MACRO- AND MICRO-PROPAGATION OF *MELIA VOLKENSII* GÜRKE (MELIACEAE): AN INDIGENOUS MULTIPURPOSE DRYLANDS TREE SPECIES

BY

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university or any other award.

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DEDICATION

This thesis is dedicated to my family; Joseck and Selpha Abwao, who have worked tirelessly to see me excel and, Ekongo, Abisai, Osome and the late Omenya whom I have shared the highs and lows of my life.

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ABSTRACT

Melia volkensii Gürke (Meliaceae) is a popular indigenous multipurpose tree species endemic in the arid and semi arid lands (ASALs) of East Africa. It is a prolific seeder, though its mass-multiplication has been constrained by problems in propagation through seed and conventional stem cuttings. In this study, three vegetative propagation techniques were investigated: (i) macro-propagation using 3-month old rejuvenated leafy stem cuttings, (ii) direct *in vitro* shoot multiplication using shoot tip explants and (iii) direct somatic embryogenesis using mature zygotic embryo and cotyledonary explants. The effects of fungicide pretreatment, surface sterilization and PGRs (IAA, IBA and NAA) at 0, 2, 4, 6, 8, and 10 g l^{-1} on rooting of leafy cuttings in a low cost mist propagator were evaluated. Up to 33 % rooting was achieved on cuttings treated with 8 g l^{-1} IBA and 50 % of the rooted cuttings were successfully hardened. *In vitro* experiments were conducted using Murashige and Skoog (1962) basal medium (MS). The effect of MS supplemented with BAP, kinetin and zeatin (0.25-3.0 mg l^{-1}) alone and in combination with NAA or IAA (0.1 and 0.2 mg l^{-1}) on shoot multiplication was investigated. Generally, BAP: IAA combinations induced shoot multiplication and up to 5.4 shoots per explant were achieved in BAP: IAA (0.5: 0.2 mg l^{-1}) combination. Rooting of *in vitro* multiplied shoots was achieved only in IBA treatments and up to 40 % success was attained in MS supplemented with 2.0 mg l^{-1} IBA. The effect of MS supplemented with BAP, NAA and 2,4-D (0.2-4.0 mg l^{-1}) alone and in combination on induction of direct somatic embryos was also investigated. Direct somatic embryos were initiated on 30-60 % of the cotyledonary explants from seeds stored for <3 months in BAP (0.5-4.0 mg l^{-1}) combined with 0.2 mg l^{-1} 2,4-D. Further development of somatic embryos into plantlets on MS and ½ MS (half macro-elements) without PGRs was unsuccessful but formed clusters of numerous shootlets. Nevertheless plantlets were successfully regenerated from shoot tips obtained from the shoot clusters formed. The results obtained in this study clearly demonstrated that *M. volkensii* is amenable to propagation by rejuvenated leafy stem cuttings, direct *in vitro* shoot multiplication and somatic embryogenesis. Optimization of the protocols developed in this study will open up opportunities for mass breeding, conservation and genetic manipulation of this species.

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ACRONYMS AND ABBREVIATIONS

2,4-D- 2,4-dichlorophenoxyacetic acid

ASALs- Arid and Semi-Arid Lands

ASO-Fellowship- Abdou-Salam Ouédraogo Fellowship

BAP- 6-benzylaminopurine

CBS- Central Bureau of Statistics

GDP- Gross domestic product

IAA- Indole-3-acetic acid

IBA- Indole-3-butyric acid

IPGRI-SSA- International Plant Genetic Resources Institute, Sub-Saharan Africa

ISAAA *Afri*Centre- International Service for the Acquisition of Agri-biotech Applications *Afri*Centre

KEFRI- Kenya Forestry Research Institute

KFMP- Kenya Forestry Master Plan

NAA- **α-naphthalene-acetic acid**

NWFPs- Non wood forest products

PGRs- Plant Growth Regulators

CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

1.1 Background information on *Melia volkensii* Gürke (Meliaceae)

Melia volkensii Gürke (Meliaceae) is an indigenous multipurpose tree species endemic in the arid and semi arid lands (ASALs) of East Africa. Its native range extends from Pare Mountains in northern Tanzania to Elwak in Kenya and southern Somalia. However less dense populations have been reported in eastern Ethiopia (Milimo, 1994). The species is found growing in regions with altitudes of 350-1700 m and annual rainfall of 300-800 mm (Dale and Greenway, 1961). It grows on most soils; sandy, clay and shallow stony soils, but preferably with good drainage (Milimo, 1994). The native range of *M. volkensii* in Kenya is shown in Fig. 1.

M. volkensii is commonly found in association with *Acacia* and *Commiphora* vegetation, where it is usually the emergent species (Tedd, 1997). Three distinct populations have been identified occurring in the Kenya-Tanzania border, eastern Kenya and Somalia (Milimo, 1994). Between these populations there are regions where the species is absent, which has been attributed mainly to unfavorable environmental conditions and impact of human settlements (Milimo, 1994, Kidundo, 1997). Distinct natural stands are few and are found mainly in protected National Parks and Nature Reserves (Milimo, 1994). Currently there are no planted forests of *M. volkensii*, though majority of trees are found on farmlands (Tedd, 1997; Mulatya, 2000).

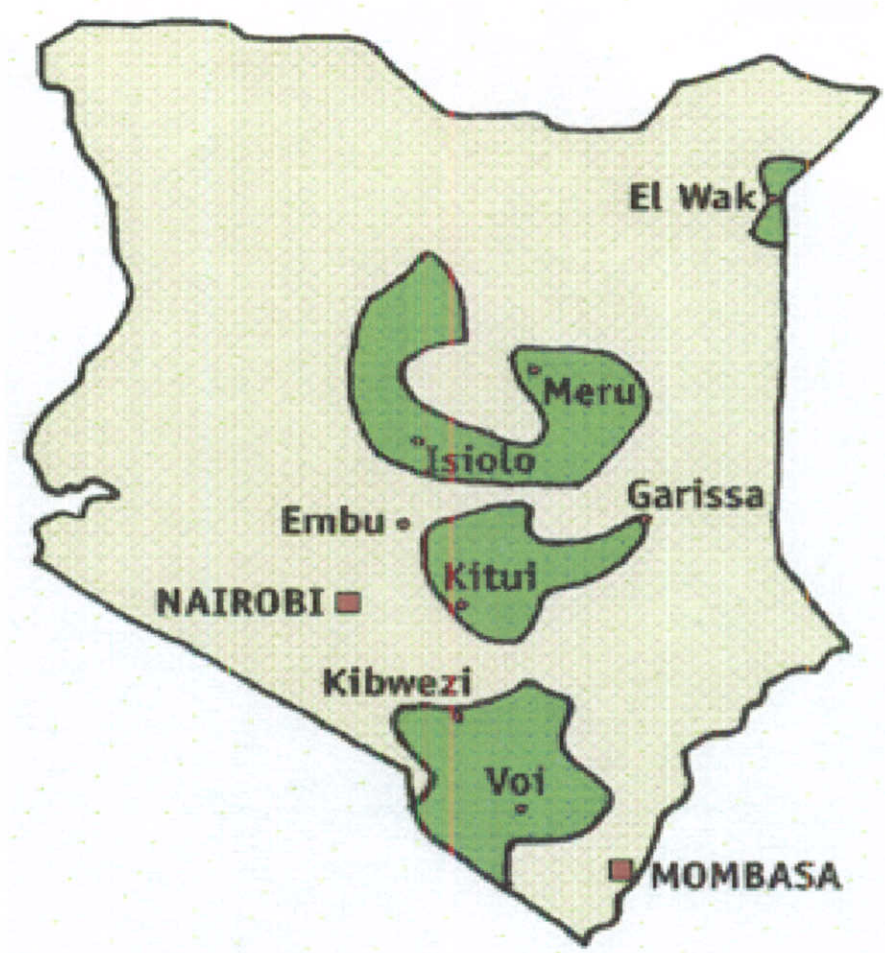

Figure 1. The distribution of *Melia volkensii* in Kenya. Regions shaded green represent the native range of *M. volkensii*.

