B: Elongation with multiple shoot induction after 6 days(A) and 12 days (B) on ½ MS + 0.1mg/l BAP+ 10% coconut water; cumulative ages = 84 and 90 days respectively. C: Another set of shoots after 16 days on same elongation medium; cumulative age = 94 days. D: A phenotypically normal shoot after 25 days on the same elongation medium; cumulative age = 103 days.

4.1.5 TDZ-induced Direct Somatic Embryogenesis and Shoot Regeneration in Cotyledon Explants

4.1.5.1 Effects of Thidiazuron Concentration and Ecotype

A high frequency of direct shoot regeneration was observed in the cotyledon explants, with the regeneration occurring on the upper, light-facing surfaces of the explants. This polarized pattern of regeneration was observed irrespective of whether the light-facing surface was abaxial or adaxial. Dense clusters of globular somatic embryos were formed (Plate 20).

Within 5 to 8 days of culture, the cotyledons showed variable extents of greening with numerous tiny pimple-like structures on the illuminated surfaces. In all three ecotypes (Mbeere, Mwingi and Kitui), the greening of explants and regeneration were limited to the upper, light-facing surfaces.

Another characteristic feature of the response of cotyledons to *in vitro* culture was curving of explants and lifting of mid-sections away from the surface of the medium with only the proximal and distal ends in contact with the medium. Some of the curved cotyledons subsequently fell on their lateral sides when the bottles were moved but this did not interfere with the pattern of regeneration as only surfaces that were facing upwards before the fall continued with regeneration.

The greening and curving responses occurred in media supplemented with TDZ as well as in the TDZ-free controls, showing that these responses were not dependent on the presence of TDZ. However, explants in the TDZ-free medium remained non-morphogenic before turning brown and undergoing necrosis after 35 days of culture. Morphogenesis was only observed in TDZ-treated medium, showing that the presence of TDZ was essential for regeneration.

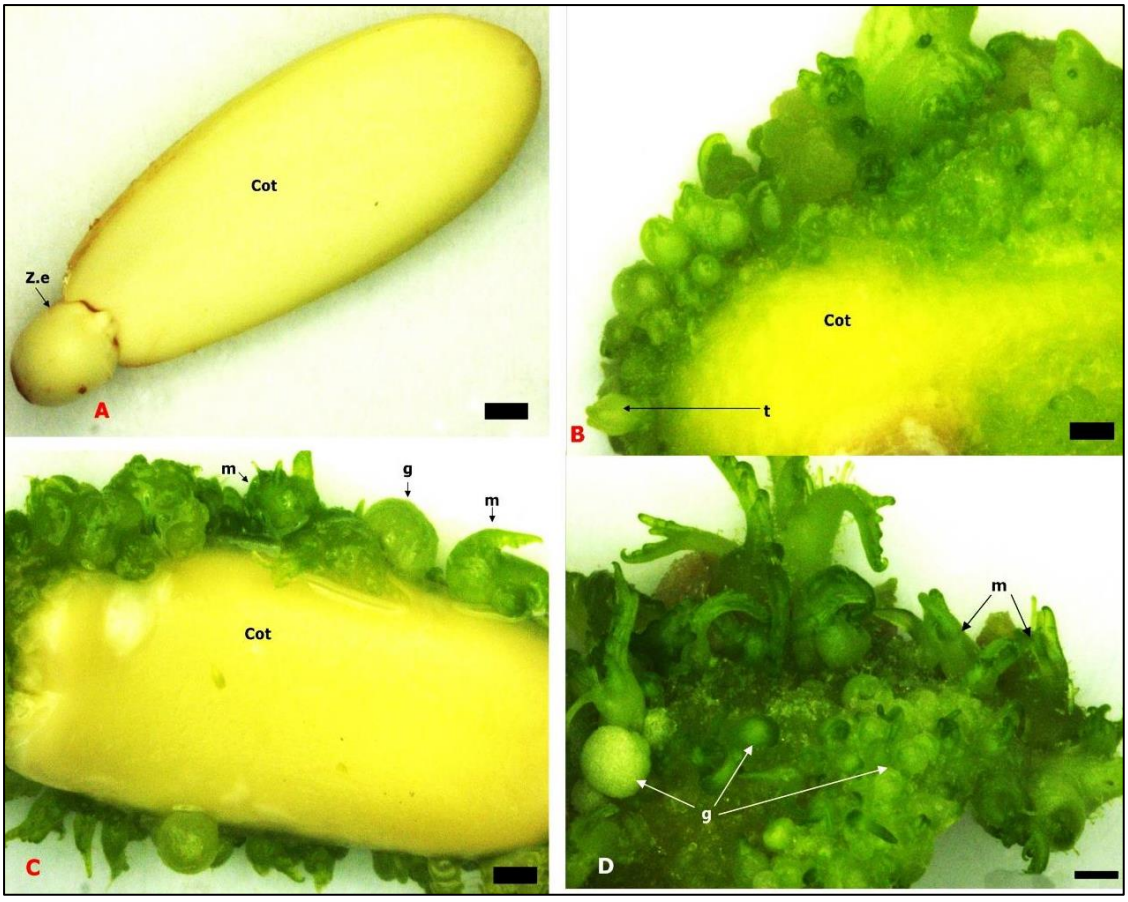


*Plate 20: Direct somatic embryogenesis in Melia volkensii cotyledons, with developmental stages shown by arrows. A: A mature zygotic embryo (**z.e**) attached to the cotyledon (**cot**). B–D: Cotyledon explants in various stages of somatic embryogenesis after 20 days of culture on ½ MS + 0.125mg/l TDZ, showing globular (**g**) and torpedo (**t**) stages and mature embryos in early stages of conversion to microshoots (**m**). C: Explant turned upside down to show absence of regeneration on the surface in contact with medium. Scale bars = 1mm.*

Development of somatic embryos was evident within 7 to 10 days with explants forming numerous tiny green nodular structures. Within 10 to 16 days, these green nodules gave rise to well defined globular somatic embryos ranging from 200 to 1500µm in diameter (Plate 20). These globular somatic embryos formed directly from the surfaces of explants without an intervening callus phase, except at the low TDZ concentrations of 0.05 and 0.125 mg/l where slight callusing occurred at the proximal ends and along the margins of cotyledons before the globular embryos emerged.

The somatic embryos had remarkable similarity in size and morphology to the zygotic embryos of mature seeds (Plate 20A). Regeneration efficiency was high with 78 - 100 % of cotyledon explants forming dense clusters of somatic embryos in medium containing TDZ at concentrations of 0.125 – 4 mg/l.

TDZ concentration and ecotype both had significant effects ($p < 0.001$) on fresh mass per cotyledon and percentage of cotyledons with somatic embryos (Figure 6A & Table 12). In all three ecotypes, fresh mass yield increased with increase in concentration of TDZ up to 0.25 mg/l, after which a gradual decrease occurred (Figure 6B). The optimal TDZ concentration for formation of fresh biomass was 0.25 mg/l of TDZ. The ecotype from Mwingi produced significantly higher fresh masses than the other two ecotypes from Mbeere and Kitui at all TDZ concentrations used. Interaction between TDZ concentration and ecotype in fresh biomass formation was also significant ($p < 0.001$).

In general, the frequency of cotyledon explants with somatic embryos was high, with the range of response for Mbeere and Kitui ecotypes being 75.32 to 100% and 88.88 to 100% respectively. Response in Mwingi was 100% in all the TDZ concentrations from 0.05 mg/l to 4 mg/l. Explants cultured on control media were non-morphogenic.

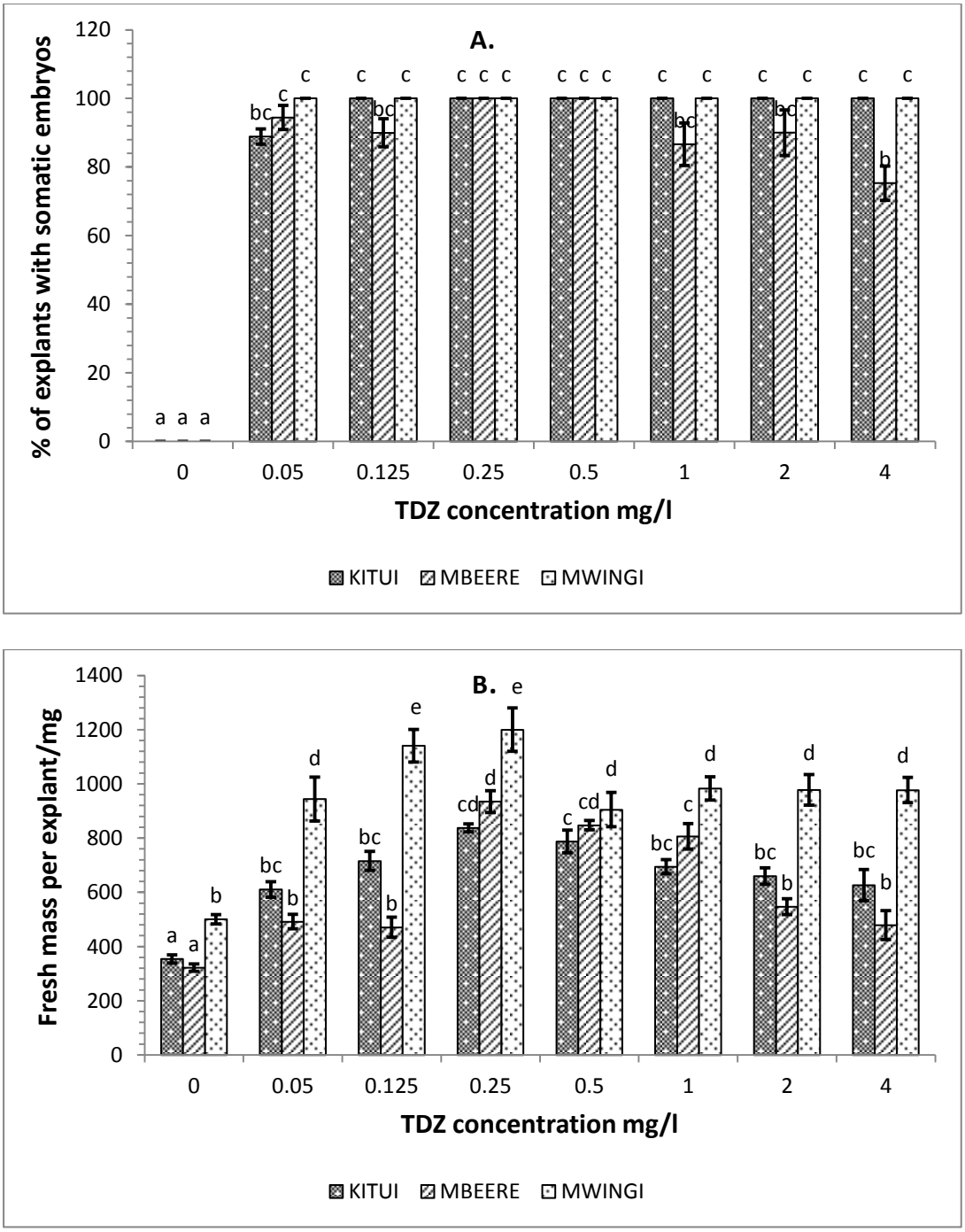


Figure 6: Effect of Thidiazuron concentration and ecotype on somatic embryo regeneration in Melia volkensii cotyledons; A: Induction of somatic embryos, and B: Fresh mass of cotyledons, after 21 days on induction medium. Number of explants per ecotype per treatment = 54; Bars with same letter do not differ using significantly using Tukey's HSD at p≤ 0.05.

Table 12:Two-way Anova output for effects of TDZ concentration and ecotype on somatic embryogenesis in Melia volkensii cotyledon explants

Source	% of cotyledons with somatic embryos			
	d.f.	F-value	Significance (p value)	%CV
TDZ concentration	(7,106)	752.43	<0.001	6.31
Ecotype	(2,106)	28.36	<0.001	
TDZ x Ecotype	(14,106)	5.80	<0.001	
Source	Fresh mass per cotyledon/mg			
	d.f.	F-value	Significance (p value)	%CV
TDZ concentration	(7,106)	52.88	<0.001	13.48
Ecotype	(2,106)	142.64	<0.001	
TDZ x Ecotype	(14,106)	6.27	<0.001	

4.1.5.2 Conversion of Direct Somatic embryos to Microshoots

After forming directly from the cotyledon explants, the somatic embryos continued to develop and converted into microshoots within 15 to 20 days of culture on the induction medium (Plate 21). They did not require to be separated from the mother explants for further development.

All seven concentrations of TDZ used supported, to different extents, the conversion of somatic embryos to microshoots. Half MS medium containing 0.25 mg/l TDZ had the best overall percentage (78.47% for Kitui, 86.10% for Mbeere and 83.33% for Mwingi) of explants with microshoots ≥ 5 mm length after 30 days in

induction medium (Figure 7). Both TDZ concentration and ecotype had significant effects ($p < 0.001$) on the percentage of explants with microshoots. Interaction between TDZ concentration and ecotype was also significant ($p < 0.001$) (Table 13)

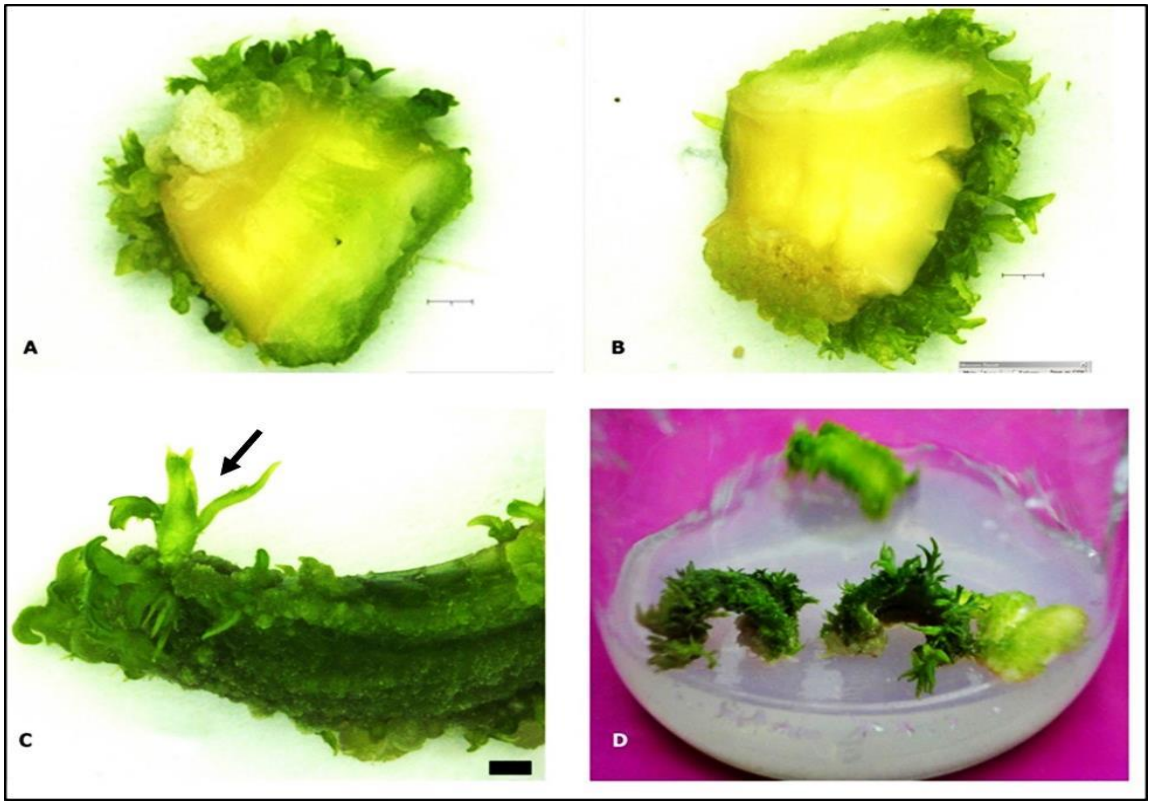


*Plate 21: Microshoot formation in Melia volkensii cotyledon explants. A & B: Photomicrographs showing shoots regenerated after 16 days on half-strength MS + 0.5mg/l TDZ; C: A well-defined microshoot (**Arrow**) formed after 20 days on half-strength MS + 0.05mg/l TDZ; D: Macroscopic view of cotyledon explants showing shoot regeneration after 24 days on half-strength MS + 0.5 mg/l TDZ induction medium. (For A, B and C scale bars = 1 mm).*

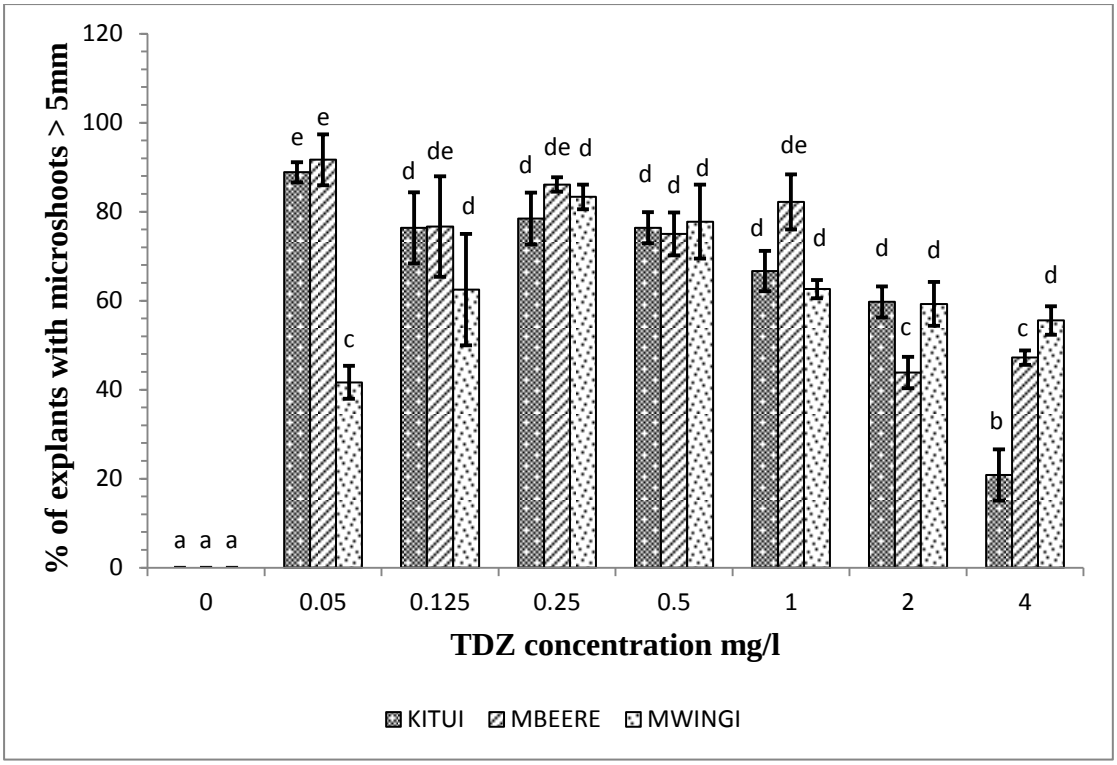


Figure 7: Effects of TDZ concentration and ecotype on percentage of Melia volkensii cotyledon explants converting somatic embryos to microshoots. Values with same letter do not differ using significantly using Tukey's HSD at $p \leq 0.05$.

Table 13:Two-way ANOVA output for effects of TDZ concentration and ecotype conversion of somatic embryos to microshoots in Melia volkensii cotyledon explants

Source	% of cotyledons with microshoots ≥ 5mm			
TDZ concentration	(7,106)	72.74	<0.001	22.83
Ecotype	(2,106)	13.08	<0.001	
TDZ x Ecotype	(14,106)	4.76	<0.001	

4.1.5.3. Elongation of Microshoots Regenerated from Cotyledons

Elongation of microshoots was achieved when whole or half segments of cotyledons having microshoots were subcultured on to half-strength MS supplemented with either 0.1mg l^{-1} BAP, 0.1 mg l^{-1} BAP plus 0.01 mg l^{-1} IAA or 0.1 mg l^{-1} BAP plus 10% coconut water (CW) (Table 14 & Plate 22). Among the four types of elongation media

tested, the mean shoot height obtained after 30 days ranged from 8.0 mm for those on hormone free ½ MS medium to 18.17mm for those on ½ MS plus 0.1 mg/l BAP and 0.01 mg/l IAA (Table 14). The combination of ½ MS plus 0.1 mg/l BAP and 0.01 mg/l IAA gave the best elongation response (18.17 ± 1.58 mm). Elongation continued after subculture of the cotyledons to fresh media of the same composition, with a mean height of 42.20 ± 5.10 mm attained after 45 cumulative days from day of induction (Plate 22B). The mean number of leaf nodes per shoot was 7.94 ± 0.60.

Table 14: Effect of growth regulators on elongation of M. volkensii microshoots

PGR combination mg/l	Days in medium	Mean Shoot length/ mm (± s.e.m)
0 PGR	22	8.00 ± 1.08^{a}
0.1 BAP	22	16.16 ± 1.94^{b}
0.1 BAP + 10% CW	24	15.57 ± 1.85^{b}
0.1 BAP + 0.01 IAA	24	18.17 ± 1.58^{b}

Values with the same superscript in a column do not differ significantly using Tukey's HSD test at $p \leq 0.05$.

The concentration of TDZ in the induction medium had a significant effect on the number of elongated shoots per cotyledon (Table 15). Optimal mean number of elongated shoots (11.25) was attained with 0.25 mg/l TDZ in the induction medium. Presence of lower or higher concentrations of TDZ in the induction medium reduced the number of elongated shoots obtained.

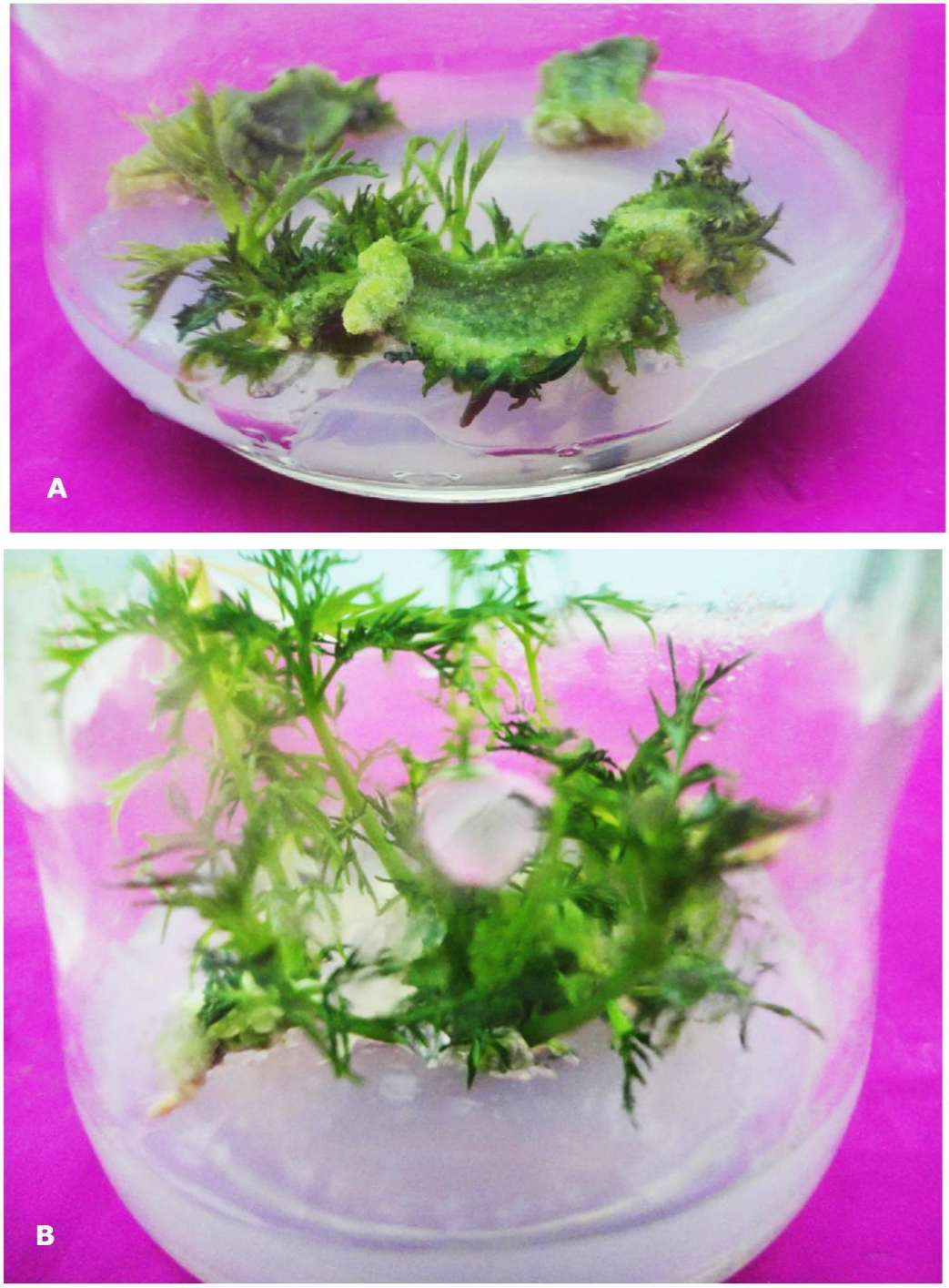


Plate 22: Elongation of Melia volkensii microshoots regenerated from cotyledons; A: after 25 days on ½ MS + 0.1mg/l BAP + 0.01mg/l IAA. B: after 40 cumulative days on same type of medium. (Initial culture was on ½ MS + 0.125 mg/l TDZ).

Table 15: Effect of induction-stage Thidiazuron concentration on yield of shoots in Melia volkensii cotyledon explants

TDZ concentration in induction media mg/l	Number of elongated shoots per cotyledon
0	0^{a}
0.05	4.67 ± 0.42^{b}
0.125	8.70 ± 1.33^{c}
0.25	11.25 ± 2.80^{c}
0.5	10.00 ± 1.75^{c}
1.0	7.80 ± 1.17^{c}
2.0	9.44 ± 1.52^{c}
4.0	5.44 ± 0.97^{b}

Data recorded after 30 days on shoot elongation medium. Values with same superscript are not significantly different using Tukey's HSD test at $p \leq 0.05$.

4.1.6 Rooting

Adverse effects such as callusing of stem bases, yellowing and leaf abscission were observed when shoots regenerated indirectly from zygotic embryos or directly from cotyledons using TDZ were planted into rooting media containing 0.05 to 1.00 mg/l IBA (Plate 23). A general difficulty was observed in rooting of the shoots in media containing IBA. Some initial rooting was attained at 0.2 mg/l IBA but the roots also callused.

Callusing of stem bases was also observed in rooting media containing 10mg/l of the polyamine putrescine (Plate 23B). Both callusing and total leaf abscission were observed and in rooting media containing 0.2 - 1 mg/l IBA (Plates 23C & 24). No callusing or leaf abscission was observed in shoots cultured in PGR-free media and in

shoots cultured on medium containing 500mg/l activated charcoal (Plate 23D). Thus addition putrescine and moderate to high levels of IBA produced adverse callusing effects, while activated charcoal had the beneficial effect of eliminating callusing of stem bases and leaf abscission (Plate 23D).

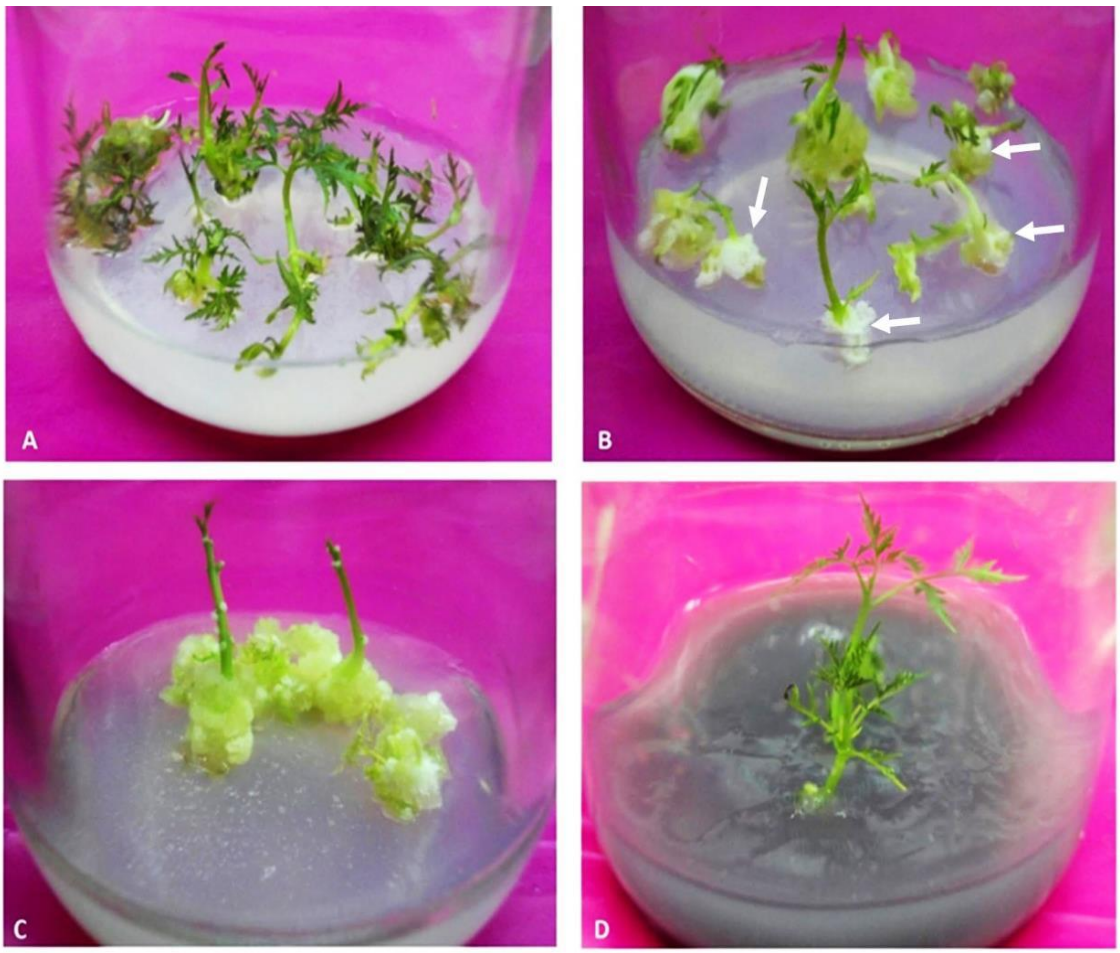


*Plate 23:Beneficial and adverse effects of different types of rooting media on Melia volkensii shoots cultured on ½ MS with; A: No PGRs, showing absence of callus after 15 days. B: 10 mg/l Putrescine, showing callusing of stems (**arrows**) after 9 days. C: 0.2 mg/l IBA, showing profuse callusing of stem bases and total leaf abscission after 38 days. D:1% (w/v), showing beneficial effect (removal of callusing and leaf abscission) of addition of 1% (w/v) activated charcoal to ½ MS medium containing 0.2 mg/l IBA.*

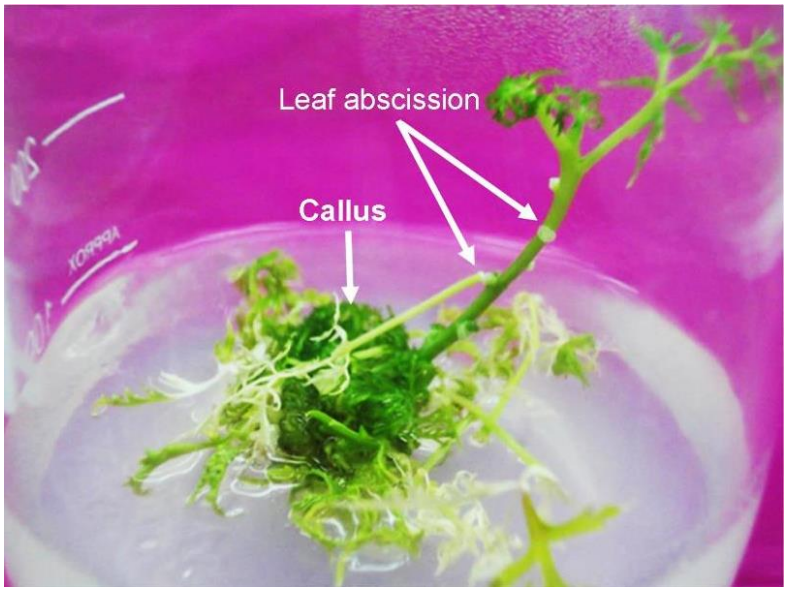


Plate 24: Adverse leaf abscission and callusing in Melia volkensii shoots after 30 days in ½ MS rooting medium containing 0.2 mg/l IBA.

Significant rooting (F test, $p < 0.001$) was only observed in medium containing 0.2 mg l^{-1} NAA alone or 0.1 mg l^{-1} NAA in combination with 0.1 mg l^{-1} IBA (Table 16). Although NAA was superior to IBA in root induction, rooting medium containing 0.05 to 0.2 mg l^{-1} NAA was not as effective in supporting continued elongation of shoots as was medium with IBA. In addition, media containing NAA also caused significant leaf chlorosis in the shoots. In general, IBA induced single taproots while NAA induced multiple roots (Table 16 & Plate 25).

Table 16: Effects of NAA and IBA on rooting of Melia volkensii shoots after 22 days in rooting medium

PGR mg l^{-1}	Days to start of rooting	% rooting [Mean ±S.E]	Number of roots per shoot [Mean± S.E]
0	12	5.71 ± 3.69^{a}	1.00 ± 0.00^{a}
0.05 NAA	-	0^{a}	0^{a}
0.1 NAA	8	8.88 ± 5.87^{a}	5.40 ± 1.29^{b}
0.2 NAA	8	32.40 ± 8.90^{b}	4.65 ± 0.67^{b}
0.05 IBA	-	0^{a}	0^{a}
0.1 IBA	10	6.67 ± 4.22^{a}	1.00 ± 0.00^{a}
0.2 IBA	-	0^{a}	0^{a}
0.1 NAA + 0.1 IBA	9	39.31 ± 6.14^{b}	5.64 ± 0.86^{b}

All rooting medium consisted of ½ MS. Values with the same superscript in a column do not differ significantly using Tukey's HSD test at $p \leq 0.05$.

Plate 25: Comparison of rooting patterns in Melia volkensii shoots caused by IBA and NAA; A. Single taproot in ½ MS + 0.1 mg/l IBA; B. Multiple roots in ½ MS +0.1 mg/l NAA. Both were observed after 20 days in rooting medium.

4.1.7. Acclimatization and Hardening

Rooted plantlets with a height of 3 - 4.5 cm established well in honey jars containing sterile vermiculite irrigated with basal ½ MS after removal from agar medium (Plates 26 & 27). Addition of 0.2 % (m/v) of urea to the basal ½ MS had a promotive effect on

shoot elongation in vermiculite. Only shoots with undamaged roots survived the transfer from agar media to vermiculite, with survival rates in the range of 85 – 90 %. Progressive opening of the honey jar lids was an effective means of hardening of shoots against water loss as evidenced by lack of wilting when the lids were completely removed. Without such progressive acclimatization, shoots wilted rapidly, with severe effects on survival when exposed to the atmosphere. Shoots that stayed in vermiculite for 25 days or more were more successful in establishing in soil.

Plate 26: Regenerated Melia volkensii plants establishing in vermiculitesupplemented with ½ MS basal medium after transfer from agar medium.

Plate 27: Acclimatization and hardening of Melia volkensii plants; A: After 33 days in vermiculite + basal ½ MS. B: 15 days after shifting from honey jars to larger containers but still in vermiculite + basal ½ MS medium. C: Hardened plants in topless containers. D: Plants establishing in soil.

4.1.8 Influence of Polyethylene Glycol 6000 and Amino Acids on Somatic Embryo Regeneration

4.1.8.1 Polyethylene Glycol 6000 and Regeneration in Zygotic Embryos

In comparison to the control media, addition of the osmotic agent polyethylene glycol-6000 (PEG) had no effect on the mean number of somatic embryos formed per callus when used at concentrations of 5 – 15 % (m/v). However, its effect on the number of somatic embryos was beneficial when used at 20 and 30 % concentrations and inhibitory at 40 and 50 % concentrations (Figure 8). The presence of PEG in the medium also resulted in more of the torpedo stages of somatic embryos and fewer globular stages (Plate 28).

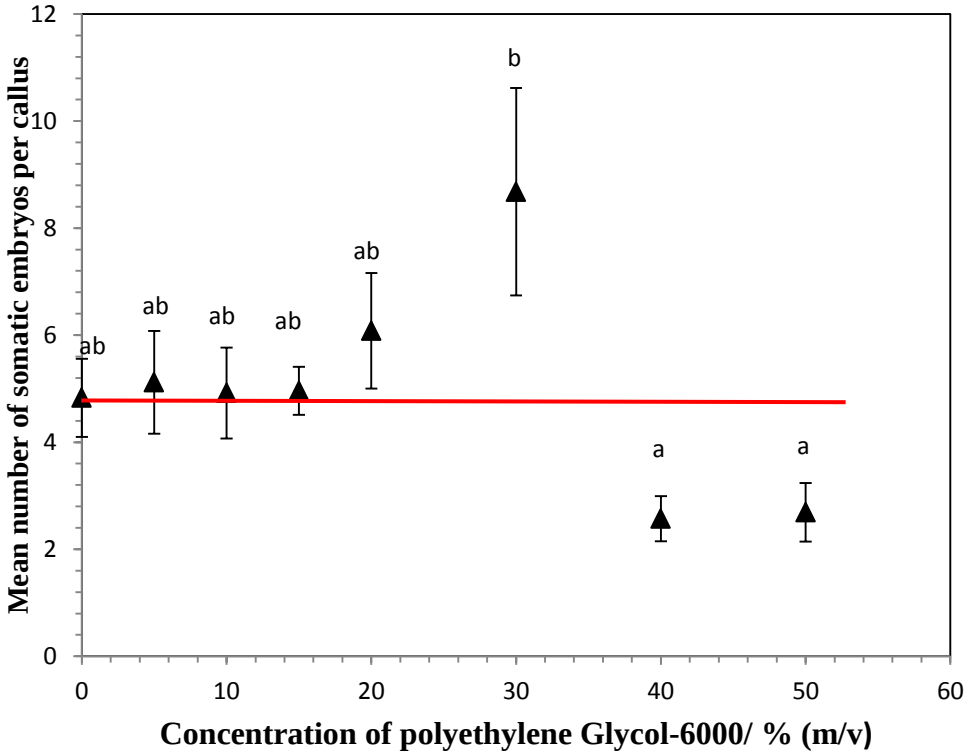


Figure 8: Effect of concentration of polyethylene glycol-6000 (PEG) on yield of somatic embryos per callus in Melia volkensii zygotic embryo explants. Horizontal line = mean response in control medium lacking PEG. Values with the same letter do not differ significantly using Tukey's HSD test at p≤ 0.05.