The ASALs cover about 88 % of Kenya's total land area (KFMP, 1994). It was estimated that 20 % of Kenya's population lived in these areas and natural resources were able to sustain human and livestock populations (CBS, 1990). However, due to migration of people from densely populated highlands, it was estimated that ASALs supported 25 to 30 % of the country's population as well as half of the livestock populations (Chikamai, 2004). Trees and shrubs in the ASALs of Kenya are important as they are in the humid highlands. They provide wood and non wood-forest products (NWFPs), such as gums and resins, honey and medicine as well as controlling soil erosion and maintenance of soil fertility (Jama *et al.*, 2003; Chikamai, 2004). In spite of their importance, trees and shrubs in the ASALs have continued to experience overexploitation and loss of biological diversity. Their future therefore lies in the enhancement of sustainable utilization and conservation. There is also the need to integrate highly demanded and well-adapted indigenous trees species in reforestation and agroforestry programmes within ASALs (Jama, *et al.*, 2003).

Use of well adapted, fast growing multipurpose and already popular tree species such as *Melia volkensii* in agroforestry systems in ASALs of eastern Africa can provide significant income to small-scale farmers (Kidundo, 1997; Mulatya, 2000). Despite *M. volkensii* being a highly valued, drought resistant and fast growing indigenous tree species, large-scale cultivation has been constrained by difficulty in propagation by both seeds and conventional stem cuttings. On the other hand, overexploitation of *M. volkensii* has also caused great concern due to loss of elite germplasm and genetic diversity. Hence

there is need to develop alternative propagation techniques for purposes of enhancing domestication, sustainable utilization and conservation of this species.

1.2 Classification and botanical description of *M. volkensii*

Melia volkensii belongs to the Meliaceae family, Meliodeae subfamily and Meliaeae tribe. The family is sub-divided into 4 sub-families, containing 51 genera and approximately 800 species. Some notable members of the family include *Melia bambola* and *Melia azedarach* (Mabberley, 1997). *M. volkensii* in Kenya is locally known by different names:, mukau (Kamba and Kikuyu), bamba (Oromo and Borana), maramurui (Samburu), baba (Somali), Mukowe (Taita) and tree of knowledge (English) (Beentje, 1994).

M. volkensii seedlings when young have a greenish stem, with leaves alternately arranged, elliptical and entirely or fairly serrated, while the tap root is fleshy (Milimo, 1989b). Mature trees (Plate 1) are deciduous, 6-20 m tall open crowned and laxly branched (Riley and Bronkenshaw, 1988; Albrecht, 1993). *M. volkensii* bark is described as gray fairly smooth but furrowed with age and sometimes reticulate gray (Beentje, 1994). The diameter is typically about 25 cm, while the leaves are bi-pinnate, pale bright green up to 35 cm long and compound with 3-7 leaflets (Albrecht, 1993).

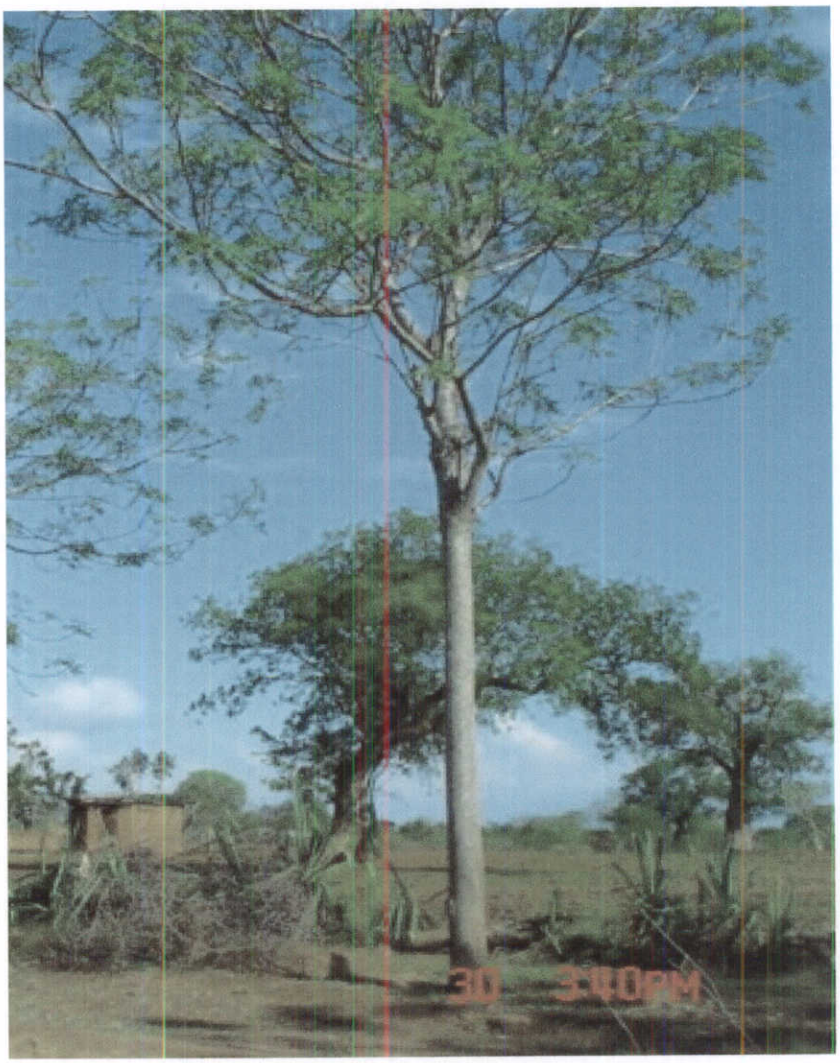

Plate 1. Mature *Melia volkensii* tree. Open crowned and laxly branched tree on a farmer's field in Kibwezi.

The leaflets are oval to lanceolate in shape, with a tapering apex and their margins are serrated (Riley and Bronkenshaw, 1988). The flowers are small, white-fragrant and arranged in loose inflorescences (Albrecht, 1993; Beentje, 1994). Flower petals are 4-5 merous, white and free, while the stamens are as many or sometimes twice the number of petals (Albrecht, 1993). The fruits are drupe–like, oval in shape, about 4 cm long with a thick bonny endocarp (Milimo and Hellum, 1989). The seeds are oval, about 2 cm long; 0.5 cm wide and the microplylar end consist of an appendage known as caruncle (Milimo, 1989b).

1.3 Phenology and life cycle

M. volkensii is a fast growing tree species, which sheds its leaves twice a year and flushes new leaves towards the end of dry season before the on-set of rains (Stewart and Blomley, 1994). The flowers and fruits are also produced twice in a year with fruits becoming ripe at the end of the dry season when the leaves emerge (Kidundo, 1997). Milimo and Hellum (1989) have indicated that fruiting of the tree is throughout the year. Flowering starts as early as 2.5 years of age (Stewart and Chirchir, 1992).

The pollination mechanism of the species is unclear and there have been conflicting reports. However, bees have been reported to visit the flowers, an indication of probable insect pollination (Teel, 1985; Albrecht, 1993). Other reports indicate that the species could be self-pollinating (Milimo, 1994). The fruit development takes 12 to 13 months from the onset of flowers to maturity (Milimo and Hellum, 1989). Fruit development lacks seasonal pattern, such that it is possible to have trees on the same site flowering and

fruiting at different times of the year (Stewart and Blomley, 1994; Kidundo, 1997). In addition, fruits at different developmental stages can also occur on the same branch making it difficult to differentiate mature from immature fruits (Kindundo, 1997). *M. volkensii* is also a very fecund species, with seasonal yield of between 600-100,000 viable seeds per tree (Milimo, 1994). The fleshy fruits are eaten by wild and domestic animals (Tedd, 1997; Kidundo, 1997). The seed nuts are mainly dispersed by animal and human (Riley and Bronkenshaw, 1988; Shepherd, 1990) and can be dormant for 2-5 years before germinating naturally (Stewart and Blomley, 1994).

1.4 Propagation of *M. volkensii*

1.4.1 Seed storage and germination

Currently there are no well-defined protocols for long-term storage of *M. volkensii* seeds. However, there are reports indicating that seeds with 10-15 % moisture content can be viably stored at 3°C for years (Jøker, 2003). Omondi (2004) also reported that mature and properly dried seed nuts could be stored in airtight containers at 3°C for years without damage.

Germination of *M. volkensii* seeds is epigeal, where two opposite simple primary leaves are stalked and develop from the cotyledons (Milimo, 1989b). Seeds are usually difficult to germinate and are first extracted from the seed nut and scarified to enhance germination. Several scarification protocols have been described (Kidundo, 1997). The protocol that has so far produced the best germination rates (> 60 %), involves breaking the seed caruncle, soaking in water overnight, longitudinal slitting at the micropylar end

and germination of seeds at 25-37°C (Milimo, 1989b). However, under ordinary tree nursery conditions, the method has produced less than 4 % germination success (Stewart and Blomley, 1994). Other widely used traditional scarification methods by local farmers entail use of fires on fleshy fruits or de-pulped seed nuts producing poor germination rates or no germination at all (Kidundo, 1997).

Factors responsible for poor germination of *M. volkensii* seeds during and after storage are not well understood, but they have been attributed to both physical and physiological factors. Generally seeds that can be dried to sufficiently low moisture content to permit low temperature storage are referred to as orthodox. Those that cannot tolerate desiccation to low moisture content and remain viable for few days to weeks are referred to as recalcitrant (Ellis *et al.*, 1990). Initially *M. volkensii* seeds were described as recalcitrant (Milimo, 1989a; Kidundo, 1997), however they have recently been described as intermediate seeds (Omondi, 2004). Apart from poor germination, seed extraction is also difficult, while extracted seeds and young seedlings are highly susceptible to fungal diseases (Jøker, 2003; Njuguna *et al.*, 2004).

1.4.2 Vegetative propagation of *M volkensii*

Propagation through vegetative means has been faced with difficulty too and there has been little success using conventional woody stem cuttings. Milimo (1989a) achieved a maximum of 50 % rooting with juvenile (3 months old) seedling stem cuttings and less than 9 % with conventional woody stem cuttings. Root cuttings have produced better results (55 %) relative to conventional cuttings from mature trees (Kidundo, 1997). However, harvesting of root cuttings is cumbersome and renders the trees susceptible to wind fall (Stewart and Blomley, 1994) at the same time tree formed from root cuttings do not establish well and are also prone to wind fall (KEFRI, unpublished)

1.5 Silviculture

There are no commercial plantations of *M. volkensii* except for research stands at Kitui and Meru (KEFRI unpublished; Stewart and Blomley, 1994). The survival and growth of *M. volkensii* in well-managed research stands is high (Stewart and Chirchir, 1992). Currently spacing trials are going on at KEFRI-Kitui, Tiva nursery. Preliminary results after 3 years of growth indicate that spacing of 3.5 × 3.5 m was optimal at this early growth stage (Kimondo and Ouma, 2004). *M. vokensii* trees growing on farmer's fields are irregularly dispersed at spacing of 10-15 m (Stewart and Blomley, 1994). Their growth is also fast compared to the trees in natural stands; this is because they are managed together with food crops (Mulatya *et al.*, 2002). Most of *M. volkensii* trees on farmers' fields are mainly natural regenerates or root suckers allowed to grow into trees. However some farmers collect wildings, while very few use seedlings raised in a nursery. Natural regenerations are irregular in terms of time and place where they occur. There are

also reports that some farmers plant seed nuts directly after scarification with fire (Kidundo, 1997).

Early regular pruning of young *M. volkensii* trees is required and it involves removal of lateral buds, as they appear in order to attain straight clean boles (Kimondo and Ouma, 2004). Pruning and thinning at later stages (>4 year old) has also been recommended when used in agroforestry systems, because of *M. volkensii* branching habits and canopy (Mulatya, *et al.*, 2002). Some farmers prune the crown, while others pollard for purposes of reducing shading of intercrops under, harvesting fodder and increasing the bole diameter (Stewart and Blomley, 1994; Tedd, 1997). Seedlings and mature trees are susceptible to fungal pathogens particularly *Fusarium* and *Colletricum* species. After a few weeks of growth, seedlings are prone to damping off, especially if the growth substrate is waterlogged. Application of broad-spectrum fungicides such as Benlate® have been successfully used to control these fungal pathogens (Njuguna, *et al.*, 2004).

1.6 Genetic diversity of *M. volkensii*

Genetic variation occurs over time due to selection, mutation, gene flow and genetic drift (Hamrick, 1989). The level of genetic variation differs among species within different geographical range, life forms and taxonomic affinities (Loveless, 1992). The improvement of any organism depends on the genetic variability available for selection, conservation and manipulation. Thus partitioning of the genetic variability within and between populations will influence breeding and conservation strategies (Chalmers *et al.*, 1992).

Currently there are few reports on genetic diversity of *M. volkesnsii*. Attempts have been made to study genetic diversity using isoenzymes analysis and glasshouse screening for genotype variation (Milimo, 1994). However use of isoenzyme to detect variations in population-based studies is limited (Dawson *et al.*, 1993). *M. volkensii* populations in Kenya have also been distinguished by their eco-climatic zones as eastern and coastal populations using the RAPD marker technique by Runo *et al.* (2004). Work by Runo *et al.* (2004) further revealed that higher genetic diversity was found in farmland populations than natural stands.

1.7 Economic importance

M. volkensii is among the most popular indigenous tree species in ASALs of Kenya (Tedd, 1997; Broadhead *et al.*, 2003). It is fast growing, tolerant to dry conditions and is also compatible with most crops. However root and crown pruning are required to reduce competition with crops (Mulatya *et al.*, 2002). The species is widely utilized as a multipurpose tree resource supplying both wood and NFWPs throughout the year (Stewart and Blomley, 1994).

M. volkensii produces high quality timber and poles apart from being the principal species used for making log hives (Kidundo, 1997). The leaves and fruits are used as fodder during dry seasons, while leaf preparations are used for medicinal purposes (Kokwaro, 1993). Fruit pulp contain 10 % crude fat, >10 % crude protein, while leaves contain 5 % crude fat, > 21 % crude proteins (Milimo, 1994). The flowers provide excellent bee forage (Stewart and Blomley, 1994), while seed extracts posses anti-feedant

and growth inhibitors against the desert locust (*Schistocera gregaria*) and mosquito (*Culex papiens* and *Anopheles arabiensis*) larvae (Rajab and Bentley, 1988; Sharook *et al.*, 1991), diamond back moth (*Plutella xylostella*) and cabbage hoper (*Trichoplusia ni*) (Aktar and Isman, 2004).

1.6 Vegetative propagation of tree species

Vegetative propagation methods have been employed in tree improvement programmes such as establishment of clonal seed orchard and multiplication of selected genotypes that offer quick productive gains (Palanisamy *et al.*, 1998). Natural regeneration by seed of some tropical woody species is faced with problems such as long breeding cycles, seed recalcitrance and dormancy. In such cases vegetative propagation methods have successfully been employed to circumvent these problems (Jain and Babbar, 2003). Traditionally seeds have been used for *ex-situ* conservation of species in gene banks. However in recent years, the advances in vegetative propagation techniques have made it possible for species with recalcitrant seeds to be conserved *ex-situ* through vegetative means (Krishnapillay, 2000).

Vegetative propagation methods, on the other hand offer several advantages over sexual propagation. They have facilitated breeders to propagate selected true to type elite clones, infertile hybrids and polyploids easily (Bonga, 1987; Longman and Wilson, 1993). However vegetative propagation methods are also faced with problems such as variation in response to a technique due to clonal, species, type, age and source of explant, and propagation conditions (George *et al.*, 1988; Leakey *et al.*, 1990). Thus exhaustive

research is required in order to determine optimum conditions for each species or variety. Vegetative techniques commonly used fall under two broad categories of micro- and macro-propagation. These techniques are utilized depending on the breeding objectives to be achieved (Savill and Kanowski, 1993).

1.6.1 Micro-propagation of tree species

Micro-propagation is a term used to refer to plant tissue culture, which is a blanket term for protoplast, cell, tissue and organ culture under aseptic conditions. These techniques are based on the concept that individual plant cells are totipotent. Vegetative propagation by means of cell and tissue culture is a powerful tool for plant germplasm conservation, rapid clonal multiplication for reforestation and tree improvement programmes (Reddy *et al.*, 2001; Nunes *et al.*, 2003). These techniques have also shown great potentials in domestication and genetic manipulation of forest species (Warrag *et al.*, 1990; Ndoye, *et al.*, 2003). Shoot tip or auxiliary bud cultures are usually convenient choice for tree species such as *Poplar*, because explants larger than single meristem region grow at higher frequencies (Confalonieri, 2003).