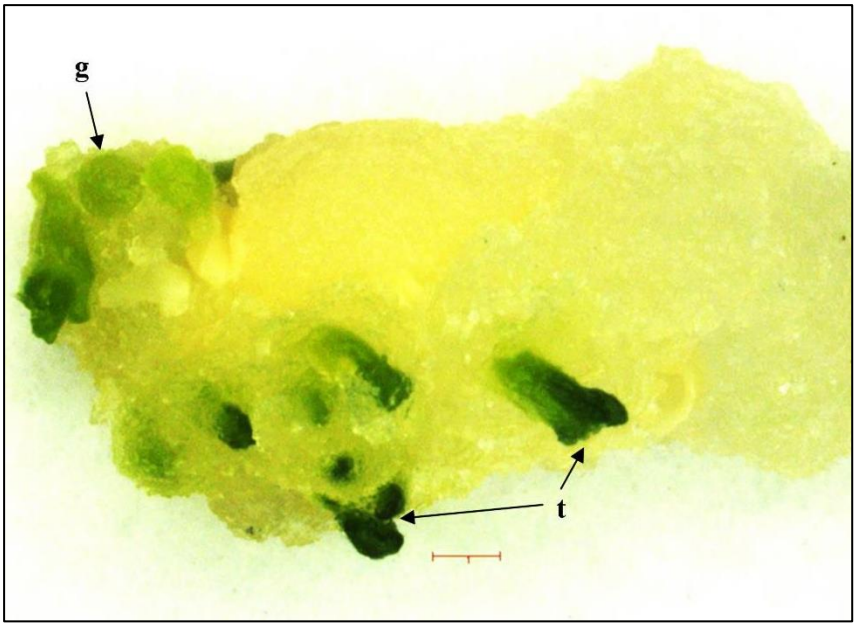


*Plate 28: Effect of Polyethylene glycol on somatic embryogenesis in Melia volkensii zygotic embryo callus. Globular stage (**g**) and torpedo (**t**) stages of somatic embryos after 15 days on ½ MS +0 regeneration medium. Previously on induction medium of ½ MS + 0.05 mg/l TDZ + 15% (m/v) PEG. Cumulative age = 36 days.*

4.1.8.2 Effect of Amino Acids on Regeneration in Zygotic Embryos

At the two concentrations tested (50 and 100 mg/l), L-glutamine and L-tryptophan were superior to L-arginine, L-proline and L-ornithine in production of fresh biomass and induction of somatic embryos. Between the two amino acids that showed promotory effect, L-tryptophan supported a higher regeneration vigour and quality of somatic embryos than L-glutamine (Plate 29). L-arginine and L-proline were the least effective in production of fresh biomass of callus and induction of somatic embryos.

The amino acid type and concentration had no significant effect on the percentage of zygotic embryos that callused in ½ MS + 0.05 mg/l TDZ (Figure 9A). However, significant effects were seen in mean fresh mass per callus (Figure 9B) and the percentage of callus with somatic embryos (Figure 9C).

(a) 100mg/l L-glutamine (b) 100mg/l L-Tryptophan

Plate 29: The effects of L-glutamine and L-tryptophan on somatic embryogenesis in Melia volkensii zygotic embryo callus; (a) L-glutamine and (b) L-tryptophan on regeneration in zygotic embryo calluses 10 days after subculture to ½ MS +0 PGR; cumulative age: 35 days.

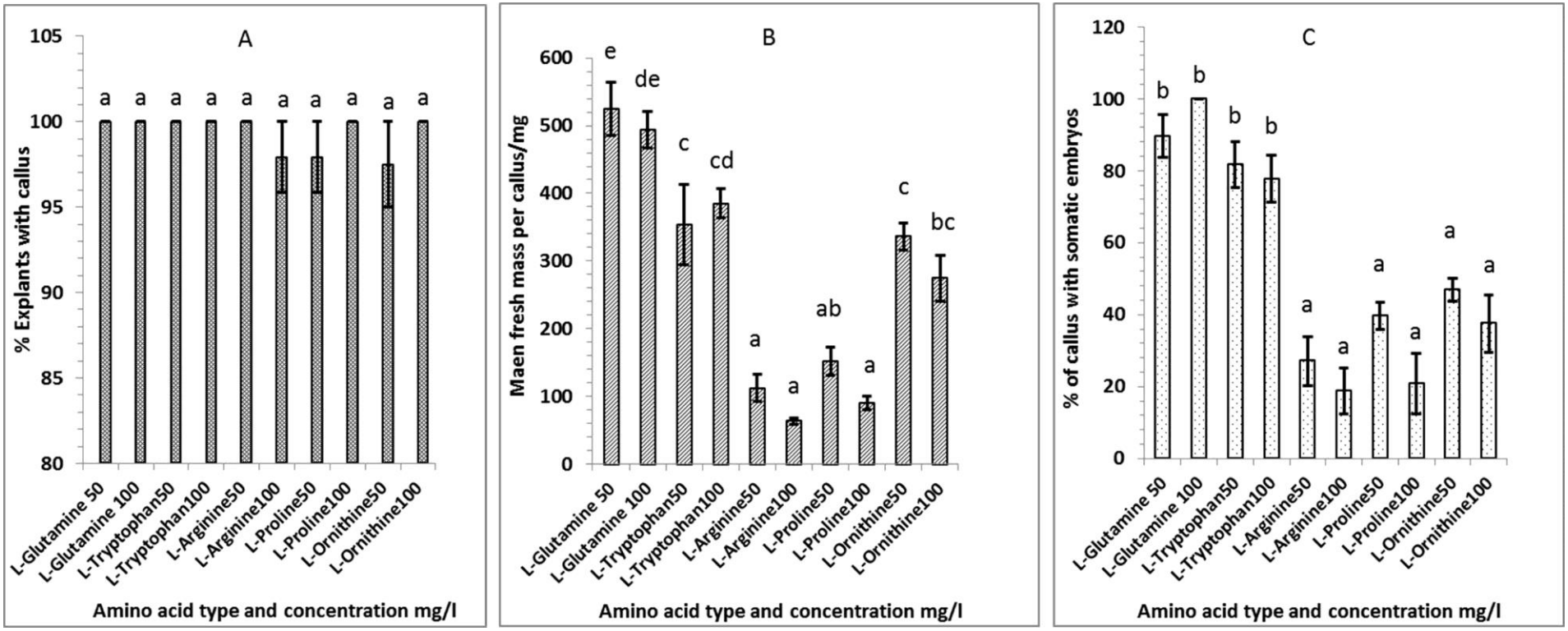


Figure 9: Effects of amino acid type and concentration on callus and somatic embryo induction in Melia volkensii; (A) Percentage of zygotic embryos with callus, (B) Callus fresh mass and (C) Percentage of callus with somatic embryos. All after 21 days on induction medium (½ MS + 0.05mg/l TDZ) with respective amino acid. Values with the same letter(s) do not differ significantly using Tukey's HSD test at $p \leq 0.05$.

4.1.8.3 Effect of Amino Acids on Regeneration in Cotyledons

L-glutamine, L-tryptophan, L-proline and L-ornithine all supported the formation of significantly higher ($p< 0.001$) fresh mass per cotyledon explant than L-arginine (Figure 10). The fresh biomass formed on medium containing L-arginine ranged from one-fifth to one-quarter of the biomass formed in media containing any of the other four amino acid types. Amino acid type also had significant effects ($p< 0.001$) on the percentage of cotyledon explants with shoots ≥ 5mm height. L-tryptophan and L-proline were the most effective amino acids in formation of microshoots (Figure 10). However, with the exception of L-tryptophan, raising the concentration of amino acid from 50 to 100mg/l had no significant effect on the percentage of explants forming microshoots (Figure 10). Overall, L-tryptophan at 100mg/l concentration had the most promotive effect on number of microshoots per explant. The second best amino acid for microshoot formation was proline, at either 50 or 100 mg/l.

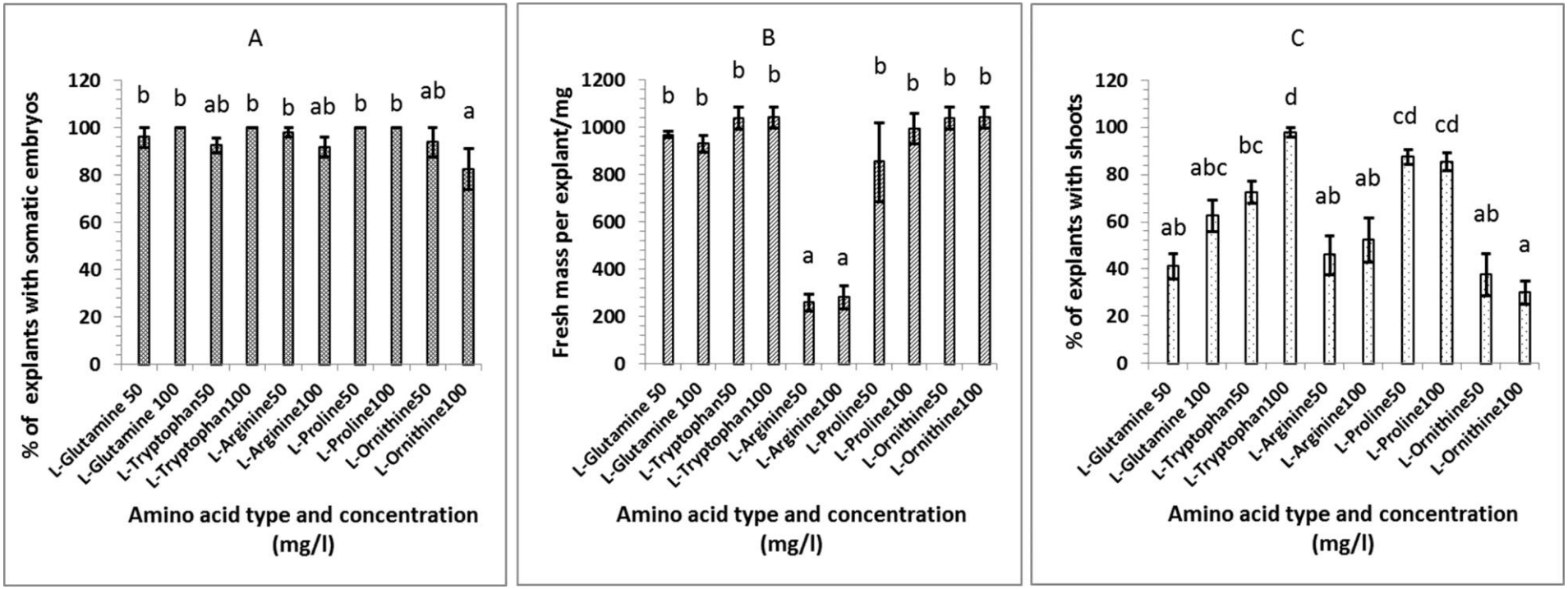


Figure 10: Effects of amino acid type and concentration on somatic embryo and shoot regeneration in Melia volkensii cotyledons; (A) Mean % cotyledons with direct somatic embryos and (B) Mean fresh mass per cotyledon explant and (C) Mean % of cotyledons with microshoots. All recorded after 21 days on induction medium (½ MS + 0.05mg/l TDZ and respective amino acid). Values with the same letter(s) were not significantly different using Tukey's HSD test at $p \leq 0.05$.

4.2. Histological Observation of mode of regeneration

4.2.1 Embryogenesis in Zygotic Embryo Callus

The regenerating callus masses used for the histology were in the same developmental stages as those described earlier in section 4.1.4 (Plate 17A, B, C). The histological examination revealed spongy tissue with loosely packed, lightly-stained cells and large air spaces in the cortical and central parts of the callus and closely packed, densely staining epidermal and sub-epidermal cells on the surface (Plate 30A). The pre-embryonic cell masses from which the somatic embryos developed arose from these epidermal and sub-epidermalcells.

These pro-embryonic masses were packed with densely stained isodiametric cells surrounded by a clearly defined protodermal layer (Plate 30B). The cell masses gave rise to globular, heart-shaped and torpedo-shaped stages of somatic embryos (Plate 30 C-E). Mature somatic embryos had a clear polarity with a shoot pole consisting of a shoot meristem and leaf primordia at one end and a root pole at the opposite end (Plate 30F). The root poles were loosely attached to the callus, which explains the ease with which the somatic embryos could detach from the callus. The embryos lacked vascular connection to the mother explant but some of them had well-defined procambium strands (Plate 30 C & F).

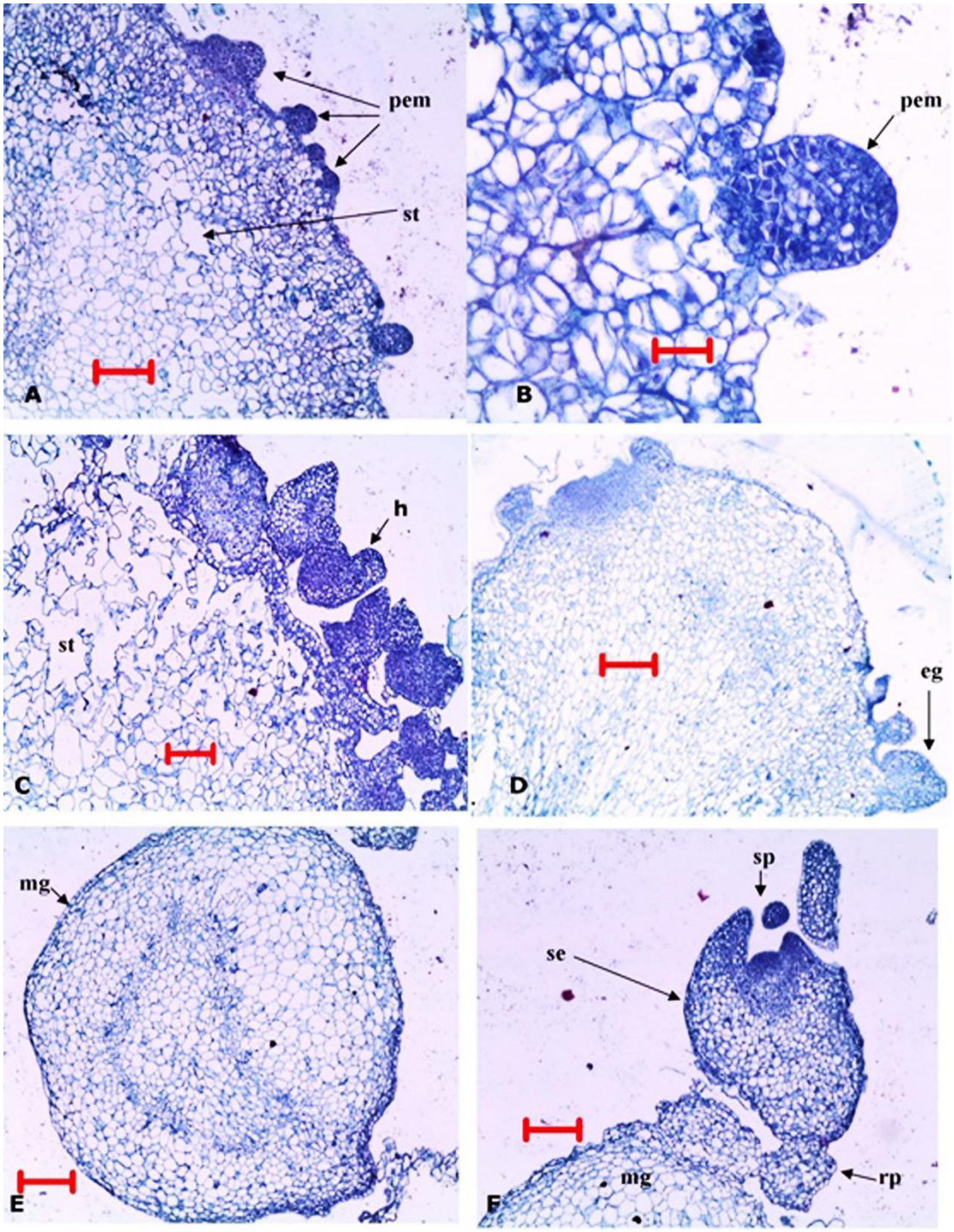


*Plate 30: Histology of somatic embryogenesis in Melia volkensii zygotic embryo callus. Developmental stages are shown by arrows. A/B: Loose spongy callus tissue (**st**) at centre with closely packed cells on the outer parts and epidermal globular proembryonic masses (**pem**). C: Spongy callus with multiple somatic embryos including the heart-shaped stage (**h**). D: Early globular stages (**eg**) developed from the proembryonic masses. E: Mature globular embryo (**mg**) in detail. F: Bipolar somatic embryo with a shoot pole (**sp**) and a root pole (**rp**). Scale bars A, C, D, E, F = 140 µm; B = 30 µm.*

4.2.2 Embryogenesis in Cotyledons

The cotyledon explants used for histology were in direct regeneration stages identical to those described earlier in section 4.1.5. (Plate 20 B,C,D). The histological examination revealed that somatic embryos (SEs) originated as nodular structures formed directly from epidermal and sub-epidermal cells. The nodular growths consisted of a distinct protoderm covering a dome-shaped mass of ground meristem cells. These meristematic cells were small, isodiametric, with densely staining cytoplasm (Plate 31 A). The nodular structures gave rise to globular, heart-shaped, torpedo and mature stages of somatic embryos that could be easily separated from the mother explant (Plate 31 B-E).

Mature somatic embryos were bipolar, with a well-developed shoot apical meristem and a root meristem at opposite poles (Plate 31F). Leaf primordia were evident at the plumular poles of the mature embryos. The embryos lacked vascular connection to the mother explant but most of them had well-defined procambium strands extending from the apical meristem to the radicular pole (Plate 31B & E). Some heterogeneity was observed in the size and morphology of somatic embryos.

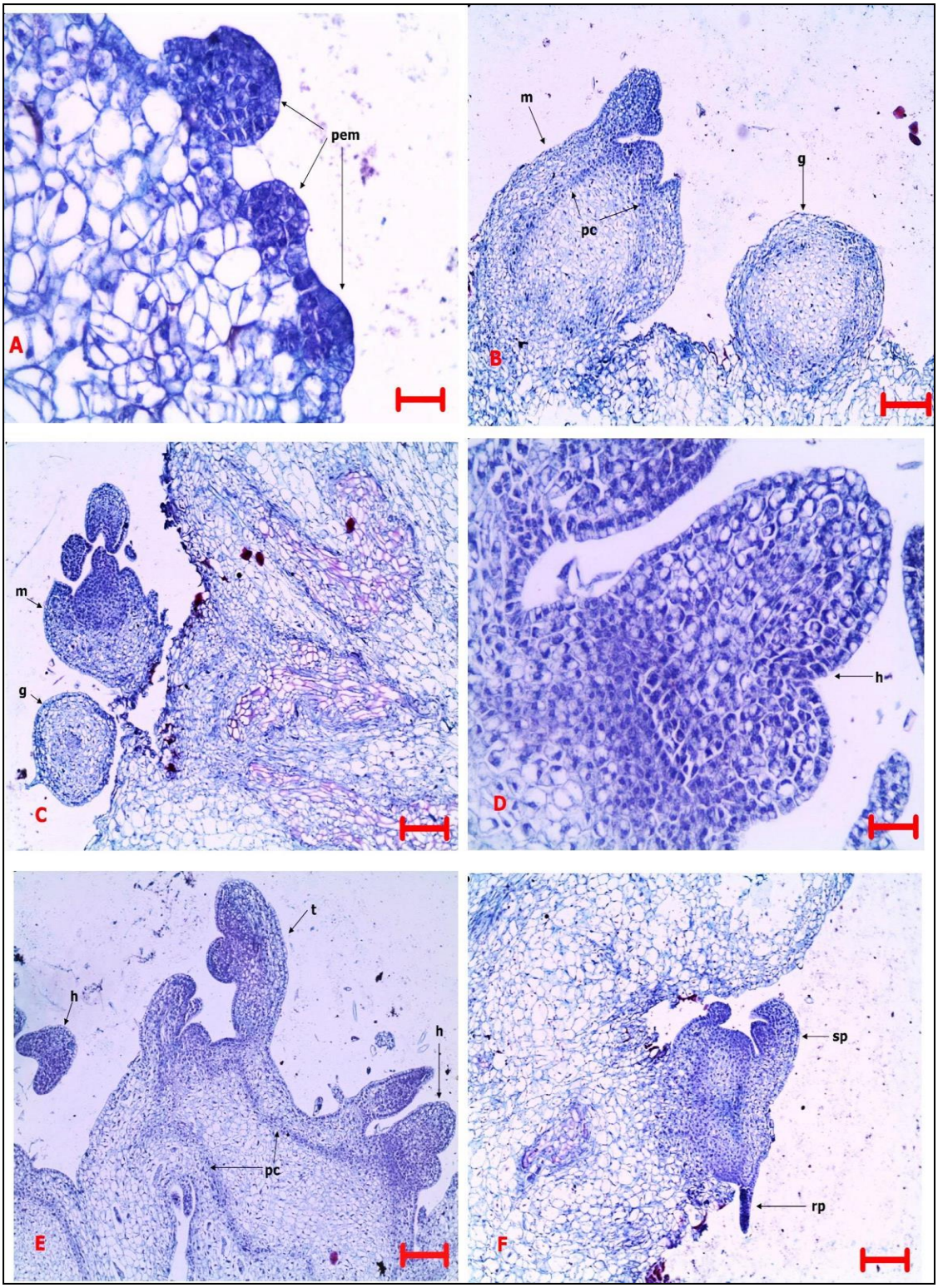


*Plate 31: Histology of direct somatic embryogenesis in Melia volkensii cotyledons. Developmental stages are shown by arrows. A: pro-embryonic masses (**pem**) arising from isodiametric, densely staining epidermal and sub-epidermal cells; B to E: globular (**g**), heart-shaped (**h**) and torpedo (**t**) stages of somatic embryogenesis and some mature somatic embryos (**m**) with well-defined procambial strands (**pc**); F: Embryo showing clear polarity with a distinct shoot pole (**sp**) and root pole (**rp**). Scale bars 30µm for A & D and 140µm for B, C, E & F.*

4.3 Morphological Characterization

4.3.1 General Variation in Morphological Characters

Significant morphological variation (F test, $p < 0.001$) was observed between the tissue culture regenerants and seed derived (wild type) seedlings in morphometric and meristic traits (Plate 32 & Table 17). In morphometric characters, the regenerants differed significantly from wild type seedlings in all the six traits measured (Table 17). Significant stunting of shoots occurred in both zygotic embryo (indirect) derived regenerants and cotyledon (direct) derived regenerants. Mean shoot heights (43.83 and 51 mm) of the regenerants were less than one-third of the mean shoot height of wild type seedlings (187.81 mm). Mean internode lengths (4.68 and 5.54 mm) of the regenerants ranged between one-fifth andone-sixth of those for wild type seedlings (Table 17). The leaves of regenerants were also smaller in leaflet length (c.a 30 mm) and mid-leaflet diameter (c.a 21-22 mm) in comparison to the wild type seedlings whose mean leaflet length was approximately 73 mm with mid-leaflet diameter of 64mm. However, the greatest difference in morphometric traits was seen in the mean length of taproots, with taproots of wild seedlings (131.2 mm) being 16 times longer than those of zygotic embryo regenerants (8.33 mm) and nearly 5 times longer than those of cotyledon regenerants (27.41 mm).

As for meristic characters, out of the five meristic traits used, four of them namely number of leaves per shoot, number of leaflets per rachis, number of leaf pinnules per leaflet and number of axillary bud sprouts were generally similar between regenerants and wild type seedlings. The means of these four traits generally overlapped when the standard errors were taken into account, indicating no significant differences. Only one meristic trait, mean number of lateral roots per plant, was different between the regenerants and the wild type seedlings. Lateral roots were

abundant in the wild type seedlings (mean of 24 per root) but significantly fewer (c.a 3 per root) in cotyledon regenerants and totally absent in zygotic embyo regenerants (Table 17). Therefore the inhibition of lateral root development was greater in *in vitro* plants regenerated via callus from zygotic embryos than those regenerated directly from cotyledon explants.

*Plate 32: Phenotypic comparison between tissue-cultured (TC) and wild type (seed derived) Melia volkensii seedlings (**WS**) from same parent tree. Age = 3 ½ months of growth in soil (Scale bar = 5 mm).*

Table 17: Descriptive statistics for morphological characters of tissue-cultured and normal Melia volkensii seedlings

(a) Morphometric measurements /mm (Mean ± s.e.m)						
Population	SH	IL	LR	LL	LD	LTP
ZE regenerants	43.83 ± 4.88[a]	4.68 ± 0.76[a]	15.28 ± 1.16[a]	30.44 ± 1.92[a]	21.18 ± 1.66[a]	8.33 ± 8.33[a]
Cotyledon regenerants	51.00 ± 3.58[a]	5.54 ± 0.56[a]	14.04 ± 1.85[a]	30.65 ± 1.96[a]	22.60 ± 1.15[a]	27.41 ± 8.17[b]
Wild type	187.81 ± 8.07[b]	28.06 ± 2.68[b]	31.78 ± 1.43[b]	72.50 ± 2.28[b]	63.79 ± 2.55[b]	131.2 ± 3.59[c]

(b) Meristic counts (Mean ± s.e.m)					
Population	NL	NLT	NLB	NAB	NLR
ZE regenerants	10.06 ± 0.30[a]	6.20 ± 0.33[a]	7.01 ± 0.24[a]	0.06 ± 0.06[a]	0[a]
Cotyledon regenerants	9.88 ± 0.29[a]	5.77 ± 0.27[a]	6.47 ± 0.21[b]	0.06 ± 0.06[a]	2.88 ± 1.04[b]
Wild type	8.41 ± 0.13[b]	5.14 ± 0.18[b]	7.64 ± 0.18[a]	0.21 ± 0.12[b]	24.11 ± 1.34[c]

***SH**= Shoot length, **IL**= Internode length, **LR**= Leaf rachis length, **LD**= Mid-of-leaf width, **LL**= Leaflet length, **LTP**= Length of taproot. (b) **NL** = Number of leaf nodes per shoot, **NLT**= Number of leaflets per compound leaf, **NLB**= Number of lobes/pinnules per leaf, **NAB**= Number of axillary bud sprouts per shoot, **NLR**= Number of lateral roots per root. For each character, values with the same superscript in a column do not differ significantly using Tukey's HSD test at $p \leq 0.05$.*

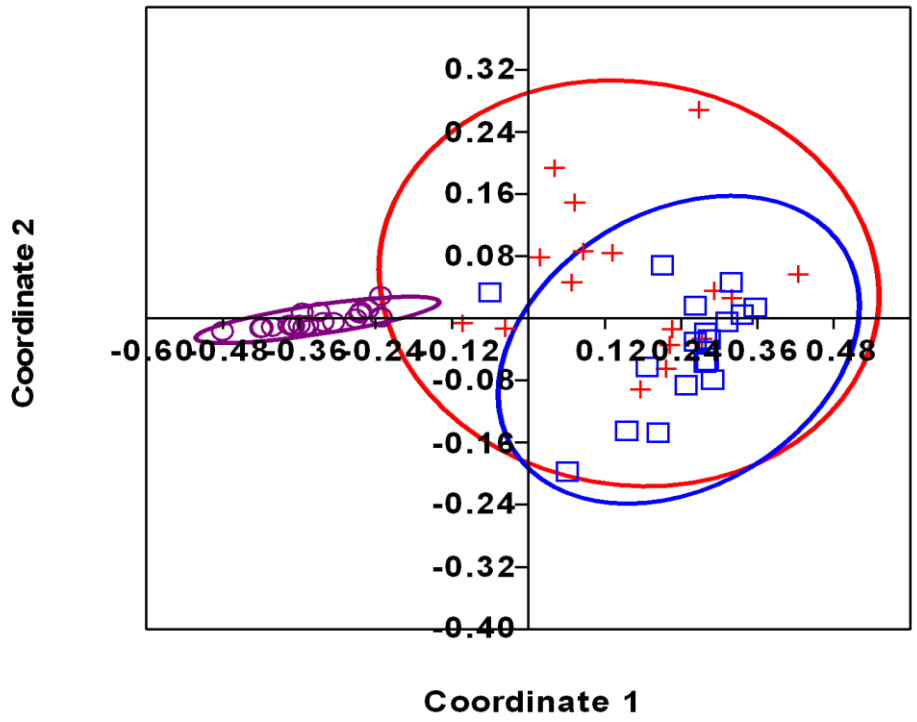
4.3.2. PCOORDA Analysis for Morphological Characters

Principal Coordinate Analysis (PCOORDA) for the morphological characters revealed a clear separation of the regenerants from the wild type for both the morphometric and meristic traits (Figure 11).

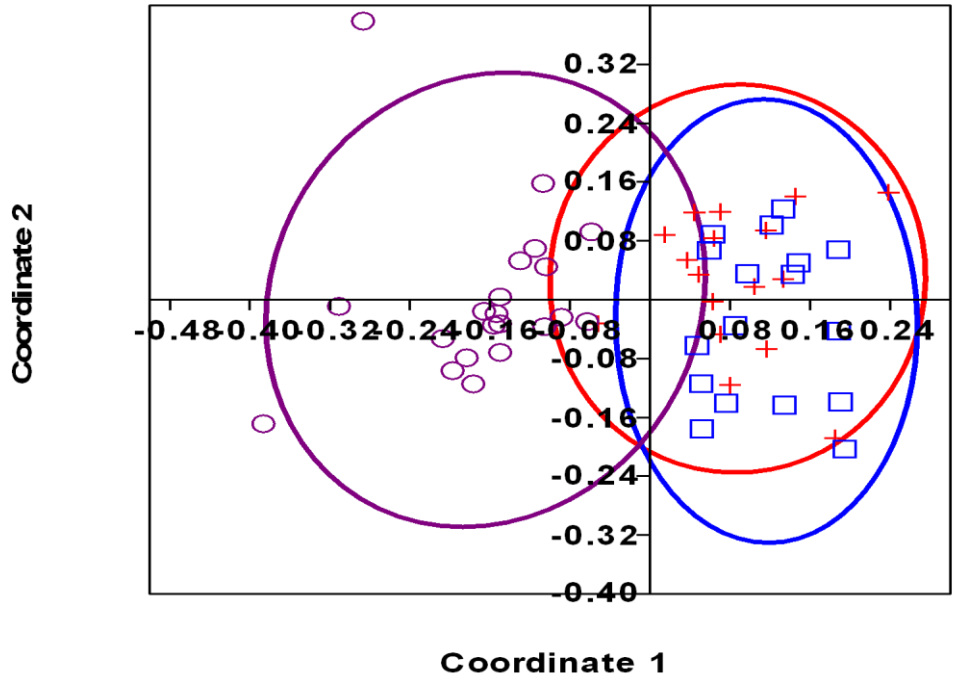
The 95% confidence ellipses of morphometric characters showed a complete separation between wild type seedlings and zygotic embryo regenerants, little overlap between wild type seedlings and cotyledons regenerants and a complete overlap betweeen cotyledon and zygotic embryo regenerants (Figure 11 A). This shows that the morphometric characters of the regenerants differed from those of the wild type seedlings but were similar between the two types of regenerants.

PCOORDA plots for the meristic characters showed increased overlap between the wild type seedlings and cotyledon regenerants (Figure 11B) and, unlike those of the morphometric traits, also gave an overlap between the wild type seedlings and zygotic embryo regenerants. This showed that the regenerants had a greater resemblance to wild type seddlings in the meristic characters than in morphometric ones. However, the two regenerants were similar in the measured meristic characters, just as they were similar in the morphometric characters.

In general, the PCOORDA plots revealed a greater difference between the regenerants and wild type seedlings in morphometric traits than in meristic traits. For both types of morphological characters, the largest separation from the wild type was seen in regenerants obtained from zygotic embryos via callus-mediated (indirect) somatic embryogenesis. Plants regenerated from cotyledons via direct somatic embryogenesis were morphologically closer to the wild type as shown by the larger overlap of the 95% confidence ellipses.



(A)



(B)

Figure 11: PCOORDA scatterplots and 95% confidence ellipses for regenerated and wild-type Melia volkensii (a) morphometric characters and (b) meristic characters, using the Gower measure of distance; Wild type (○) Cotyledon regenerants (+) and zygotic embryo regenerants (□).

The principal axes 1 and 2 accounted for 81.87% of the variation in morphometric traits (Table 18), showing that the separation by principal coordinates was successful. However, in the case of meristic traits, the principal axes 1 and 2 accounted for a much lower contribution (48.42%) to the total variation. This also indicates that, among the two morphological traits, there were significant differences in morphometric but not in meristic traits.

Table 18: Eigenvalues and variance contributions for morphological characters

Category	Axis	Eigenvalue	Contribution %	Total contribution %
Morphometrics	1	4.331	76.03	81.87
	2	0.293	5.34	
Meristics	1	1.173	31.83	48.42
	2	0.611	16.59	

4.3.3 Cluster Analysis for Morphological Characters

Cluster analysis also showed a clear separation of the regenerants from wild type seedlings based on morphological characters (Figure 12). When clustered based on morphometric traits, there were two distinct clusters. Wild type seedlings formed a distinct cluster with 84% bootstrap reliability (Figure 12) whilst the two types of regenerants clustered together in the second cluster, with a lower bootstrap value of 48%. There was a high level of within-cluster overlap among and between the two types of regenerants, showing high similarity and little differentiation between the regenerants with respect to morphometric traits.

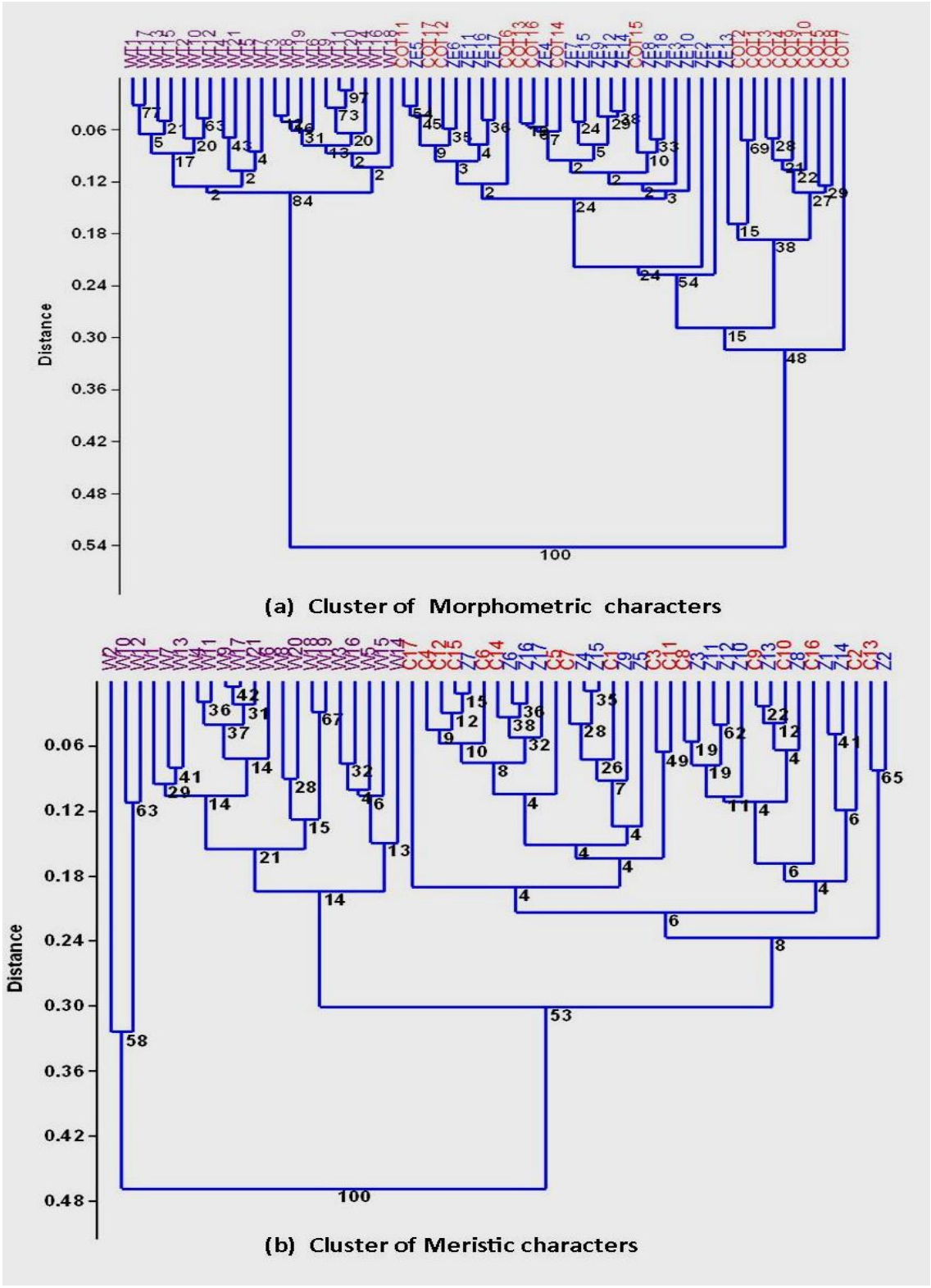


*Figure 12:Paired-group dendrogram for morphological characters (a) Morphometric traits and (b) meristic traits. Node values are bootstrap percentages for 1000 replications.**WT** or **W** = wild type seedlings, **COT or C** = cotyledon regenerants and **ZE or Z** = zygotic embryo.*

Clustering using meristic traits also produced two significant clades, a smaller clade with 58% bootstrap reliability consisting of only three wild type seedlings and a much larger clade with 53% bootstrap reliability, in which most of the wild type seedlings and the two types of regenerants were clustered (Figure 12). Within-cluster overlap among and between the wild type and the two types of regenerants was greater in meristic traits than in morphometric ones. This indicates that the wild type seedlings and regenerants had a higher level of similarity in the meristic traits than in morphometric ones.

4.3.4 Analysis of Similarity (Anosim) and Similarity Percentages (SIMPER) Analysis for Morphological Characters

Anosim results further validated the PCOORDA and cluster analysis results**.** Morphometric and meristic traits gave Anosim R-values of 0.778 ($p < 0.0001$) and 0.688 ($p < 0.0001$) respectively, indicating significant morphological dissimilarity (or distance) between the regenerants and wild seedlings (Table 19).

Table 19:One-way Anosim statistics for morphological traits of regenerated and wild-type Melia volkensii seedlings

Anosim test results					
Test Permutations	Mean rank within	Mean rank between	R statistic	*p*(same)	Inference
10,000	365.8	964.5	0.778	0.000099	Significant dissimilarity between groups
10,000	397.8	908.0	0.688	0.000099	Significant dissimilarity between groups

The Similarity Percentages (SIMPER) analysis revealed that pair-wise morphological dissimilarities were generally greater between wild seedlings and regenerants than between the two types of regenerants themselves (Table 20). Morphological dissimilarities were greatest between wild type seedlings and zygotic embryo (indirect) regenerants, at 27.10% for morphometric and 41.47% for meristic traits respectively. Conversely, this means that the least pair-wise percentage similarity values in morphological traits were 72.90% (for morphometrics) and 58.53% (for meristics), which occurred between the wild type seedlings and zygotic embryo regenerants.

Table 20:Pair-wise SIMPER dissimilarity percentages for morphological characters

(a) Morphometric characters (% dissimilarity)			
	Cotyledon regenerants	Zygotic embryo regenerants	Wild type
Cotyledon regenerants	0		
ZE regenerants	12.92	0	
Wild type	20.92	27.10	0
(b) Meristic characters (% dissimilarity)			
	Cotyledon regenerants	Zygotic embryo regenerants	Wild type
Cotyledon regenerants	0		
ZE regenerants	13.44	0	
Wild type	35.15	41.47	0

Out of the overall average morphometric dissimilarity observed, length of taproot alone accounted for 41.52% (Table 21) of the dissimilarity. This was followed by internode length (17.25%) and shoot height (13.60%). These three traits cumulatively accounted for 72.37% of the overall dissimilarity in morphometric characters.