Woody taxa are generally difficult to regenerate via tissue culture methods especially for explants derived from mature trees (George *et al.*, 1988; Swamy *et al.*, 2004). This is mainly due to sporadic growth patterns, recalcitrant nature, high incidence of microbial contamination and polyphenol exudation (Zimmerman, 1986; Jain and Babbar, 2003). However, seasonal explant harvesting and rejuvenation, use of polyvinyl pyrrolidone (PVP) and activated charcoal among others, have been used in addressing recalcitrance

and polyphenol problems (Quraishi *et al.*, 2004; Bhatt and Dhar, 2004). Despite problems associated with mature explants, success has been reported in a number of woody species such as *Acacia mearnsii* (Sascha *et al.*, 2000) and *Quercus robur* (Toribio *et al.*, 2004).

The plant regenerative routes of micro-propagation may involve two or more culture steps, each with its individual culture requirements, such as explant type, plant growth regulators (PGRs) concentration and type, which are often species or variety specific (George *et al.*, 1988; Hartman *et al.*, 1997; Salvi *et al.*, 2001). The generally accepted methods of *in vitro* plant multiplication are direct and indirect, where plant regeneration are achieved with and without intervening callus phase, respectively. Murashige (1974) defined the stages of micro-propagation as (i) explant establishment, (ii) multiplication of propagules and (iii) plant rooting and transfer to soils.

1.6.1.1 *In vitro* shoot multiplication

Adventitious organogenesis or direct regeneration usually occurs on original explants or on primary cultures derived from original explants (Schestibratov, *et al.*, 2003). Direct regeneration can be achieved on many plant species, especially those generally propagated adventitiously. Success with these species is mainly associated with changes in the level of endogenous PGRs, when explants are detached from the parent plants (Haines, 1992). Direct regeneration methods are based on induction of primordial bud growth by supplying exogenous cytokinins. This results in formation of miniature shoot clusters directly from explants tissues, which are subdivided and cultured into fresh medium for root induction or continued multiplication (Yeoman, 1986).

Generally higher ratios of cytokinin to auxins have been employed in direct regeneration protocols using explants such as axiliary, apical and adventitious meristems. However, the rates of induction vary with species, explant type and culture condition (Salvi *et al.*, 2001; Swamy *et al.*, 2004). Several protocols using the direct regeneration method have been described for species such as *Tectona grandis* L. (Teak), *Balanites aegyptiaca* by Tiwari *et al.*, (2002) and Ndoye *et al.* (2003), respectively. The type of explant to be used is determined by the objective, for example meristem cultures have been used mainly for virus elimination (Nehra and Kartha, 1994; Sascha *et al.*, 2000) and rejuvenation purposes (Franclet *et al.*, 1987; Bon and Monteuuis, 1991).

Each explant type has its own shortcomings and advantages, for example; meristem cultures have been associated with difficulties during promotion of shoot elongation (Barakat and El-Lakany, 1992). Though shoot tips and auxiliary buds have been successful in direct regeneration of woody species, they are difficult to decontaminate and are generally slow to respond, especially if obtained from mature trees (Jain and Babbar, 2003). Direct regeneration protocol is preferred for multiplication of clones; this is because the method is stable and less prone to genetic instability (Timmis *et al.*, 1987). Application of direct regeneration has been very successful with juvenile materials. However, successful cloning of mature trees of known characteristics can be achieved by significantly reducing problems associated with mature explants (Sascha *et al.*, 2000; Toribio, *et al.*, 2004).

1.6.1.2 Callus cultures and somatic embryogenesis

Higher levels of PGRs mainly auxins than is necessary for stimulation of adventitious roots or shoots results in proliferation of callus. Callus can be induced from a number of explants, however juvenile tissues are the most responsive. Generally, shoots or embryos can be initiated when callus is subcultured into medium with low levels of PGRs, especially auxins (Bacwar *et al.*, 1988; Hsiang-Chi *et al.*, 1997; Moser *et al.*, 2004). Friable callus are usually initiated by extremely high level of auxin such as 2,4-dichloropynoxyacetic acid (2,4-D) and when agitated in liquid media they form cell suspension cultures. Suspension cultures have been used to prepare protoplast cultures or induction of somatic embryos (Nanda and Rout, 2003; Schestibratov, *et al.*, 2003) and production of useful secondary metabolites such as azadirachtin (Kearney *et al.*, 1994; Allan *et al.*, 1994). Callus organogenesis is not common, but has been successful in palm tree (*Areca catechu*) and *Pinus radiata* (Tang *et al.*, 1998; Schestibratov *et al.*, 2003).

Asexual reproduction of some species and hybrids has been difficult to achieve by cuttings or root suckers. Regeneration through somatic embryogenesis can be a useful and most efficient method for clonal or mass propagation of such species (Confalonieri, 2003). It is also a useful tool for breeding programmes, genetic transformation and germplasm cryopreservation (Minocha and Jain, 2000; Toribio *et al.*, 2004; Arnold *et al.*, 2005). Somatic embryos have been initiated from a wide range of explant sources such as cotyledons, zygotic embryos, young seedlings, leaves and anthers (Jörgensen, 1993; Wilhem, 2000; Toribio *et al.*, 2000).

Regeneration of plants through somatic embryogenesis can be achieved through direct and indirect techniques. Direct techniques usually do not have an intervening callus phase, takes a shorter time and has reduced risks for somaclonal variations as opposed to the indirect technique. Induction of somatic embryogenesis has been accomplished in many forest species (Thorpe *et al.*, 1991; Dustan *et al.*, 1995). However in some species, such as Lychee fruit tree (*Litchi chinensis* Sonn.), maturation of embryos and plant recovery has been difficult (Puchooa, 2004).

1.6.2 Macro-propagation of tree species

Macro-propagation technique involves use of semi-lignified and lignified cuttings (Hartman *et al.*, 1997). Vegetative propagation through cuttings is one of the fastest and easiest ways to produce seedlings and had been initially confined to easily rooted trees such as *Poplar* and *Eucalyptus* species (Leakey *et al.*, 1994; Jinks, 1995). In vegetative propagation by cuttings, a portion of the stem, root or leaf is cut from the parent or stock plant and then induced to form shoots or roots by chemical or environmental manipulations (Longman and Wilson, 1993; Hartman *et al.*, 1990). PGRs, mainly auxins (IAA, IBA and NAA) have been used to induce adventitious roots in forestry and agriculture. Successful propagation by cuttings is dependent on rooting ability of the tree, which varies among species, provenance, age and rooting medium. In addition other factors namely; propagation environment, cutting type, size, insertion method and, PGRs type and concentration applied to induce roots are equally important (Longman and Wilson, 1993; Jinks, 1995).

Cuttings have been classified according to the part of the plant in which they have been obtained; stems (hardwood, semi-hardwood, softwood and herbaceous), leaves, leaf buds and roots (Leakey *et al.*, 1990; Hartman et al., 1997). Although stem cuttings can be used to propagate many species easily, some can be difficult too. Use of rejuvenated stem and in particular leafy stem cuttings has been found to be more promising for such species (Longman and Wilson, 1993). The retention of juvenile features in shoot apices near the root system has facilitated rejuvenation and hence commercial propagation of selected tree clones (Longman and Wilson, 1993; Hartman *et al.*, 1997). On the other hand, root cuttings have also been used to propagate species that easily form adventitious buds, which become suckers. When roots of these species are dug, removed and cut into pieces, buds are likely to form (Leakey *et al.*, 1990; Hartman *et al.*, 1997).

1.7 Justification of the study

M. volkensii is a commercially viable indigenous tree species which thrive well in ASALs conditions. However, overexploitation and settlement in its native range has led to loss and fragmentation of natural populations (Milimo, 1994). This has caused great concerns due to loss of genetic diversity, disruption of gene flow and isolation of remaining populations (Odee *et al.*, 2004). The problem has been exacerbated by lack of enough planting materials due to problems associated with propagation of this species.

Mass propagation of *M. volkensii* by seeds is difficult, mainly due to problems in seed extraction and germination (Teel 1985; Kidundo, 1997; Omondi, 2004). Seed propagation protocols developed so far are cumbersome and difficult to optimize, and have produced poor results (<4 % germination) under nursery conditions (Stewart and Blomely, 1994). Vegetative propagation by conventional stem cuttings, on the other hand has also produced erratic and extremely low success rates (<10 % rooting) (Kidundo, 1997; Mulatya, 2000). Though root cuttings has been reported as a promising alternative to conventional stem cuttings (Kidundo, 1997), the method entails destructive harvesting of roots, which is cumbersome to implement. Hence there is a need to investigate alternative propagations methods.