As for the meristic traits, the mean number of lateral roots was the single most important source of dissimilarity, accounting for 77% of the dissimilarity, followed by the mean number of leaves and leaflets at 7.82 and 7.17% respectively. These three traits accounted for 91.99% of the overall dissimilarity in meristic characters.

Table 21: SIMPER ranking of individual and cumulative contributions of morphological characters to overall average dissimilarity between regenerated and wild-type Melia volkensii seedlings.

(a) Morphometric characters			
Character	Average dissimilarity	Contribution %	Cumulative contribution %
1. Length of tap root	8.64	41.52	41.52
2. Internode length	3.59	17.25	58.77
3. Shoot height	2.83	13.60	72.37
4. Mid-of-leaf diameter	2.15	10.33	82.70
5. Leaf length	1.82	8.75	91.45
6. Leaf rachis length	1.78	8.55	100
Overall average dissimilarity	20.81	-	-
(b) Meristic characters			
Character	Average dissimilarity	Contribution %	Cumulative contribution %
1. Number of lateral roots	24.26	77.02	77.02
2. Mean number of leaf nodes per shoot	2.46	7.81	84.83
3. Mean number of leaflets per leaf	2.26	7.17	92.00
4. Mean number of pinnules/lobes on leaflets	2.12	6.73	98.73
5. Number of axillary bud sprouts on shoot	0.40	1.27	100
Overall average dissimilarity	31.50	-	-

4.4 Molecular Characterization using RAPD Markers

4.4.1 Quality and Yield of Extracted DNA

The DNA extraction method used in this study produced good quality, high molecular weight genomic DNA (gDNA) with lengths greater than 10.0 kilobases (Plate33). The gDNA bands were highly resolved, with minimal smears in most of the lanes. The gel image also showed a high level of similarity in molecular lengths of the gDNA extracted from tissue cultured plants and the normal seedlings (wild type).

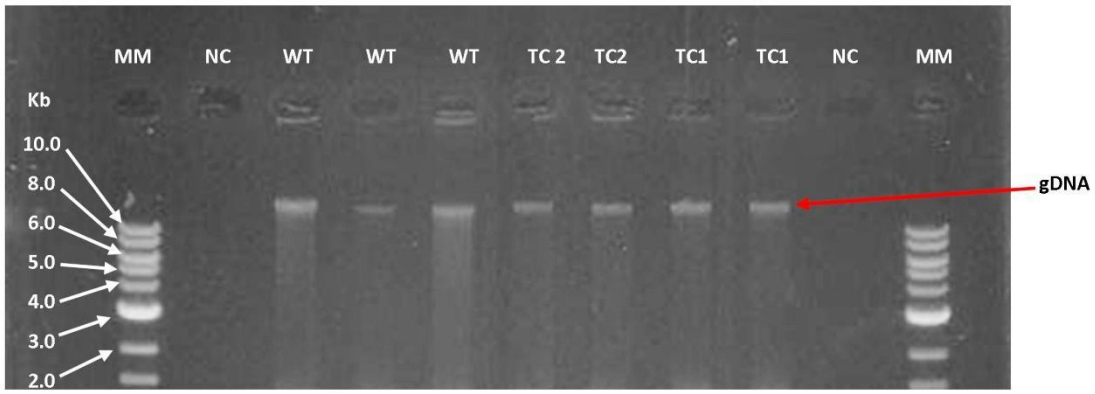


Plate 33: Gel profile for Melia volkensii genomic DNA samples (1-7) after 45 minute run at 75V in 0.8% agarose gel stained with ethidium bromide. MM = molecular markers in kilobases (1kb Bioline Hyperladder®).

The yield and purity levels of the extracted DNA were good (Table 22). The A_{260}/A_{280} ratios for DNA of cotyledon regenerants (1.81) and wild type seedlings (1.83) were close to the ideal ratio of 1.80 expected for pure DNA. However, the A_{260}/A_{280} ratio for zygotic embryo regenerants (1.60) was unexpectedly lower than ideal, indicating more contamination with macromolecules in the DNA extracted from the zygotic embryo callus. A_{260}/A_{230} ratios obtained for the three groups of plants ranged from 0.68 to 1.69 and were generally much lower than the ideal ratio of 2.0-2.2.

Table 22: Concentration and purity ratios for extracted Melia volkensii DNA

Plant type	**DNA concentration ng/µl**	**A_{260}/A_{280} ratio**	**A_{260}/A_{230} ratio**
Cotyledon regenerants	915.40 ± 86.59	1.81 ± 0.04	1.33 ± 0.28
Zygotic embryo regenerants	379.70 ± 104.04	1.60 ± 0.02	0.68 ± 0.03
Wild type seedlings	1528.32 ± 148.60	1.83 ± 0.03	1.69 ± 0.08

Values are means ± standard error

4.4.2. Primer Selection

All the 8 candidate primers, Mel-1 to Mel-8, amplified *M. volkensii* g-DNA, generating a total of 371 monomorphic and polymorphic RAPD bands/markers (Plate 35). The overall amplification frequency for the 80 PCR-RAPD reactions carried out during primer screening was 69 out of 80 (86.25%). The mean number of bands/loci per lane was 5.03±1.39. Mel-5 primer, with an oligonucleotide sequence of 5' TGC CGA GCT G 3', produced the maximum number of highly resolved bands (9) in some of the amplified samples (Plate 34), largest mean number of bands or loci per lane (7.11±2.52) and second highest overall percentage of polymorphic loci (23.33) (Table 23). Altogether, the eight screened primers produced a total of 30 polymorphic RAPD bands/markers from the 69 amplified DNA samples.

From the primer screening test using the eight candidate primers, Mel-5 was the primer with the most number of amplicons followed by Mel- 4, Mel-6 and Mel-8, in that order. On the basis of the quantitative yield (number) and quality (band distinctiveness) of the amplicons in both the wild type and tissue-cultured plants, Mel-

5 was selected, as the most suitable primer among the eight candidate primers for random amplified polymorphic DNA (RAPD) analysis in *M. volkensii*.

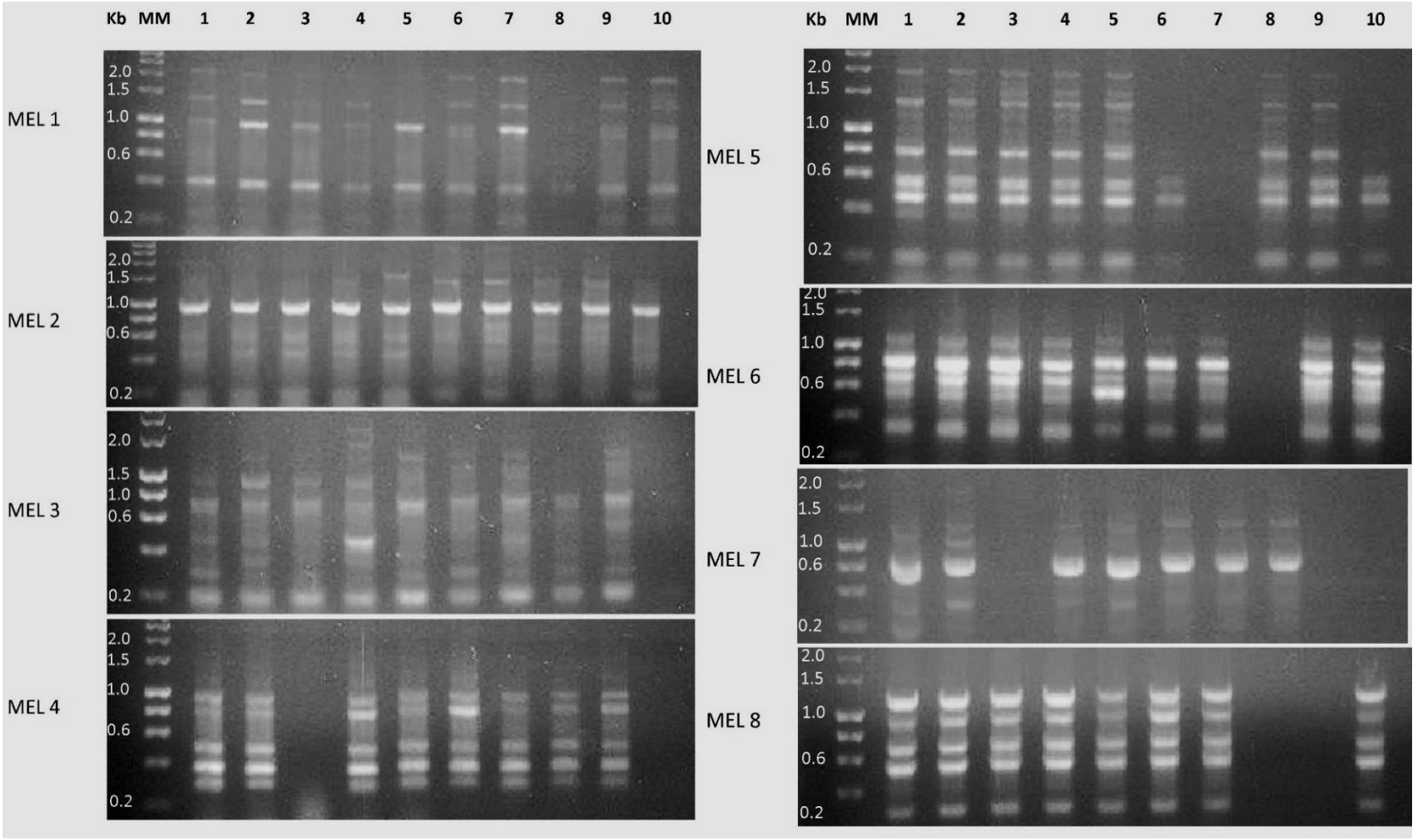


Plate 34:PCR-RAPD profiles for Melia volkensii genomic DNA with eight primers; showing amplified and non-amplified lanes for 10 samples of g-DNA. ***MM****= molecular markers (Bioline Hyperladder®),* ***lanes 1-5*** *= DNA of tissue cultured plants,* ***lanes 6 -10*** *= DNA of wild seedlings.* ***MEL 1-8*** *= code names of the primers used.*

Table 23: Descriptive statistics for Melia volkensii genic variation characters revealed by the candidate primers with 10 samples of g-DNA

Primer	Bands/loci resolution	Bands/loci per lane	N_a	N_e	H	I	N_p	P_p
Mel-1	Low	4.89 ± 1.05	1.30 ± 0.47	1.17 ± 0.34	0.09 ± 0.18	0.15 ± 0.25	9	30.00
Mel-2	Low	4.40 ± 0.69	1.13 ± 0.35	1.05 ± 0.18	0.03 ± 0.11	0.05 ± 0.16	4	13.33
Mel-3	Low	5.56 ± 0.88	1.17 ± 0.38	1.10 ± 0.28	0.06 ± 0.15	0.09 ± 0.21	5	16.67
Mel-4	High	5.00 ± 0.00	1.10 ± 0.31	1.05 ± 0.19	0.03 ± 0.09	0.04 ± 0.14	3	10.00
Mel-5	High	7.11 ± 2.52	1.23 ± 0.43	1.16 ± 0.35	0.09 ± 0.18	0.13 ± 0.25	7	23.33
Mel-6	High	5.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0	0.00
Mel-7	Low	3.29 ± 0.49	1.03 ± 0.18	1.01 ± 0.06	0.01 ± 0.04	0.01 ± 0.07	1	3.33
Mel-8	High	5.00 ± 0.00	1.00 ± 0.00	1.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0	0.00

*Total number of bands/markers by molecular sizes = 30. N_a= observed number of alleles, N_e=Effective number of alleles, **H**=Nei's gene diversity index, **I**=Shannon's information index, N_p= Number of polymorphic alleles, P_p= Percentage polymorphism (Means ± standard deviation).*

4.4.3. RAPD-marker genic variation

Amplification frequency using the selected primer (Mel-5) was 107 out a total of 130 PCR reactions (82.31%) for the regenerants (Plates 35 and 36) and wild seedlings (Plate 37) combined. From the 107 PCR-RAPD reactions, this primer generated 19 distinct band sizes or loci ranging from of 200 to 2000 base pairs, with a total of 759 monomorphic and polymorphic RAPD bands.

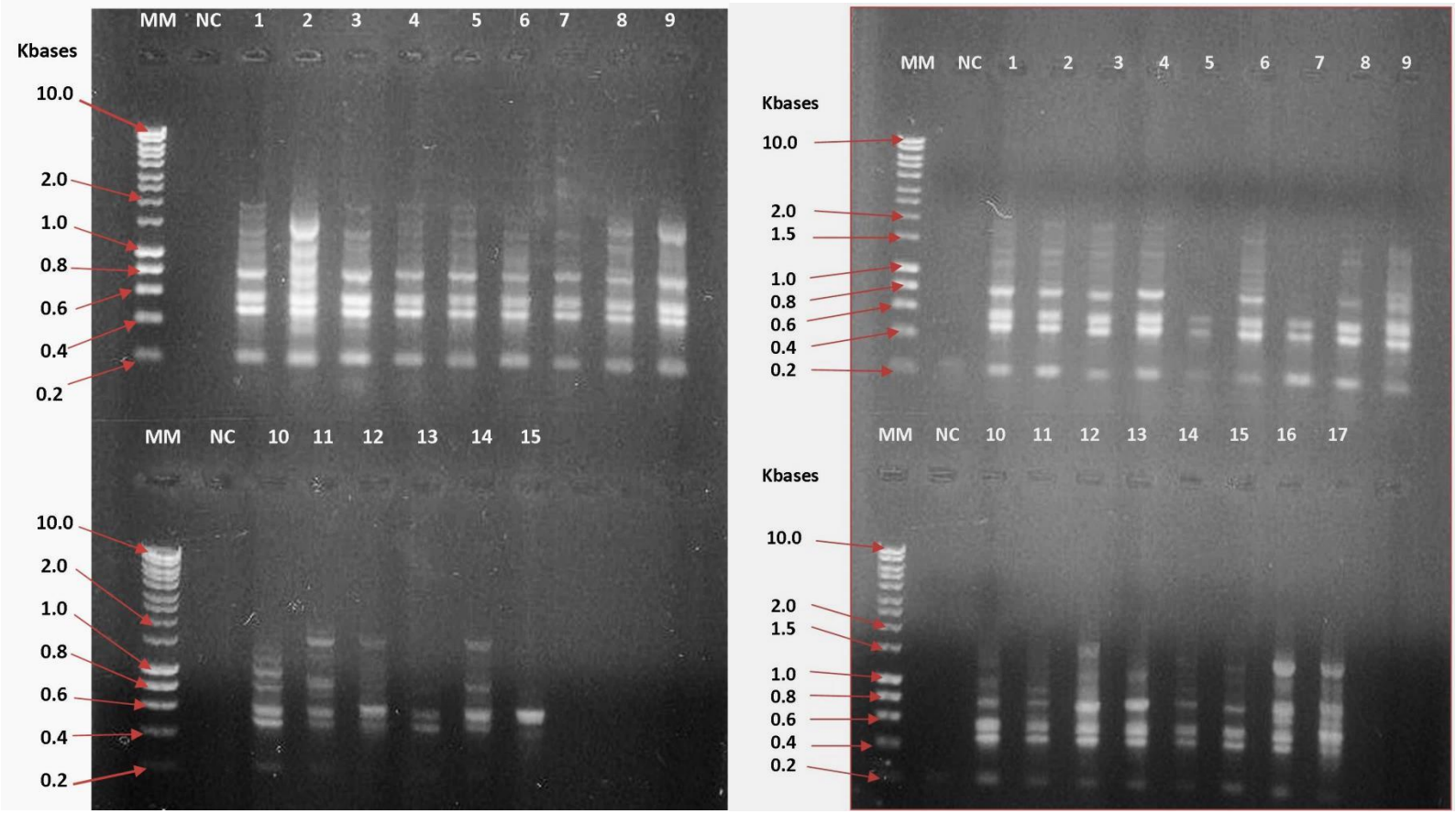
Two genic characters, the observed number of alleles (N_a) and effective number of alleles (N_e), showed little difference between the regenerants and wild (seed derived) seedlings (Table 24). However, Nei's gene diversity index (H) and Shannon's information index (I) were considerably greater in the regenerants than in the wild type seedlings. In addition, the two measures of diversity (H and I) were generally higher in the non-clone regenerants than in the cloned ones. Nei's gene diversity index was 9-9.5 and 4.5-6.5 times greater, respectively, in non-cloned and cloned regenerants than in wild type seedlings (Table 24). Shannon's information index was 9-9.3 and 4.3-6 times greater, respectively, in non-cloned and cloned regenerants than in wild type seedlings. The number of polymorphic loci and percentage of polymorphic loci were both 9-11 and 4-5 times greater, respectively, in non-cloned and cloned regenerants than in wild type seedlings.

Among the tissue-cultured plants, the non-clones showed greater gene diversity than the clones. Gene diversity was also greater in the plants obtained from zygotic embryos via indirect regeneration than those obtained from cotyledons via direct regeneration.

Table 24: RAPD-based genic characters of tissue-cultured and normal Melia volkensii seedlings

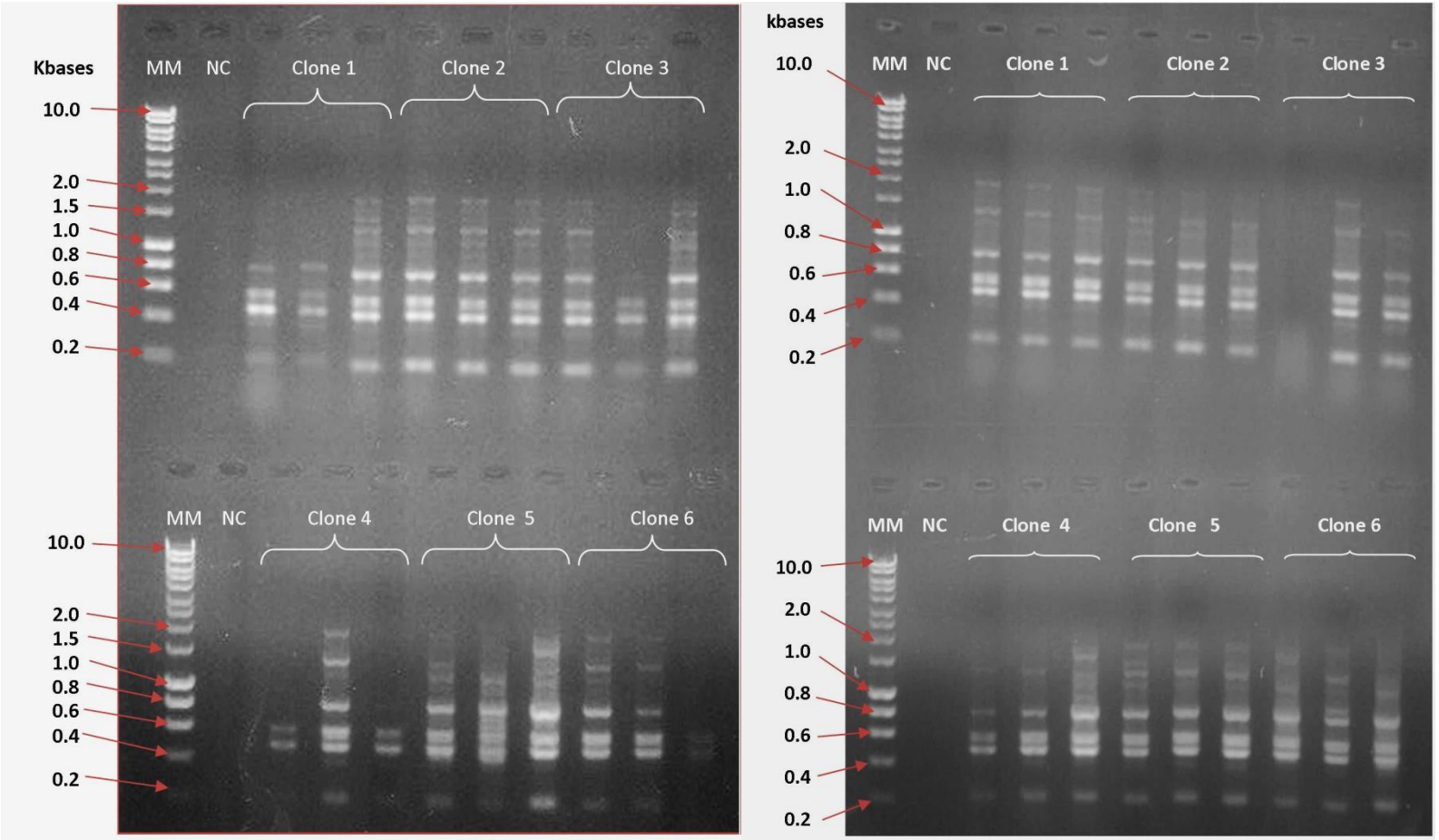
Population	**Amplified/Total DNA samples**	**N_a**	**N_e**	**H**	**I**	**N_p**	**P_p (%)**
ZE non-clones	19/20	1.58 ± 0.51	1.30 ± 0.37	0.18 ± 0.19	0.28 ± 0.28	11	57.89
Cotyledon non-clones	17/20	1.47 ± 0.51	1.33 ± 0.41	0.19 ± 0.22	0.27 ± 0.31	9	47.37
ZE clones	22/30	1.26 ± 0.45	1.25 ± 0.42	0.13 ± 0.22	0.18 ± 0.31	5	26.32
Cotyledon clones	21/30	1.21 ± 0.42	1.16 ± 0.33	0.09 ± 0.18	0.13 ± 0.26	4	21.05
Wild type	28/31	1.05 ± 0.23	1.03 ± 0.12	0.02 ± 0.07	0.03 ± 0.12	1	5.26
Overall	107/131	1.90 ± 0.03	1.45 ± 0.04	0.26 ± 0.02	0.39 ± 0.03	30	31.58

*Total number of bands/markers by molecular sizes =19. **ZE**= Zygotic embryo. N_a= observed number of alleles, N_e=Effective number of alleles, **H**=Nei's gene diversity index, **I**=Shannon's information index, N_p= Number of polymorphic alleles, P_p= Percentage polymorphism (Means ± standard deviation).*



(A) *(B)*

Plate 35: RAPD profiles of non-clones of Melia volkensii in vitro plants regenerated from zygotic embryos and cotyledons, Mbeere ecotype. PCR carried out with MEL-5 primer. ***MM*** *= molecular size ladder in kilobases,* ***NC*** *= negative control, 1-15 = different in vitro plants. Four other amplified samples not shown in gel (A).*



(A) *(B)*

Plate 36: RAPD profiles of 18 cloned Melia volkensii in vitro plants regenerated from zygotic embryos and cotyledons, Mbeere ecotype. PCR carried out with MEL-5 primer. ***MM*** *= molecular size ladder in kilobases,* ***NC*** *= negative control. Results for clones 7 to 10 are not shown in the gels.*

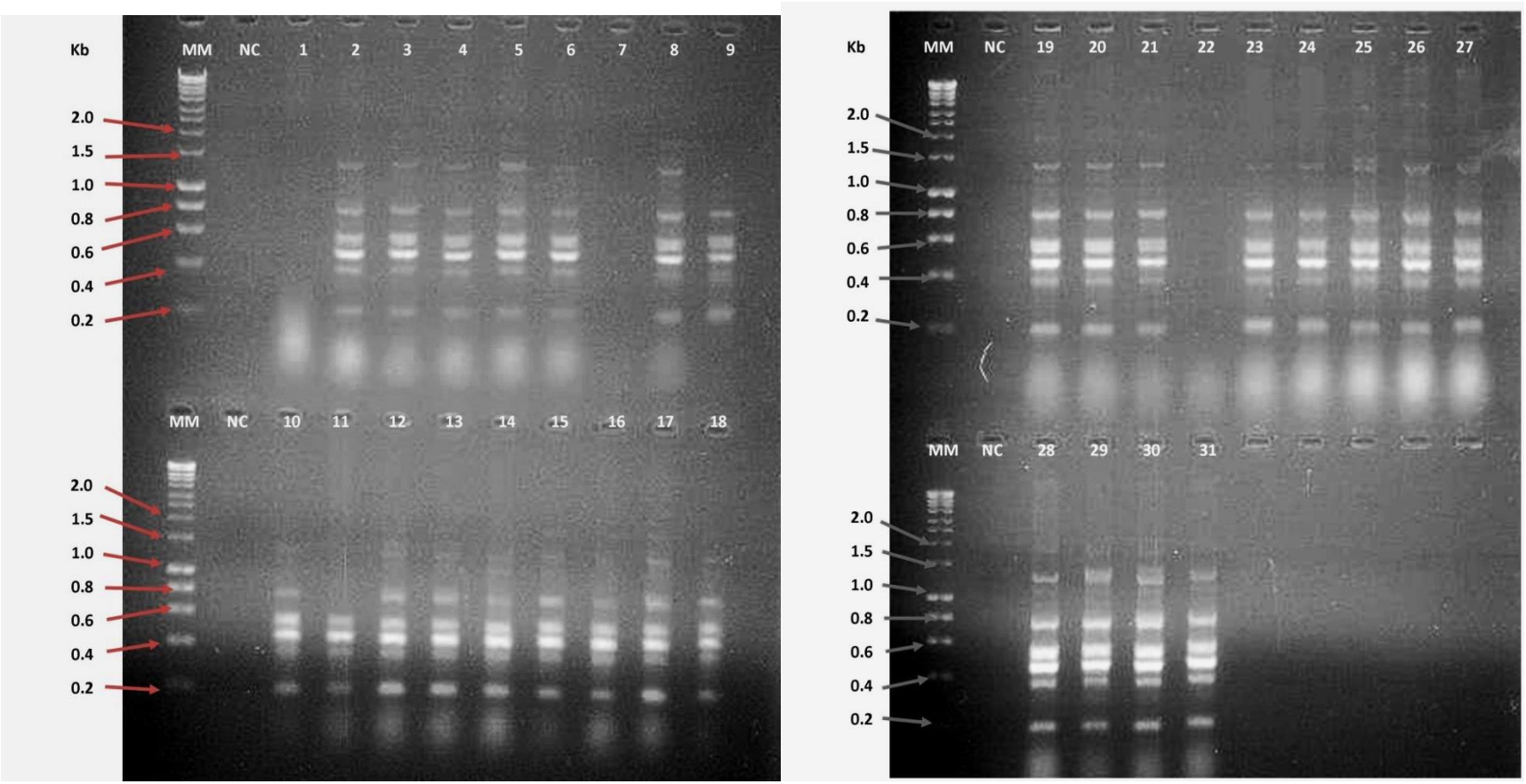


Plate 37: RAPD profiles of 31 normal Melia volkensii seedlings showing amplified and non-amplified lanes. PCR carried out with MEL-5 primer. ***MM*** *= molecular size ladder in kilobases,* ***NC*** *= negative control. Primer = MEL-5.*

4.4.4. PCOORDA analysis for genic variation characters

The PCOORDA plot for RAPD-based genic variation characters showed little or no genetic differentiation between the regenerants and the wild type plants. There were very large overlaps in the 95% confidence ellipses of the regenerants and wild type seedlings (Figure 13).

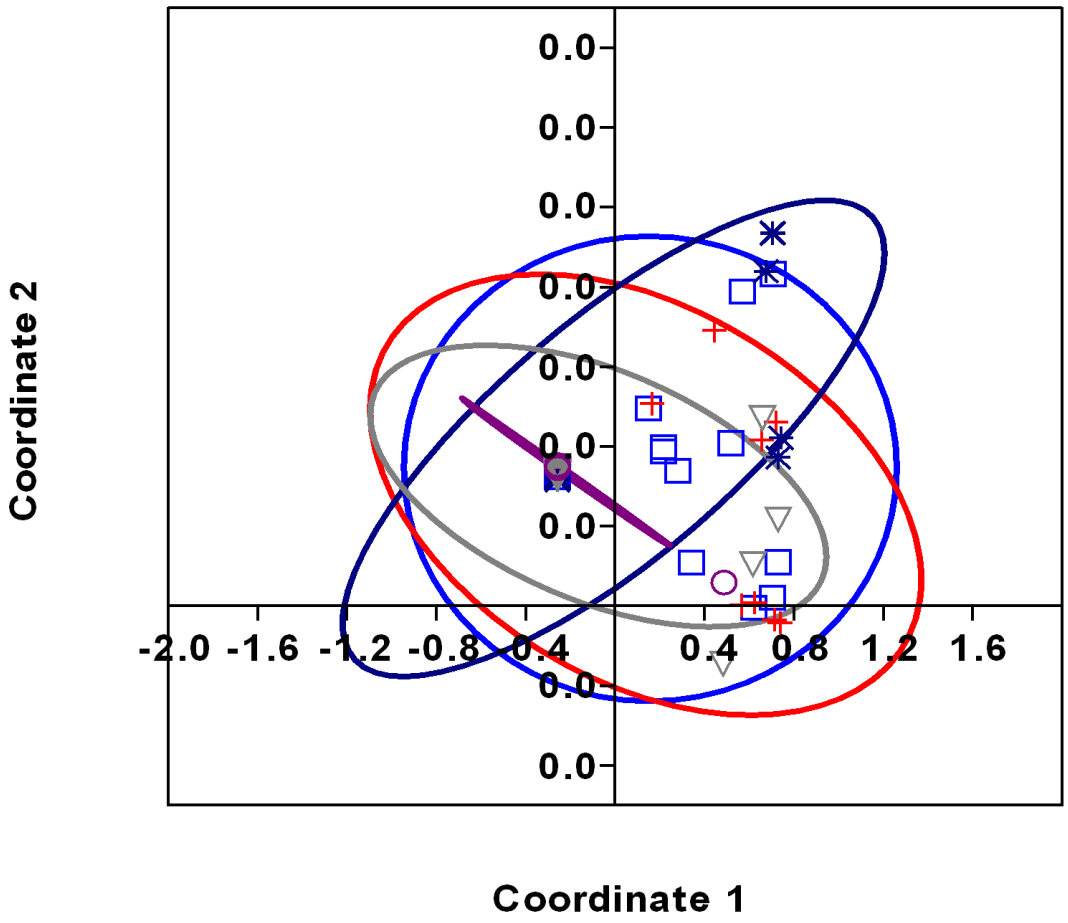


Figure 13: PCOORDA scatter plots and 95% confidence ellipses for genic characters, using Gower measure of distance. Zygotic embryo non-clones (□), zygotic embryo clones (✳), cotyledon non-clones (▽), Cotyledon clones (+), wild type (○).

4.4.5 Cluster Analysis for Genic Variation Characters

Clustering based on genic variation characters revealed lack of genetic distance in 66 out of the total of 96 (68.75%) plants tested and presence of some genetic differentiation in the remainder (31.25%) (Figure 14). The no-distance clade, represented in the dendrogram by a horizontal line at 0.0 distance, consisted of 94.7% of all the wild type DNA samples tested, 71.4% of the entire DNA from cotyledon clones, 63.6% of the entire DNA from zygotic embryo clones, 52.6% of DNA from cotyledon non-clones and 42.9% of DNA from zygotic embryo non-clones.

Based on this clustering, it can be seen that zygotic embryo non-clones showed the largest genetic differentiation between regenerants and wild type seedlings at 57.1%. These were followed by cotyledon non-clones at 47.4%. Cloned plants had lower genetic differentiation percentages with zygotic embryo clones having 36.4% genetic differentiation from wild type whilst the value for cotyledon clones was 28.4%. Consequently cotyledon clones were identified as the group of regenerants that was closest to the wild type seedlings in genetic similarity.

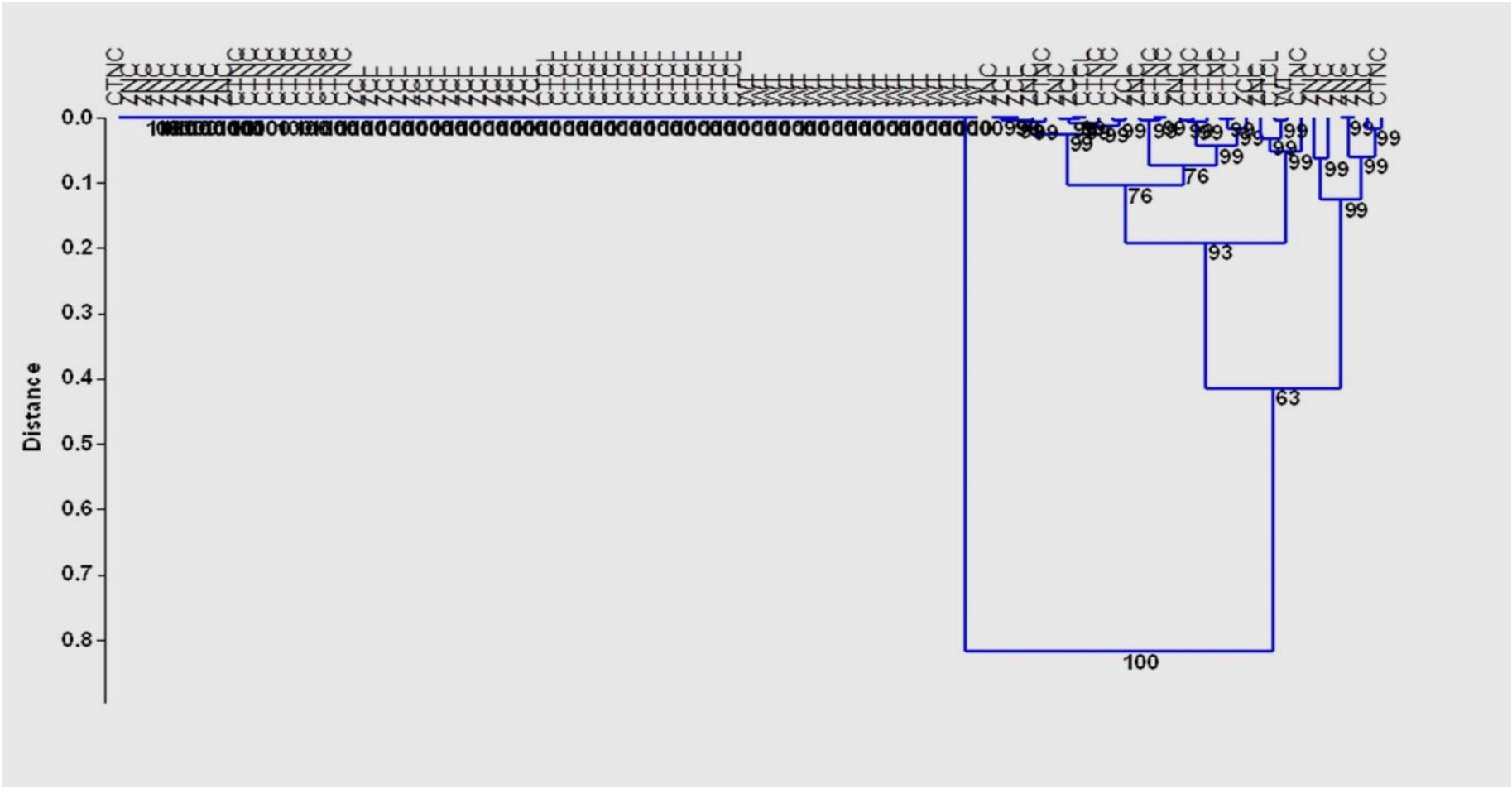


Figure 14: Paired-group dendrogram for genic characters of regenerated and wild-type Melia volkensii seedlings. Node values are bootstrap percentages for 1000 replications. ***CTNC****= Cotyledon non-clones,* ***ZNC****=Zygotic embryo non-clones,* ***ZCL****=Zygotic embryo clones,* ***CTCL*** *= Cotyledon clones,* ***WT****=Wild type.*

4.4.6 Analysis of Similarity (Anosim) and Similarity Percentages (SIMPER) for Genic Characters

For the genic characters, Anosim results also validated the PCOORDA and cluster analysis results**.** The RAPD-based genic variation characters gave an Anosim R-value of 0.047, showing no significant genetic dissimilarity (significant similarity) between the regenerants and wild seedlings (Table 25).

Table 25:One-way Anosim for genic characters of regenerated and wild-type Melia volkensii

Statistic	**RAPD-marker genic variation characters**
Mean Rank within	2147
Mean Rank between	2253
R value	0.047
p(same)	0.0077
Inference	Significant similarity between groups

According to the SIMPER test, dissimilarity percentages for genic variation characters averaged around 20% between wild type seedlings and non-clone regenerants on one hand and 9.82-15.61% between wild type seedlings and cloned regenerants on the other hand (Table 26). This indicates high levels of genetic similarity between the *in vitro* plants and wild seedlings as measured by the RAPD markers. Based on these values, similarity between non-clone regenerants and wild type seedlings was in the range of 79.20 to 80.99%. That between cloned regenerants and wild type seedlings was in the range of 87.59 to 90.18%.

Apportionment of contributions of each genic character to the observed overall dissimilarity among the genic characters by the SIMPER test revealed that observed number of alleles was the single most important source of dissimilarity among these characters, accounting for 37.66% of the overall average dissimilarity (Table 27). This was followed by effective number of alleles and Shannon's information index at 26.22% and 21.44% respectively. These three variables cumulatively accounted for 85.32% of the overall dissimilarity in genic characters. On its own, Nei's gene diversity index accounted for only 14.68% of the overall average dissimilarity. This shows that, using Nei's gene diversity index alone, the overall genetic similarity between the regenerants and wild type seedlings can be estimated at 85.32%.

Table 26:Pair-wise SIMPER disimilarity percentages for genic characters of regenerated and wild-type Melia volkensii seedlings.

Genic variation characters (% dissimilarity)					
	Cotyledon non-clones	Z.E non-clones	Cotyledon clones	Z.E clones	Wild type
Cotyledon non-clones	0				
Z.E non-clones	21.17	0			
Cotyledon clones	19.90	21.13	0		
Z.E clones	20.50	21.59	15.61	0	
Wild type	19.01	20.80	12.41	9.82	0

Table 27: SIMPER ranking of individual and cumulative contributions of genic characters of regenerated and wild-type Melia volkensii seedlings

Genic variation characters			
Character	Average dissimilarity	Contribution %	Cumulative contribution %
1. Observed number of alleles	6.85	37.66	37.66
2. Effective number of alleles	4.77	26.22	63.88
3. Shannon's information index	3.90	21.44	85.32
4. Nei's gene diversity	2.67	14.68	100
Overall average dissimilarity	18.19	-	-

CHAPTER FIVE: DISCUSSION

5.1 *In Vitro* Regeneration

5.1.1 Suitability of Explants for *In Vitro* Culture and Regeneration

In the present study, *M. volkensii* axillary buds excised from semi-hard cuttings and segments of the nine different stages of immature fruits used were non-responsive. They browned and failed to show either callogenesis or regeneration. This is probably due to either low totipotency or the release of polyphenolic compounds from the cut surfaces of explants. Such compounds get oxidized by polyphenol oxidase enzymes forming substances that are widely reported to cause inhibition of enzymes, cell death and browning of explants (Arnaldos *et al*., 2001; Chawla, 2004; Ko *et al.*, 2009; Amalia *et al*., 2014).

Carpels, leaves, petioles/rachis, mature zygotic embryos, root segments and seedling epicotyls readily formed callus in a variety of media. Ease of callusing varied between explants with highest ability recorded in zygotic embryos and root segments and lowest ability in petioles and rachis explants. However, the response of cotyledon explants was uniquely different from that of all other explants. No callus induction occurred in the cotyledons. Instead, nodular growths formed directly from the explants and shoots were produced from the nodular masses. Similar variability in callus induction and regeneration ability between explant types is reported in some members of Meliaceae such as *Melia azedarach* L., (Vila *et al*., 2004), *Azadirachta indica* (Kota *et al*., 2006) and *Cedrela montana* (Basto *et al*., 2012). Reasons for this phenomenon include differences in endogenous levels of plant growth regulators, variations in optimal dosages of plant growth regulators needed

for induction of callusing and regeneration, physiological state of explant and different sensitivities of explants to surface disinfection agents.