Advances in biotechnology have generated new opportunities for alleviation of propagation difficulties at the same time offering opportunities for genetic resource conservation (Kameswara, 2004). This is of significant importance especially for species that are difficult to conserve or propagate by conventional methods (seeds and cuttings) such as *M. volkensii*. There are no reports in literature on propagation of *M. volkensii* by tissue culture and rejuvenated leafy stem cuttings. The main objective of this study was to develop vegetative propagation protocols for *Melia volkensii* Gürke, focusing on direct micro-propagation (shoot multiplication and rooting; somatic embryogenesis) and macro-propagation (rejuvenated leafy stem cuttings) techniques.

1.8 Research hypotheses

i. *M. volkensii* is amenable to micro-propagation techniques (direct *in vitro* shoot multiplication and rooting, and direct somatic embryogenesis).

ii. *M. volkensii* can be propagated through leafy stem cuttings from rejuvenated mature trees.

1.9 Objective

1.9.1 General objective

The main purpose of this study was to develop alternative vegetative propagation protocols for *Melia volkensii* Gürke, focusing on micro- and macro-propagation techniques.

1.9.2 Specific objectives

i. To determine optimum concentrations and combinations of plant growth regulators on induction of direct *in vitro* shoot multiplication of *M. volkensii* using shoot tip explants.

ii. To compare response of shoot tip explants from mature rejuvenated tree and seedlings.

iii. To regenerate *M. volkensii* plantlets through direct somatic embryogenesis.

iv. To determine effect of pretreatment and surface sterilization on survival and subsequent rooting of rejuvenated leafy stem cuttings of *M. volkensii.*

v. To determine the effects of auxin type and concentration on rooting of rejuvenated leafy stem cuttings of *M. volkensii*.

1.10 Expected outputs of the study

The development of alternative vegetative propagation (micro and macro-propagation) protocols will facilitate large-scale recruitment, sustainable use and conservation of *M. volkensii* germplasm. In view of the fact that seeds are recalcitrant or intermediate, long-term *ex situ* storage is difficult. Robust vegetative regeneration protocols for *M. volkensii* will form the basis for development of *ex situ* or *in situ* conservation strategies using either *ex vitro* or *in vitro* propagation techniques. The development of robust vegetative propagation protocols will also facilitate rapid multiplication, genetic manipulation/ transformation, open up opportunities for domestication and development of new cultivars. Subsequently, this will lead to making available high quality planting materials to farmers and other forestry stakeholders, and incorporate this species in agroforestry, reforestation and afforestation programs in the ASALs.

CHAPTER TWO: MATERIALS AND METHODS

2.1 Macro-propagation

2.1.1 Selection and rejuvenation of Mature *Melia volkensii* trees

A total of twelve mature *M. volkensii* trees were randomly selected from six farms in Kibwezi and Kitui Districts, located in the ASALs of eastern Kenya. All the selected trees were >5 years old (as informed by farmers) and with >3 meters of straight bole. Six trees were selected per site, in each site three trees were rejuvenated by stem coppicing and remainder by root pruning. Rejuvenation was carried out during the short rainy season of October to December 2004. Trees were coppiced at a height of 30-45 cm above the ground and two to three roots with diameter ≥10 cm per tree were pruned at a distance of one meter away from the tree trunk. Root pruning was achieved by completely removing a root segment of 5-10 cm in length.

2.1.2 Collection and preparation of leafy stem cuttings

The leafy root suckers and stem coppices were collected three months after rejuvenation (MAR). The 3-month old coppice (Plate 2A) and sucker stems (Plate 2B) were harvested early in the morning, sprayed with water, placed in polythene bags and transported to the laboratory in cool boxes. Suckers and coppices showing moderate growth (30-40 cm tall) and with basal diameter of 3-4 mm were harvested. The extremely thin, stressed, diseased and thick vigorously growing shoots were avoided. Overstuffing of suckers and coppices in polythene bags and direct contact with ice in cool boxes was also avoided during transportation. In the laboratory, two to three node leafy stem cuttings of 50-70 mm in

length were prepared. The basal cuts were made at about one centimeter below the lower node and all leaves were removed except one leaf with two half trimmed leaflets on the upper side (Plate 2C). Later 'clean cuts' were made with a sharp sterile scalpel blade just below the lower node, before leafy cuttings were treated with the PGRs (IBA) and insertion into rooting medium (2D). During preparation the cuttings were constantly sprayed with water to maintain leaf turgidity.

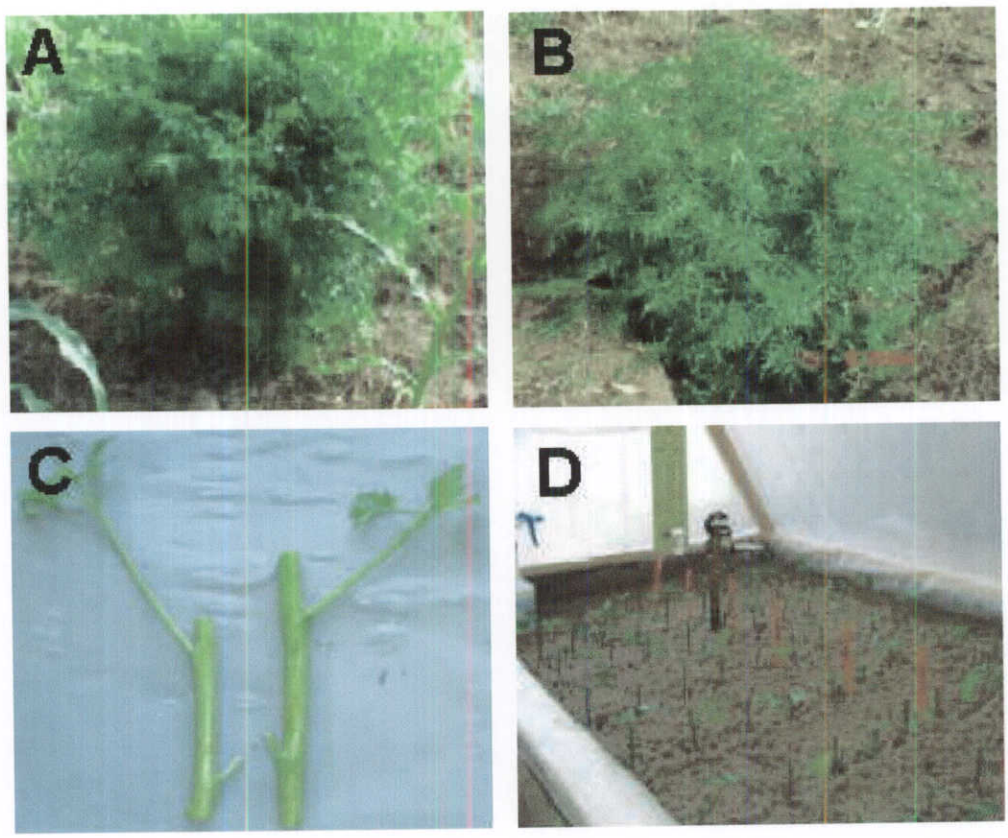

Plate 2. Rejuvination, preparation and rooting of *Melia volkensii* leafy stem cuttings. (A) Stem coppices and (B) Root suckers, produced at 3 months after rejuvenation of mature trees; (C) Two-node leafy stem cuttings before 'cleaning' the cuts made by secateurs with a sharp sterile scalpel blade; (D) Leafy stem cuttings inserted in sand rooting substrate in a low cost mist propagator at 15 days after insertion (DAI).

2.1.3 Rooting of rejuvenated leafy stem cuttings

2.1.3.1 Effect of fungicide pretreatment and surface sterilization

The influence of pretreatment with fungicide and surface sterilization on survival and subsequent rooting of rejuvenated leafy stem cuttings was investigated. The leafy stem cuttings were prepared using sucker stems collected from Kitui i.e. single provenance. Two trees with enough suckers were selected; the suckers of one tree (S1) were pretreated with 0.2 % Benomyl® fungicide, seven days prior to harvesting. Suckers of the second tree (S2), were not subjected to any treatment before or after harvesting. The harvested S1 suckers were surface sterilized with 0.2 % sodium hypochlorite for two to three minutes and rinsed in water before preparation of two to three node leafy stem cuttings. Prior to treatment with rooting hormones and insertion into rooting substrate, the S1 cuttings were also immersed into 0.2 % Benomyl® fungicide and allowed to drip for three to five minutes.

The S1 and S2 cuttings were treated with IBA prepared and used as described by Hartman *et al.* (1997) (appendix 1). The concentrations tested for root induction were 0, 2, 4, 6, 8 and 10 g l^{-1} and plain distilled water was also included as a second control treatment, since 0 g l^{-1} IBA consisted of 50 % ethanol. The cuttings were inserted into sand rooting substrate (washed and sterilized at 121°C, 1.06 kg cm^{-2} for 60 minutes) in a low-cost mist propagator covered with 50 % shade cloth. Cuttings were rooted for two months under mean relative humidity (RH) of 70-90 % and mean temperatures of 22°-28°C. High relative humidity was maintained by spraying fine mist using a hand sprayer thrice per day.

2.1.3.2 Effect of auxin type and concentration

Leafy stem cuttings prepared from root suckers and coppices collected from Kitui and Kibwezi were used to evaluate the effect of auxin type and concentration on induction of adventitious roots. They were prepared as described for S1 cuttings in section 2.1.3.1. Three PGRs (IBA, IAA and NAA) were tested at 0, 2, 4, 6, 8 and 10 g l^{-1} and distilled water. The cuttings were rooted under similar structure, rooting media and duration as described in section 2.1.3.1. The mean RH and temperature recorded during the course of the experiment were 65-80 % and 23-27°C, respectively.

2.1.3.3 Weaning and hardening of rooted cuttings

The rooted cuttings in section 2.1.3.1 and 2.1.3.2 were transplanted into potted nursery soil (3: 1, soil: sand) and allowed to establish under rooting conditions for a period of 2 weeks. During this period, they were weaned with Broughton and Dillworth plus N plant nutrient solution (Appendix 2) as described by Somasegaran and Hoben (1985) twice per week. During the third and fourth week, conditions in the propagator were gradually reduced, then cuttings were finally removed and allowed to grow under normal glasshouse conditions.

2.1.4 Experimental design, assessment and data analysis

The treatments in all rooting experiments conducted had five replicates each consisting of six cuttings randomized within the rooting bed (30 cuttings per treatment). However in the experiment investigating the effects of PGRs type and concentration (section 2.1.3.2), each cutting was labeled based on provenance (Kitui or Kibwezi) and source (sucker or coppice cutting). The numbers of buds and size, surviving cuttings and diseased cuttings were assessed at 15, 30, 45 and 60 days after insertion (DAI) into the rooting substrate, while the percentage rooting, number and length of roots per cutting were assessed at 60 DAI. Two factor analysis of variance (ANOVA) was conducted on data sets to investigate the effects of; (i) PGR type × Concentration and, (ii) Provenance × cutting source. The Newman-Keuls test ($p<0.05$) was used to compare the treatment means. Data on percentage rooting and number of roots per cutting were transformed [$X' = \sqrt{(X + 0.5)}$] prior to the statistical analysis (Zar, 1974).

2.2 Micro-propagation

2.2.1 Raising and maintenance of glasshouse stock plants

Undamaged *M. volkensii* seed nuts were obtained from Kenya Forestry Seed Centre, Muguga. Seeds were extracted from nuts (Plate 3A) and their caruncles broken. They were then surface sterilized with 10 % sodium hypochlorite for 15 minutes and rinsed in three changes of sterile distilled water. Sterile seeds were soaked overnight in 1 % sodium hypochlorite before longitudinal slits were made at the micropylar end, as described by Milimo (1989b). The slitted seeds were then pre-germinated for 10-15 days in sterile moist vermiculite incubated at 28±2°C and 16 h photoperiod. The germinated

seeds were transplanted into potted nursery soil (soil: sand, 2:1) and raised under normal glasshouse conditions at KEFRI-Muguga.

The seedlings were watered twice weekly and water logging was avoided. Glasshouse pests mainly red spider mites and scales, were controlled using 0.25 % Diazinon®. Shoot tip explants (2-3 cm tall) for shoot multiplication experiments were first harvested at three months after transplanting of the pre-germinated seeds. The non-lignified re-growths were consistently harvested after attaining heights of 5-10 cm (Plate 3B) to maintain juvenility of the explants obtained.

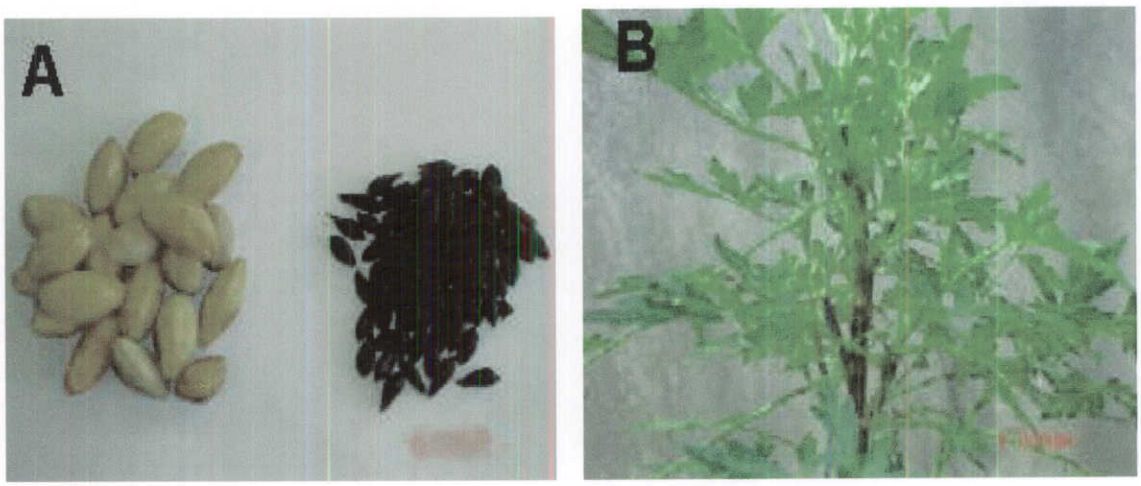

Plate 3. Raising and maintenance of glasshouse explant source for *in vitro* shoot multiplication. Raising and maintenance of *M. volkensii* seedlings explant sources for *in vitro* shoot multiplication. (A) *Melia volkensii* seed nuts (left) and extracted seeds (right); (B) Shoot re-growths on glasshouse seedling stock plant ready for harvesting of shoot tips.

2.2.2 Explant sterilization and culture conditions

Defoliated shoot tips were surface sterilized with 6 % sodium hypochlorite solution containing two drops of Tween® detergent for 10 minutes and then rinsed in three changes of sterile distilled water. Sterile tips were re-cut into sizes of 3-5 mm and inoculated into multiplication medium. Explants from rejuvenated trees (suckers and coppices) in Kitui and Kibwezi were collected 7 days after pretreatment with 0.2 % Benomyl®. The shoot tip explants (about 2-3 cm) were sprayed with clean water in sealable polythene tubes and transported to the laboratory in a cool box. Rejuvenation and pretreatment with fungicide is as described in section 2.1.3. In the laboratory, shoot tip explants were soaked overnight in 5 mg l^{-1} ampicillin, rinsed in distilled water and then subjected to similar sterilization protocol as glasshouse sourced explants.

The multiplication medium consisted Murashige and Skoog (1962) basal salt mixture and vitamins augmented with 30 g l^{-1} sucrose (MS), supplemented with PGRs alone and in combination. In all the experiments control treatments, consisted of MS devoid of PGRs, while pH was adjusted to 5.8 prior to addition of 0.8 % agar, using 1 M NaOH or HCl. Approximately 40 ml of medium formulation were dispensed in 500 ml glass jars and sterilized at 121°C, 1.06 kg cm^{-2} for 15 minutes. Two explants were inoculated per jar and were incubated in a growth chamber maintained at 28±2°C under a 16:8 h photoperiod with cool white fluorescent lamps providing light intensity of 70 µmol $m^{-2}s^{1}$.