In this study, only callus of zygotic embryos and root callus were morphogenic. Failure of calli obtained from the other explants to show any morphogenic events contrasts with previous reports of successful regeneration from similar explantsin other members of the meliaceae family. These include shoot regeneration from unpollinated ovaries, nodal segments, roots and excised axillary buds of neem, *Azadirachta indica* A. Juss (Akula *et al.*, 2003; Quraishi *et al.,2004*; Srinidhi *et al*., 2008; Srivastava *et al*., 2009), leaf cuttings of Sentang, *Azadirachta excelsa* (Jack) Jacobs (Foan and Othman, 2006), nodal segments and epicotyls of big-leaf mahogany, *Swietenia macrophylla*King (Cerdas *et al*., 1998; Scottz *et al.*, 2007) and apical meristems of purple lilac *Melia azedarach* L.(Vila *et al.*, 2002). It is probable that endogenous levels of plant growth regulators vary significantly between families and genera, requiring different regeneration protocols for each species.

Consequently, the results of explant screening identified mature zygotic embryos and cotyledons as the most suitable explants for regeneration of *M. volkensii*. These two types of explants have also been successfully used in regeneration of other members of the meliaceae family.Immature zygotic embryos were used in regeneration of Neem, *Azadirachta indica* (Murthy and Saxena, 1998; Rout, 2005) and the Persian lilac tree *Melia azedarach*L. (Vila *et al.*, 2003; 2010). Regeneration from mature cotyledons is reported in *Azadirachta indica* (Murthy and Saxena, 1998; Gairi and Rashid, 2004; Artigas *et al.*, 2015) and in *Melia volkensii* **(**Indieka *et al.*, 2007).

5.1.2 Responses of Explants to Cytokinins BAP, Kinetin and Thidiazuron

From the initial studies aimed at determining the most suitable cytokinins for regeneration from *M. volkensii* zygotic embryos and cotyledons, TDZ outperformed both BAP and Kinetin in induction of somatic embryos and shoots. This finding is in agreement with the documented superiority of urea-based cytokinins such as TDZ and CPPU (Forchlorofenuron) over the adenine-based cytokinins (BAP, Kinetin and Zeatin) for plant regeneration (Huetemann and Preece, 1993; Kaneda *et al.,* 1997; Gu and Zhang, 2005; Sunagawa *et al.,* 2007; Guo *et al.,* 2011). There are three probable explanations for this; first the urea-based cytokinins are not degraded by the cytokinin oxidase enzymes present in plants (Makara *et al.,* 2010) and so they retain their molecular and functional stability over a longer period. Two, TDZ is thought to modulate both auxin and cytokinin effects (Guo *et al.,* 2011), whereas adenine-based cytokinins act only through cytokinin-mediated processes. Evidence also suggests that TDZ causes accumulation of endogenous adenine-based cytokinins by preventing their degradation through inhibition of cytokinin oxidase (Guo *et al.,* 2011).

The occasional fasciation of shoots observed in the present study, especially with higher levels of BAP and Kinetin, has also been described in previous studies (Bairu and Kane, 2011). This is a physiological disorder resulting in abnormal appearance of plant organs such as stems, flowers, fruits and roots. Organs appear to be more than usual in number and general morphology and are often fused together. Sinuishin and Gostimskii (2008) attributed fasciation to uncontrolled activity in apical or lateral meristems and also identified a recessive mutation responsible for fasciation in garden pea (*Pisum sativum* L.),

suggesting a probable genetic basis for the disorder. It is probable that tissue culture-related fasciation arises from *de novo* mutations induced by mutagenic nature of plant tissue culture.

Cytokinin-related hyperhydricity was also observed in this study. Use of BAP and coconut water was associated with a higher incidence of this condition. Replacement of coconut water with 0.01% IAA had the beneficial effect of eliminating this undesirable condition. The exact mechanism is not yet clear but appears to originate from failure of processes that regulate osmotic uptake of water and the stress that results from water-logging of tissues (Rojas-Martinez *et al*., 2010). Similar hyperhydricity has been reported in other studies (Huetteman and Preece, 1993; Kevers *et al.,* 2004; Bosela and Michler, 2008; Guo *et al.,* 2011; Ruzic *et al.,* 2011).

5.1.3 Effects of Media Type and Strength

Although full-strength MS medium supported a higher percentage induction of somatic embryos and shoots from zygotic embryos and cotyledons explants than either B5 and lower strengths of MS, shoots obtained in MS medium had a relatively high frequency of defects such as hyperhydicity and *in vitro* flowering. A reduction in MS nutrients generally improved the phenotypic appearance of the shoots. Media of low macro and micronutrient content such as ¼ and ½ MS were very favourable for regeneration of normal shoots. These findings are in agreement with the general observation of Saad and Elshahed (2012), that for some plant species, reduction of salts to ½ or ¼ strength gives better results in *in vitro* growth. Media composition has also been shown to influence regeneration of other plants such as cowpea (Brar *et al.*, 1997), rice (Khanna and Raina, 1998) and the egg plant (Kaur *et al.*, 2013).

The low frequency of precocious *in vitro* flowering observed in the present study is not unusual. The highest frequency of *in vitro* flowering was 3.63% in ¾ MS medium followed by 0.8% on full-strength MS, both supplemented with 0.05% TDZ. Sato and Esquibel (1995) reported similar frequencies of *in vitro* flowering from hypocotyl segments of *Melia azedarach* L. grown in basal MS medium. Handro and Floh (2001) also reported low-frequency (5%) flowering in hypocotyl explants of *Melia azedarach* L. in basal MS.

The role of PGRs in *in vitro* flowering appears to vary between species. Peeters *et al.* (1991) found the cytokinin BAP to be crucial for *in vitro* flower bud induction in tobacco plants. Auxin was only essential for stimulation of differentiation and further development of the initiated flower buds. In general gibberellins and cytokinins, alone or in combination, have been shown to enhance *in vitro* flowering in many plant species (Ziv and Naor, 2006). Although *in vitro* flowering has the undesirable effect of terminating apical growth of the shoots, it offers an ideal system for the study of flower morphogenesis as suggested by Ziv and Naor (2006). It can also be exploited for future development of *in vitro* fruit and seed production systems in this and other species as suggested by Sharma *et al*. (2014).

5.1.4. Thidiazuron-induced Indirect Somatic Embryogenesis in Zygotic Embryos

With regard to contribution, the present study produced the first report of TDZ-mediated regeneration of *M. volkensii* from mature zygotic embryos using ½ MS and B5 media (Appendix 3, number 1). In addition, it demonstrated for the first time the efficacy of unconventional low-cost agrochemical-grade TDZ in tissue culture of *M. volkensii* as a substitute for the exorbitant plant-cell-culture tested TDZ. These findings are important since an earlier attempt by Indieka *et al*. (2007) using *M. volkensii* zygotic embryos cultured on MS supplemented with combinations of BAP and 2,4-D only gave callus with no somatic

embryogenesis. This may be attributed to the different modes of action of the plant growth regulators used in the two studies.

The high frequency of callus induction obtained in this study in zygotic embryos on control media (Basal ½ MS or B5) was unexpected. This indicates that callogenesis in mature zygotic embryos of *M. volkensii* was independent of exogenous application of TDZ. It also suggests that endogenous levels of PGRs in mature zygotic embryos may be adequate for callus induction. However, only callus formed in presence of TDZ was morphogenic, showing that TDZ was essential for the observed somatic embryogenesis. Lower concentrations of TDZ (0.05 – 0.25 mg/l) were more effective in induction of somatic embryos in the callused zygotic embryos. This is supported by Vila *et al.* (2003; 2010) who reported efficient somatic embryogenesis from immature zygotic embryos of *M. azedarach* L., on MS medium with low concentrations (≤ 10μM) of TDZ as sole PGR. It is also in agreement with other reports showing very low concentrations of TDZ as favourable for shoot morphogenesis in other plant species (Mok and Mok, 1982; Huetteman and Preece, 1993; Murthy and Saxena, 1998; Guo *et al.*, 2011). In addition, callus masses in this study required a subculture to basal ½ MS or B5 medium for optimum somatic embryo induction and conversion of the embryos to shoots, indicating a need for passage time for endogeneous TDZ levels to fall to an optimumlevel.

Somatic embryos induced from the calli of zygotic embryo readily converted to microshoots on Basal ½ MS or B5 medium. However, the resulting shoots remained stunted on either basal or TDZ-supplemented MS or ½ MS medium and only elongated when subcultured to ½ MS or B5 medium containing either 0.1 mg/l BAP alone or in combination with 5 or 10 % coconut water. The presence of coconut water resulted in formation of hyperhydric shoots, which are undesirable. The observed stunting of shoots is supported by

earlier reports showing the inhibitory effects of TDZ on shoot elongation (Murthy *et al.*, 1998; Debnath, 2005; Guo *et al.*, 2011). This can be attributed to the strong carry-over effect of TDZ which causes it to continue influencing the physiological status of plants after transfer from TDZ-media and well into the *ex vitro* stages (Peddaboina *et al.*, 2006; Makara *et al.*, 2010; Roy *et al.*, 2012). Elongation of the stunted shoots with BAP or coconut water is consistent with the suggestion that TDZ-induced inhibition can be removed by sub-culturing the shoots to media containing another cytokinin, in particular zeatin or BAP (Debnath, 2005).

5.1.5. Thidiazuron-induced Direct Somatic Embryogenesis from Cotyledons

The present study also produced the second report (Appendix 3, number 2) of direct somatic embryogenesis in *M. volkensii* from mature cotyledons, after Indieka *et al.* (2007). This study describes a new protocol that significantly improved the efficiency of *M. volkensii* regeneration from 60% frequency with mean yield of 6.25 somatic embryos per explant attained by Indieka *et al.* (2007) to a highly reproducible 100% frequency with dense clusters of somatic embryos and microshoots. The main difference between the two protocols is that whereas Indieka *et al.* (2007) used MS medium with combinations of BAP (0.5 - 4 mg/l) and 2,4-D (0.2 mg/l), the present study used ½ MS medium with TDZ (0.05 - 4 mg/l) as sole PGR.

This direct mode of regeneration from *M. volkensii* cotyledons using TDZ is consistent with findings of Murthy and Saxena (1998) and Artigas *et al.* (2015) who reported direct somatic embryogenesis from mature cotyledons of *Azadirachta indica* A. Juss., using MS medium with 0.3 - 10 mg/l TDZ and Vila *et al*. (2003; 2010) who reported direct somatic embryogenesis from immature zygotic embryos of *Melia azedarach* L. (Meliaceae) using MS medium with 0.1 to 3 mg/l TDZ as the only plant growth regulator. A similarly high frequency

of direct regeneration was also reported earlier in neem (*A. indica*) cotyledons (Gairi and Rashid, 2004) using TDZ as sole plant growth regulator.

Just as in the case of zygotic embryos, regenerationin *M. volkensii* cotyledons occurred only in medium containing TDZ, confirming the presence of TDZ as essential for the process. However, somatic embryogenesis in cotyledons followed a polarized pattern where the response was restricted to the upper-facing, directly illuminated surfaces of explants suggesting a requirement for light. This observation is supported by the previous report of Baweja *et al.* (1995) which found a promotive effect of light on somatic embryogenesis in hypocotyls of *Albizia lebbeck* L., (Fabaceae). However, no such polarized regeneration was seen in the present study in callused zygotic embryos of *M. volkensii*, where somatic embryos formed over the entire surface of the callus, including surfaces in contact with the medium. This suggests a probable involvement of different trigger mechanisms in induction of direct and indirect modes of somatic embryogenesis in *M. volkensii*.

The concentration of TDZ used in the induction media significantly influenced the number of transplantable shoots obtained from the cotyledons. Cotyledons cultured initially on a medium containing 0.25 or 0.5 mg/l TDZ gave the highest mean numbers of transplantable shoots (11.25 and 10.00 respectively). This is in agreement with the reported findings of Huetteman and Preece (1993), Debnath (2005) and Guo *et al.*, 2011, which show the low TDZ concentrations promote and high concentrations inhibit shoot organogenesis or somatic embryogenesis in many plant species. Huetteman and Preece (1993) and Guo *et al*. (2011) suggested that high TDZ levels inhibit morphogenesis and shoot growth by causing adverse distortion of the delicate balance of endogenous auxins and cytokinins.

As was the case with shoots regenerated from zygotic embryos explants, shoots obtained from cotyledons also exhibited stunting. This is also supported by earlier reports on the inhibitory effects of TDZ on shoot elongation (Murthy *et al.,* 1998; Debnath, 2005; Guo *et al.,* 2011) and can also be attributed to the high stability and carry-over effect of TDZ (Peddaboina *et al.,* 2006; Makara *et al.,* 2010; Roy *et al.,* 2012). Just as in the case of shoots from zygotic embryos, these shoots also required subculture to a medium containing a second cytokinin (BAP or coconut water) for elongation to occur. The best elongation of the microshoots from both explant types was achieved on ½ MS medium containing 0.1 mg/lBAP alone or in combination with either 10% (v/v) coconut water or 0.01 mg/l IAA. This is supported by the findings of Debnath (2005) and Shahin-uz-zaman *et al.* (2008).

5.1.6 Histological Observations on Regeneration

Morphological and histological examination of the explants at various stages of *in vitro* culture confirmed the regeneration pathway as indirect somatic embryogenesisin zygotic embryos and direct somatic embryogenesis in cotyledons. The response of *M. volkensii* zygotic embryos contrasted sharply with the direct mode of somatic embryogenesis reported in immature zygotic embryos of *Melia azedarach* using TDZ (Vila *et al.,* 2003; 2010) but is not completely unexpected as the responses of explants to *in vitro* culture are highly species- and genotype-dependent (Gandonou *et al.,* 2005; Michel *et al.,* 2008).

The anatomical features of the pre-embryonic masses, globular, heart-shaped and torpedo stages of somatic embryos revealed by histology were remarkably similar to those of somatic embryos obtained from mature seeds of *Azadirachta indica* (Meliaceae) (Murthy and Saxena, 1998) and immature zygotic embryos of *Melia azedarach* (Vila *et al.* 2003), using TDZ as sole PGR. They were also similar to stages of somatic embryos obtained from mature

cotyledons of *Melia volkensii* using BAP and 2,4-D (Indieka *et al.* 2007). Finer histological details such as epidermal and sub-epidermal origin of pre-embryonic masses and anatomical features of globular, heart-shaped, torpedo and cotyledonary stages of the somatic embryos observed in this study were also remarkably similar to those obtained by Indieka *et al.* (2007) in *M. volkensii* and Vila *et al.* (2003; 2010) in *M. azedarach*.

The histological examination also revealed the bipolar nature of the somatic embryos, lack of vascular connections to the mother explants, presence of procambial strands and loose attachment of the somatic embryos to the mother explant. These characteristics have also been reported in somatic embryos of some meliaceae such as *Azadirachta indica* (Murthy and Saxena 1998) *Melia azedarach* (Vila *et al.,* 2003) *Melia volkensii* (Indieka *et al.,* 2007), and in other plant species such as coffee and grapevine (Quiroz-Figuera *et al.,* 2006) and rice (Vega *et al.*, 2009).

In contrast to the morphological homogeneity observed in somatic embryos formed from calluses of zygotic embryos, considerable heterogeneity was observed in size and morphology of somatic embryos induced from the cotyledons. This calls for further study to determine - the effect of this morphological heterogeneity on the conversion of somatic embryos into plants. However, such somatic embryo heterogeneity is not new as similar heterogeneity was reported in TDZ-induced somatic embryos of *M. azedarach* (Vila *et al.,* 2010) obtained from immature zygotic embryo explants. This also indicates that the phenomenon may not be specific to somatic embryos obtained from cotyledons.

5.1.7 Rooting

Despite the high frequencies of somatic embryogenesis and shoot regeneration obtained from both zygotic embryos and cotyledons, the present study realised low frequency of rooting of the shoots. The best rooting response (39.3.%) and the highest mean number of roots per shoot (5.64) was achieved on half-strength MS medium supplemented with 0.1 mg/l NAA and 0.1 mg/l IBA. When used separately, 0.1 mg/l IBA and 0.1 mg/l NAA only gave 6.67% and 8.88% rooting responses receptively. This is consistent with the low-frequency (25% on PGR-free ½ MS) of conversion of TDZ-induced *M.azedarach* L. somatic embryos into rooted plantlets (Vila *et al*., 2003).

Although rooting has been reported to be generally difficulty among members of the Meliaceae, some significant rooting has been attained in tissue cultured plants of some members of the family. Sharry and Silva (2006) attained 60-70% rooting of plantlets obtained from *M. azedarach* somatic embryos on PGR-free ½MS medium. However, those plants were regenerated from embryogenic callus induced on MS medium containing 3 mg/l NAA, 1m g/l BAP and 5 mg/l GA3. Sen *et al*. (2010) also reported 80% rooting of *M. azedarach* on ½MS supplemented with 1.0 mg/l IBA. Those shoots were regenerated directly from nodal segments cultured on MS medium containing 1.2 mg/l BAP, 0.1 mg/l IAA and 9 mg/l Adenine Sulphate. Whereas in *Azadirachta indica* Shahin-uz-zaman et al. (2008) attained 85% rooting of shoots on ½MS containing 0.05 mg/l IBA and 0.05 mg/l IAA, for shoots regenerated from shoot and root tip explants using combinations of BAP and NAA.

From the above, it can be seen that high rooting frequencies of $\geq$ 80% among the melicaeae have been attained mostly when the cytokinin used for plant regenerated was BAP but not with TDZ. This could be attributed to the high persistence of TDZ in plants tissues due

to inability of cytokinin oxidase to break it down (Makara *et al.*, 2010). However, one notable exception to this observation is the findings of Reddy and Rao (2006) who attained 86.6% rooting of shoots of Neem on ½MS supplemented with 2.0 µM IBA, even when the shoots had been regenerated from cotyledon nodes using 1.5 µM TDZ and 0.5 µM IBA.

The results obtained in this study indicate that rooting is yet to be optimised from the 30% previously reported by Indieka *et al*. (2007). This shows there is still need for further study for optimization of rooting which is currently the only barrier to the utilization of these protocols for mass propagation of the species.

5.1.8 Efficacy of Low-cost Agrochemical Thidiazuron

The high frequency of shoot regeneration attained from the cotyledons using the same low-cost agrochemical TDZ used for zygotic embryos further validates its efficacy as a substitute for conventional TDZ. This confirms its usefulness in tissue culture of *M. volkensii*, and could significantly cut down costs. The TDZ used in this study was sourced from Kingtai chemicals Ltd., China at the cost of US$ 160 per 700g, which is far much cheaper than Sigma-Aldrich plant-tissue-culture-tested TDZ which costed €2,025 for 0.5g in 2011 (Pers. Comm.). This study complements other local efforts in the search for low-cost tissue culture inputs for use in commercial plant propagation systems, such as use of table sugar as a substitute for plant-tissue-culture-tested sucrose (Demo *et al* 2008), cheaper gelling agents such as cassava starch (Kuria *et al.*, 2008) and cheaper macro and micronutrient sources such as agricultural fertilizers (Ogero *et al.*, 2012).

5.1.9 Effects of Polyethylene Glycol on Regeneration

Polyethylene glycol (PEG) was observed to be beneficial to somatic embryo induction in calli of zygotic embryos, but only when used at 20 or 40% (v/v) concentrations. Lower concentrations had no benefit while higher concentrations inhibted the response. Somatic embryos formed in presence of PEG exhibited more clearly defined shapes than those formed in its absence. However, regeneration from cotyledons did not benefit from the presence of PEG. The promotive role of PEG on somatic embryogenesis is well documented. Li *et al*., (1997) found that combinations of PEG and Abscissic acid (ABA) promote somatic embryogenesis in loblolly pine (*Pinus taeda* L.). PEG at 60 g/l also promoted the number, maturation and conversion of somatic embryos of papaya (*Carica papaya* L.) (Heringer *et al*., 2013). At concentrations of 5- 10 %, PEG promoted maturation of somatic embryos in hybrid fir (*Abies alba* x *Abies numidica*) (Salaj *et al.,* 2004). Other species for which PEG has shown beneficial effect are date palm (*Phoenix dactylifera* L.) (Al-Khayri, 2009; Al-Khayri and Al-Bahramy, 2012), where use of PEG allowed synchronised development of somatic embryos; soybean (Walker and Parrott, 2001); white spruce (Stasolla *et al.,* 2003), big-leaf mahogany (*Swietenia macrophylla*) (Maruyama, 2009) and tea (Saganthi *et al.,* 2012).

Polyethylene glycol (PEG) is thought to influence somatic embryogenesis through combined effects of osmotic stress and *de novo* synthesis of regulatory proteins. It is a non-plasmolysing osmoticum which enhances somatic embryo induction and maturation by inducing water stress (Salaj *et al.,* 2004). Stasolla *et al.* (2003) reported PEG-related improvement of the number and quality of somatic embryos through activation of genes involved in the formation of the embryo body plan and in control of the shoot and apical meristems. It has been also been found to increase the synthesis and accumulation of low-

molecular weight proteins involved in somatic embryo development, including the enzymes enolase, esterase and alcohol dehydrogenase 3 (Salaj *et al.*, 2004; Vale *et al.*, 2014).

5.1.10. Effects of Amino Acids on Regeneration

In zygotic embryo explants, L-glutamine and L- tryptophan significantly enhanced induction and quality of somatic embryos. However, in the cotyledon explants, the beneficial amino acids with regard to promotion of somatic embryogenesis and formation of microshoots were L-tryptophan and L-proline. Consequently, L-tryptophan promoted somatic embryogenesis in both explants while L-glutamine was only beneficial to zygotic embryos and L-proline to cotyledons. These findings support previous reports showing that supplementation of media with specific amino acids enhances induction of regeneration via somatic embryogenesis and organogenesis pathways (Caesar and Ignacimuthus, 2010). Amino acids provide a reduced form of nitrogen which can be easily assimilated by plant cells and tissues, in comparison to inorganic nitrogen sources which require metabolic conversion to reduced forms before assimilation by the plant (Quedraogo *et al*., 1998; Saad and Elshahed, 2012).

The specific type of amino acid required for enhancement of somatic embryogenesis varies between plant species. Vasanth *et al*. (2006) found that L-glutamine enhances shoot regeneration in cotyledon and embryo axis explants of peanut (*Arachis hypogea* L). L-glutamine and L-tyrosine were beneficial in shoot regeneration from cotyledon node explants of *Artemisia vulgaris* L. (Kumar and Kumari, 2010). L-glutamine and spermidine at 500mg/l produced the highest number of shoots in date palm (El-Sharabasy *et al.*, 2012); Duran *et al.*, (2013) reported significant enhancement of regeneration in wheat by L-alanine and L-

glutamine. In the aquatic plant *Lemna minor*, L-serine and L- glycine promoted regeneration from callus (Yang *et al.,* 2014).

5.2 Morphological and RAPD-marker characterization

Of the two groups of morphological characters studied (morphometric and meristic), morphometric traits showed more variability between the regenerants and normal seedlings than meristic traits. Regenerants were generally characterized by stunting of shoots, shorter internodes, smaller leaves and shorter taproots in comparison to normal seedlings of same developmental stage. Similar stunting of shoots regenerated using TDZ has been reported in grapes (Gray and Benton, 1991) and in some tree species such as *Cassia* (Parveen and Shahzad, 2010), *Myrica rubra* (Asghar *et al.*, 2013), *Calophyllum inophyllum* (Thengane *et al.*, 2006). TDZ use is also reported to cause complete or partial failure of rooting in regenerated shoots (Gray and Benton, 1991; Lu, 1993; Ranyaphia *et al.*, 2011).

In meristics, the regenerants were less dissimilar to the wild type seedlings in the number of leaves per shoot, leaflets per rachis, leaf pinnules per leaflet and axillary bud sprouts. The only meristic trait that differed significantly between the regenerants and the wild type seedlings was mean number of lateral roots per plant. Rooting and development of lateral roots was generally poor in the regenerants but vigorous in wild seedlings. The inhibitory effect of TDZ on root development was greater in plants regenerated from zygotic embryos than those regenerated from cotyledon explants. Poor root meristics in the regenerants can also be attributed to the reported inhibition of rooting (Gray and Benton, 1991; Lu, 1993; Ranyaphia *et al.,* 2011).

PCOORDA plots, Cluster analysis, Anosim and SIMPER analysis all confirmed the morphological dissimilarity between regenerants and the wild seedlings in morphometric and

meristic traits. A similar complementary role of the Anosim test to principal coordinate and cluster analyses in the study of morphological characteristics of plants is reported by Abouzied *et al.* (2013). In the present study, wild type seedlings clustered separately from the regenerants. However, the separation of regenerants from the wild type seedlings based on meristic traits was smaller than that based on morphometric traits. This was expected as meristic traits, such as the number of leaf nodes, leaflets and lobules, are fixed early in development and tend to be less prone to environment-induced variability than morphometric traits (Lawing *et al*., 2008). Therefore, morphometric traits typically exhibit continuous variation while meristics tend to show discontinuous variation.

Both the quantitative and qualitative attributes of the genomic DNA extracted from the regenerants and wild type seedlings were good, with A_{260}/A_{280} ratios (1.60 to 1.83) close to the ideal ratio of 1.80 expected for pure DNA as recommended by ThermoScientific (2014). However, A_{260}/A_{230} ratios for the DNA of the regenerants and wild seedlings (0.68 to 1.69) were much lower than the ideal ratio of 2.0-2.2 (ThermoScientific, 2014). Low A_{260}/A_{230} ratios are often encountered in plants due to a considerable carrying-over of carbohydrates and proteins during extraction. This shows that either the proteinaseK enzyme used in this extraction failed to eliminate all proteins or the ratio of CTAB to NaCl used in this study may have failed to precipitate all the carbohydrate.

In the present study, screening of the eight candidate primers produced a total 30 polymorphic RAPD bands/markers from 69 amplified PCR reactions. This is consistent with the findings of Runo *et al.* (2004), in which eight random primers generated 38 scorable polymorphic bands from 90 PCR-RAPD reactions using DNA obtained from *in situ* populations of *M. volkensii.* However, a detailed comparison of primer performance between

that study and the present one is not possible as the oligonucleotide sequences of primers used by Runo *et al.* (2004) were not revealed.

From the screening reactions, Mel-4 (5'CCT TGA CGC A 3') and Mel-5 (5'TGC CGA GCT G 3') emerged as the primers with the most resolved RAPD profiles. Both primers have been previously successful in PCR-RAPD of *Lansium domesticum* Corr., meliaceae, (Song *et al*., 2000) genomic DNA. These were followed by Mel-6 (5' GGC ATG ACC T 3'), which was successfully used in RAPD analysis of *Xylocarpus granatum* Koen., *Xylocarpus moluccensis* Lamk. and *Xylocarpus mekongensis* Pierre, meliaceae, (Pawar *et al*., 2013). Fourth was Mel-8 (5' GGC TAT CCG A 3'), an arbitrary oligonucleotide sequence used for the first time in the present study. Overall, the most successful primer in terms of number and distinctness of bands was Mel-5. This probably indicates more complementarity between the random binding sites where this primer anneals in genomic DNA of *M. volkensii* and *Lansium domesticum* (Song *et al.,* 2000) than with the other two members of meliaceae family, *Azadirachta indica* A. Juss (DaSilva *et al.,* 2013) and *Xylocarpus* species (Pawar *et al.,* 2013).

In general, the regenerants gave higher values of Nei's gene diversity and Shannon's information indices than wild type seedlings. This indicates that the direct and indirect somatic embryo regeneration systems used in the present study may have produced some de *novo* genetic variations which add to those naturally present in the species. Such tissue culture-generated genetic variations have been reported in other plant species (Larkin and Scowcroft, 1981; Neelakanda and Wang, 2012). However, the values of Nei's gene diversity (H) index obtained for the regenerants in this study (0.09 – 0.19) compare favourably with those obtained by Runo *et al.* (2004) (c.a. 0.06 – 0.12) for widely dispersed natural populations of *M. volkensii* in Kenya. This indicates that the component of genetic variability attributable to

the regeneration systems used in this study may not significantly deviate from that which is found within and between natural populations of the species. The relatively low value of Nei's gene diversity index seen in the wild type seedlings could be attributed to the fact that seeds used in raising the wild type seedlings for the RAPD analysis were from a cluster of parent trees standing on the same farm, which are probably closely related.

Several studies have used cluster analysis to complement either principal coordinate or principal component analyses of morphological or genetic traits in plants (Pratta *et al.,* 2000; Song *et al*., 2000; Sheidai *et al.,* 2008a, 2010; Valenzuela *et al.,* 2011; Abdellatif *et al.,* 2012; Gamburg and Voinikov, 2013; Plazas *et al.,* 2014). In the present study cluster analysis of the RAPD-genic variation characters validated the PCOORDA plots. Regenerants were morphologically separate from, but genetically similar to, the normal seedlings. It is probable that morphological differences observed in this study may have arisen from tissue-culture related epigenetic or physiological factors as described by Miguel and Marum (2011) and Bairu and Kane (2011).

Further molecular characterization using other markers such as microsatellites **(**Jin*et al.,* 2008) and amplified fragment length polymorphism (Li *et al.,* 2007) may be required for confirmation of the apparent genetic similarity between the regenerants and normal seedlings. In addition, since majority of epigenetic changes in tissue culture arise from DNA methylation (Miguel and Marum, 2011; Neelakanda and Wang, 2012), there is need for use of methylation-sensitive amplified polymorphism (MSAP) techniques to reveal variations in patterns of DNA methylation for detection of underlying epigenetic variations. By providing the first report of RAPD-marker characterization for tissue-cultured *M. volkensii* plants, this study paves the way for identification of RAPD markers that may be uniquely associated with

particular beneficial or harmful morphological or physiological traits and further characterization of the same through development of sequence-characterised amplified regions (SCARs).

5.3. Outputs

This study has contributed the first report of regeneration of *Melia volkensii* from zygotic embryos and high efficacy of low-cost agrochemical TDZ in tissue culture of the same species (Appendix 3, number 1). It has also contributed the second report of regeneration of *Melia volkensii* through direct somatic embryogenesis from cotyledons but with enhancement of regeneration frequency from 60% previously reported by Indieka *et al*. (2007) to 100% in the present study (Appendix 3, number 2). These two publications demonstrate the successful attainment of specific objectives (i) and (ii) of the study. This study also contributed the first report of morphological and RAPD-marker characterization of tissue-cultured *Melia volkensii* plants (Appendix 3, number 3). This addresses specific objectives (iii) and (iv) of the study. The findings obtained, which showed the regeneration methods used in the study as capable of maintaining high genetic fidelity and proximity to the parent trees, are the first such findings for *M. volkensii* and contribute significantly to the existing body of knowledge on the propagation of the species.

CHAPTER SIX: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

In general all four objectives of the study were attained. In the first objective, to determine protocols for rapid and optimized *in vitro* regeneration and propagation of *M. volkensii*, this study produced novel protocols for high-frequency regeneration of *M. volkensii* from mature zygotic embryos and cotyledons using 0.05 – 0.25 mg/l TDZ as the sole PGR. In the second objective, to determine the pathway(s) of regeneration, this study revealed direct and indirect somatic embryogenesis as the modes of regeneration. With regard to the third and fourth objectives, to evaluate and assess the morphological and genetic similarity between the regenerants and seed-derived seedlings, the study revealed significant morphological variations between the regenerants and seedlings of the same mother trees. However, RAPD markers did not reveal significant genetic difference.

In more specific terms, the following conclusions can be drawn from this study:

1. *M. volkensii* is amenable to high-frequency regeneration from mature zygotic embryos and cotyledons.
2. A two-step protocol can be used for direct regeneration from cotyledons; induction of somatic embryos and concurrent conversion to micro-shoots using ½ MS with 0.25 mg/l TDZ, followed by elongation of the micro-shoots using ½ MS with 0.1 mg/l BAP and 0.01 mg/l IAA.
3. For zygotic embryos, a three-step protocol was developed; induction of callus and somatic embryos on ½ MS with 0.05 mg/l TDZ followed by one 21-day subculture to TDZ-free ½ MS medium for optimum somatic embryogenesis and concurrent micro-

shoot formation, before elongation of the shoots using ½ MS with 0.1 mg/l BAP and 0.01 mg/l IAA.

4. Use of 0.1 mg/l BAP alone or in combination with 0.01 mg/l IAA was effective in removal of shoot stunting associated with residual TDZ carried over from induction phase.

5. High potency and usefulness of the unconventional, low-cost agrochemical Kingtai-TDZ in plant tissue culture was demonstrated in this study.

6. Rooting was optimum in ½ MS supplemented with a mixture of 0.1 mg/l IBA and 0.1 mg/l NAA. However, frequency of rooting attained was low.

7. Other than stunting of shoots, the plantlets obtained exhibited normal phenotype and were successfully transferred to soil.

8. Morphological characterization showed significant differences in morphometric and meristic characters, with the tissue cultured plants having shorter stems, internodes, leaves and taproots, and fewer lateral roots than normal seedlings. However, these morphological differences could be epigenetic or physiological rather than genetic.

9. Molecular studies involving RAPDs showed that the tissue cultured plants maintained high genetic fidelity and proximity to their parents.

6.2 Recommendations

The following are recommendations on possible applications of the findings of this study and opportunities for further work:

1. There is need for further study for optimization of rooting. Low rooting frequency is the only remaining barrier to the utilization of tissue culture for mass propagation of *M. volkensii* seedlings through tissue culture.
2. Further study on germination of isolated somatic embryos could provide a useful means of avoiding these rooting difficulties.
3. Further molecular characterization using other markers such as microsatellite (SSR) and amplified fragment length polymorphism (AFLP) may be required for revelation of other levels of genetic similarity or variation between the tissue cultured and seed derived seedlings.
4. Use of methylation-sensitive PCR methods could reveal the extent of tissue culture-induced epigenetic variation, which cannot be detected by the primers used in this study.
5. Further work should include growing the regenerated trees to maturity within the semi-arid lands and evaluation of their growth performance.

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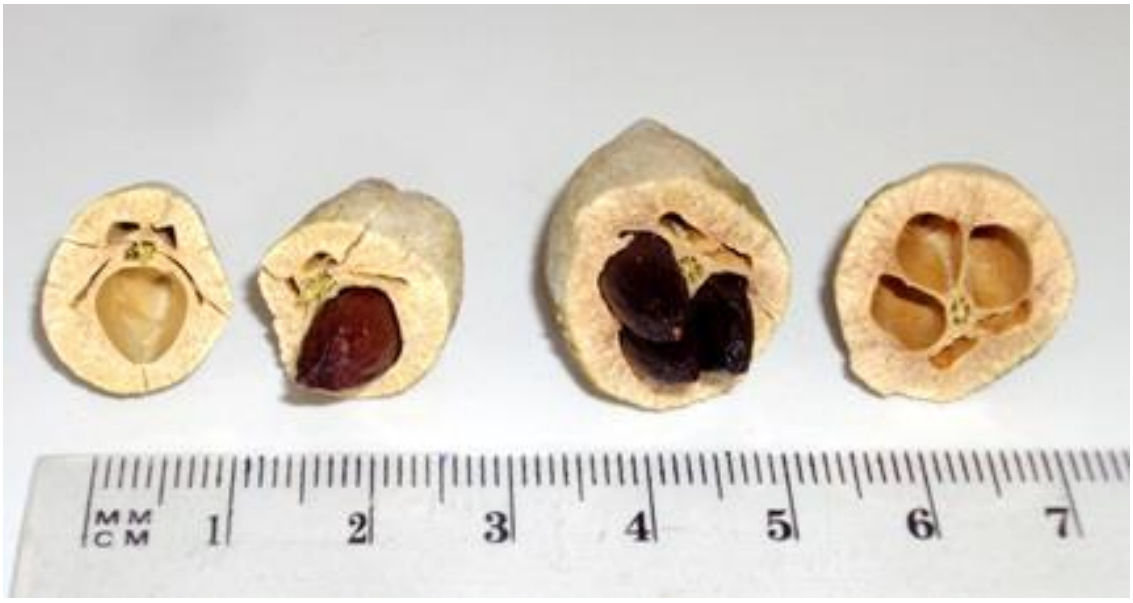
Appendix 1.Split Endocarps of *M. volkensii*



(Source: Author)

Note: Their woody nature is a barrier to seed extraction.

Appendix 2. Comparison of Strength Variants of MS Media with B5 Medium

Nutrients	MS	¾ MS	½ MS	¼ MS	B5
Macronutrients					
Ammonium Nitrate	1650	1237.5	825	412.5	-
Ammonium sulphate	-	-	-	-	134.00
Potassium Nitrate	1900	1425	950	475	2500.0
Calcium Chloride, Anhydrous	440	330	165	82.5	150.00
Magnesium Sulphate	370	277.5	185	92.5	250
Potassium Phosphate Monobasic	170	127.5	85	42.5	-
Sodium phosphate monobasic	-	-	-	-	150
Micronutrients					
Potassium Iodide	0.83	0.623	0.415	0.2075	0.75
Boric Acid	6.2	4.65	3.1	1.55	3.0
Manganese Sulphate. $4H_2O$	22.30	16.73	11.15	5.575	-
Manganese Sulphate.H_2O	-	-	-	-	10
Zinc Sulphate.$7H_2O$	8.6	6.45	4.3	2.15	2.00
Molybdic Acid. $2H_2O$	0.25	0.1875	0.125	0.0625	0.25
Cupric Sulphate.$5H_2O$	0.025	0.01875	0.0125	0.00625	0.025
Cobalt Chloride.$6H_2O$	0.025	0.01875	0.0125	0.00625	0.025
Ferrous Sulphate.$7H_2O$	27.8	20.85	13.9	6.95	27.8
Na 2 -EDTA	37.26	27.945	18.63	9.315	37.26
Vitamins					
Nicotinic Acid	0.5	0.375	0.25	0.125	-
Pyridoxine, HCl	0.5	0.375	0.25	0.125	-
Thiamine	0.1	0.075	0.05	0.025	10.0
Other components					
Myo-Inositol	100	75	50	25	100
Glycine	2	1.5	1	0.5	2

MS= Murashige and Skoog's (1962) Medium; B5= Gamborg *et al*.'s (1968) medium

Appendix 3. List of Journal Publications Arising From This Work

1. **Mulanda**, S.E., Adero, O.M., Amugune, O.N., Akunda, E., and Kinyamario, I.J. (2012). High frequency regeneration of the drought-tolerant tree *Melia volkensii* Gürke using low-cost agrochemical Thidiazuron. Biotechnology Research International Vol. 2012, doi: 10.1155/2012/818472.
2. **Mulanda,** S.E**.**, Adero, M.O., Wepukhulu, D.K., Amugune, N. O, Akunda, E., and Kinyamario, J.I. (2014). Thidiazuron-induced somatic embryogenesis and shoot regeneration in cotyledon explants of *Melia volkensii* Gürke. Propagation of Ornamental Plants 14 (1): 40 – 46.
3. **Mulanda**, S.E., Chuhila, Y., Awori, R.M., Adero, M.O., Amugune, N.O., Akunda, E. and Kinyamario, J.I. (2015). Morphological and RAPD-marker characterization of *Melia volkensii* (Gürke) *in vitro* plants regenerated via direct and indirect somatic embryogenesis. African Journal of Biotechnology 14 (15): 1261 – 1274.