2.2.3 *In vitro* shoot multiplication and rooting

2.2.3.1 Glasshouse sourced seedling explants

Three experiments were conducted to evaluate the effect of MS supplemented with PGRs alone or in combination on induction of direct shoot multiplication using shoot tip explants. The experiments were conducted as follows: (i) MS supplemented with BAP, kinetin and zeatin alone at 0, 0.25, 0.5, 1.0 and 2.0 mg l^{-1} (ii) BAP, kinetin and zeatin at 0.5, 1.0, 2.0 and 3.0 mg l^{-1} in combination with NAA (0.1 and 0.2 mg l^{-1}), (iii) BAP, kinetin and zeatin at 0, 0.5, 1.0, 2.0 and 3.0 mg l^{-1} in combination with IAA (0.1 and 0.2 mg l^{-1}). The shoot multiplication experiments were conducted in two 30-day phases of; (i) culture establishment and (ii) shoot multiplication. In culture establishment, the first generation shoot tip explants were inoculated into multiplication medium for 30 days. In the shoot multiplication phase, second generation shoot tips from phase one were harvested, cut into sizes of 3-5 mm and subcultured into respective multiplication medium.

The rooting experiments were conducted using >2 cm tall shoots multiplied using BAP (0.5 mg l^{-1}) and IAA (0.2 mg l^{-1}). Rooting media tested consisted of MS and ½ MS (half macro-nutrients) supplemented with NAA, IAA and IBA alone at 0, 0.5, 1.0 2.0 and 3.0 mg l^{-1}. After 40 days of culture, the rooted shoots were transplanted into sterile vermiculite substrate packed in plastic cup and covered with polythene bag. The substrate was moistened with Broughton and Dilworth (Appendix 2) plus N nutrient solution (Somasegaran and Hoben, 1985) prior to sterilization.

2.2.3.2 Field collected explants

Unlike glasshouse explants, 3-5 mm surface sterilized shoot tips of field-collected explants were first cultured in ½ MS for 21 days. Slightly elongated non-infected shoots tips were then trimmed into sizes of 2-3 mm and inoculated into multiplication medium. It consisted of MS supplemented with BAP: IAA (0.5: 0.2 mg l^{-1}) selected based on the results obtained with glasshouse explants. Rooting experiments for field-collected explants were similar to those described for glasshouse-sourced explants in section 2.2.3.1.

2.2.4 Direct somatic embryogenesis

2.2.4.1 Explants source, sterilization and culture conditions

Mature seeds of *M. volkensii* from Nuu provenance (Kitui district, Kenya), which had been in cold (4°C) storage for <3 and >12 months were used in this study. The outer and inner seed coats were removed, followed by separation of zygotic embryos and cotyledons. The explants were surface sterilized for 10 minutes with a solution of 6 % sodium hypochlorite containing two drops of Tween® 20 detergent and then rinsed in three changes of sterile distilled water. The cotyledons were split longitudinally into two and rinsed again in three changes of sterile distilled water. The explants were placed with abaxial surface in contact with the embryo induction medium containing MS (Murashige and Skoog, 1962) vitamins, micro- and macro-elements, 30 g l^{-1} sucrose, and supplemented with plant growth regulators (PGRs) either alone or in combination. The pH of the formulations was adjusted to 5.8 using 1 M NaOH or HCl, prior to the addition of 0.8 % agar, and sterilized at 121°C and 1.06 kg cm^{-2} for 15 minutes. Approximately 25

ml of sterile embryo induction medium was dispensed per sterile plastic Petri dish (∅ 9 cm). All the somatic embryogenesis experiments were cultured under temperature and photoperiod conditions described in section 2.2.2.

2.2.4.2 Induction of direct somatic embryos and plant regeneration

Two experiments were conducted to evaluate the response of mature cotyledonary and zygotic embryo explants on induction of direct somatic embryos. In the first experiment the effects of MS medium supplemented with PGRs using explants from seeds stored for <3 months were evaluated as follows: BAP, NAA and 2,4-D (0.5, 1.0 and 2.0 mg l^{-1}) alone, BAP (0.5, 1.0, 2.0 and 4.0 mg l^{-1}) combined with NAA (0.5 and 0.2 mg l^{-1}), and BAP (0.5, 1.0, 2.0 and 4.0 mg l^{-1}) combined with 2,4-D (0.5 and 0.2 mg l^{-1}). In the second experiment, BAP (0.5, 1.0, 2.0 and 4.0 mg l^{-1}) combined with 2,4-D (0.5 and 0.2 mg l^{-1}) were evaluated on explants from seeds stored for >12 months. All control treatments were devoid of PGRs. Two explants were placed in each dish as a replicate.

After 4 weeks of culture, sections of cotyledonary explants containing direct somatic embryos were excised and subcultured on semi solid MS and ½ MS (micro- and macro-elements) without PGRs. Shoots tips harvested from the clusters of shootlets formed on MS and ½ MS media were used for shoot multiplication on semi solid MS media supplemented with BAP (0.5 mg l^{-1}) and IAA (0.2mg l^{-1}) for 6 weeks. Shoots of ~3-5 cm long were subcultured on MS media supplemented with IBA (0.5, 1.0, 2.0 and 5.0 mg l^{-1}) for rooting.

2.2.5 Experimental design and data analysis

Direct shoot multiplication and somatic embryogenesis experiments consisted of 10 replicates (20 explants) per treatment. The rooting experiment consisted of 3 replicates each with 5 shoots (15 explants). All the experiments were laid out in the growth chamber in a completely randomized block design. The data for shoot multiplication experiments, namely; height, number of shoots per explants and quality scores (shoot form) of harvestable shoots ≥2 cm (1, 2 and 3; for low moderate and high score) were assessed at 15 and 30 days after subculture (DAS). Data on rooting (% rooting, length and number of roots per shoot), somatic embryogenesis (% somatic embryogenesis and number of embryos explant^{-1}) and callus formation were assessed after 30 days of culture incubation. Callus formation in shoot multiplication and somatic embryogenesis experiments were assessed and scored on a scale of 1, 2 and 3 representing low, moderate and high, respectively. The data sets were subjected to two-factor ANOVA to investigate the effects of; (i) PGR types × Concentrations and, (ii) PGR combinations × Concentrations. The means were compared using the Newman-Keuls Test (p<0.05). The data on number of embryos per explant, percentage somatic embryogenesis and rooting were transformed [$X' = \sqrt{(X + 0.5)}$] prior to the statistical analysis (Zar, 1974).

CHAPTER THREE: RESULTS

3.1. Effect of pretreatment and surface sterilization of leafy stem cuttings

The survival of S1 cuttings in all IBA concentration treatments by 30 DAI was above 70%. However at 45 DAI, leaves and nodal buds of some cuttings had turned yellow and upon careful lifting, their bases were noted to be rotten. Rooting of 10-30 % was achieved on cuttings treated with 4-10 g l^{-1} IBA at termination of the experiment (60 DAI). The highest rooting percentage (30 %) was achieved on cuttings treated with 8 g l^{-1} IBA and lowest (13 %) was achieved on those treated with 6 g l^{-1} IBA. There were no generalized trends observed on all parameters assessed and only percentage rooting was significantly ($p<0.05$) influenced by the concentration of IBA used, while the Newman and Keuls test ($p<0.05$) revealed that only mean percentage rooting at 8 g l^{-1} was different from other IBA concentrations. Only 5 % of all the rooted cuttings were successfully weaned and hardened.

Irrespective of IBA concentration used, 30 % of the S2 cuttings inserted had fungal infestation by 7 DAI and by 20 DAI all the remainders were also infected. Heavy fungal infestation was observed on leaf petioles, leading to loss of leaves and finally death of the cuttings. However, up to 60 % of the cuttings before death had actively growing nodal buds of 1.0-5.0 mm, especially those treated with IBA at 0-4 g l^{-1}. The experiment involving S2 cuttings was terminated at 20 DAI. The cuttings were also carefully lifted out and their bases inspected. Those treated with IBA at 0-4 g l^{-1} and distilled water had callused at their bases, while those treated with 6-10 g l^{-1} IBA did not form callus.

3.2 Influence of auxins on rooting of leafy stem cuttings

In this experiment only 15 % of the total cuttings inserted were diseased by 30 DAI and thereafter no fungal infestation on cutting was observed. In addition, cuttings treated with PGRs (IBA, IAA and NAA) at 6-10 g l^{-1}, had relatively smaller nodal buds (0.4-0.9 cm) as compared to those treated with 0-4 g l^{-1} and distilled water (1.0-1.3 cm). At termination of the experiment (60 DAI), 13-33 % rooting was achieved on cuttings treated with IBA at 4-10 g l^{-1} (Table 1). The cuttings treated with NAA and IAA, only 4 g l^{-1} and 6 g l^{-1}, respectively, induced 5 % rooting. Other NAA and IAA treatment concentrations did not induced root formation (Appendix 3).

Table 1. Effect of IBA concentration on rooting of 3-month old rejuvenated leafy stem cuttings at 60 days after insertion (DAI) in sand rooting substrate.

IBA ($g\ l^{-1}$)	No. of roots $cutting^{-1}$ *	Root length (cm) $cutting^{-1}$	% Rooting*
0	0a**	0a	0a
2	0a	0a	0a
4	1.7a	5.2b	20b
6	0.3a	4.5b	13b
8	5.3b	4.7b	33c
10	1.3a	6.5b	20b
CV %	**22.4**	**18.2**	**16.8**

*Data were transformed [$X' = \sqrt{(X + 0.5)}$] prior to ANOVA ($n = 20$). **Values in a column followed by the same letter are not significantly different according to the Newman-Keuls Test ($p<0.05$). Values in are means of data in their original scale.

At 60 DAI, some of the bases of cuttings were rotten (Plate 4A). Generally, bases of cuttings treated with distilled water, 0 and 2 g l^{-1} auxins were callused (Plate 4B), whereas the rooted (Plate 4C) and non-rooted (Plate 4D) cuttings treated with 4-10 g l^{-1} auxins had slightly furrowed and swollen bases, respectively. Irrespective of the auxin type, 80-90 % and 20-40 % of cuttings treated with 0-4 g l^{-1} and 6-10 g l^{-1}, respectively, had actively growing nodal buds.

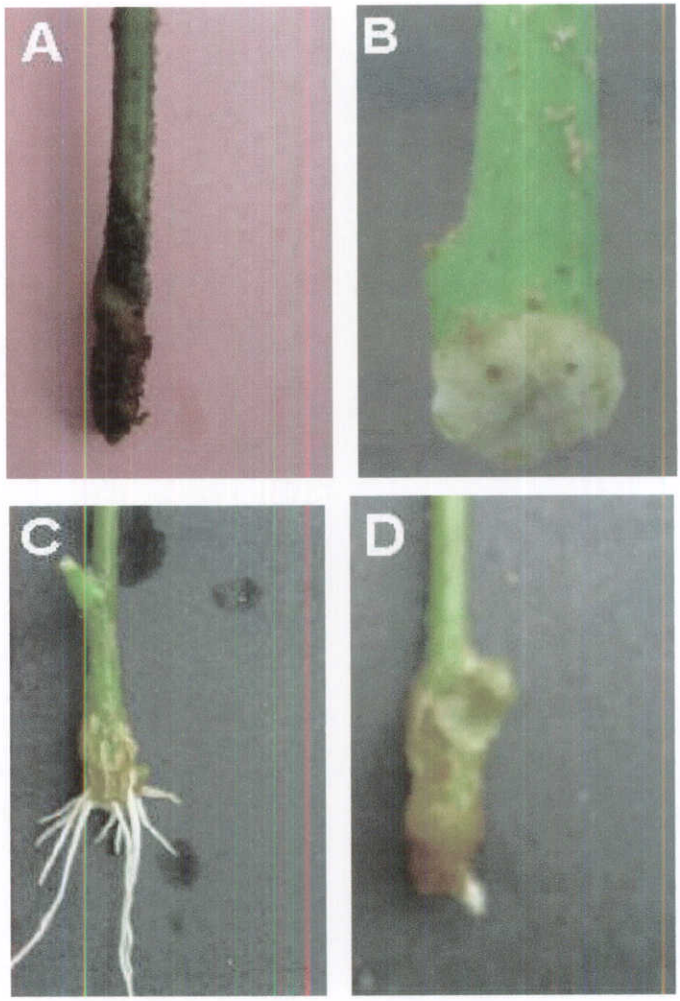

Plate 4. Effect of treatment with rooting hormones on 3-month old *M. volkensii* rejuvenated leafy stem cuttings. (A) Rotten base of a cutting observed at 45 days after insertion (DAI); (B) Callused base of a cutting treated with 2 g l^{-1} NAA; (C) Furrowed and rooted, and (D) Swollen and non-rooted base of a leafy stem cutting treated with 8 g l^{-1} IBA at 60 DAI.

There was no significant (p<0.05) influence of provenance and cutting source on rooting of leafy stem cuttings. Nonetheless, cuttings treated with IBA irrespective of the source and provenance out-performed those treated with NAA and IAA on all rooting parameters considered. The number and length of roots per cutting were significantly (p<0.05) influenced by IBA concentration. However, variation in number, length and thickness of roots within and between treatments were observed (Plate 5A and B). In the cuttings treated with 8 g l^{-1} IBA, 2-12 roots per cutting of 0.9-15 cm long were recorded. The overall best scores on all rooting parameters were achieved on cuttings treated with 8 g l^{-1} IBA (Table 1). Up to 40% of the total rooted cuttings were successful weaned, hardened and allowed to grow under normal glasshouse conditions (Plate 5C).

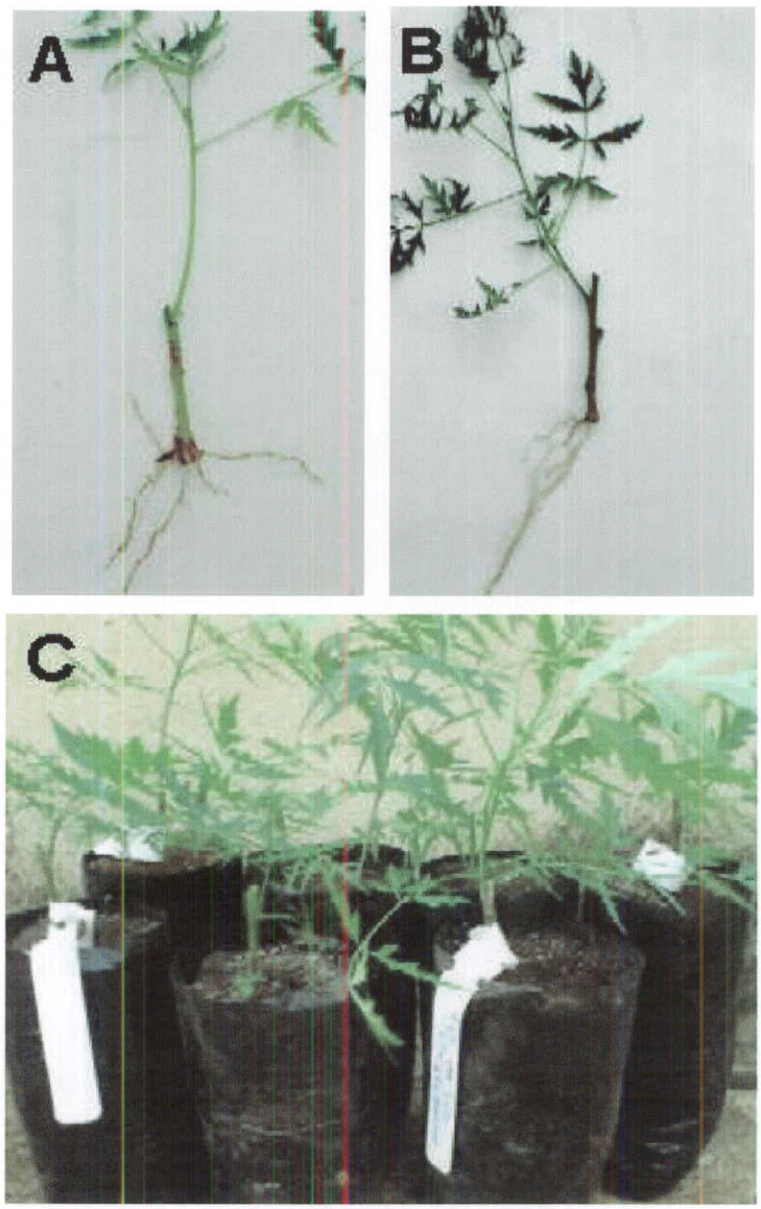

Plate 5. Variation in number, size and length of roots on cuttings treated with IBA. (A) Thick well spread roots; (B) single root with numerous fine branching roots formed on leafy stem cuttings treated with 8 g l^{-1} IBA at 60 days after insertion (DAI) in sand rooting substrate; (C) *Melia volkensii* seedlings raised from rejuvenated leafy stem cuttings at one month after transplanting, weaning and hardening.

3.3 Effect of cytokinins alone on *in vitro* shoot multiplication

The visible response of shoot tip explants on MS supplemented with cytokinins (kinetin, zeatin and BAP) alone was generally observed after 10 days of culture. Irrespective of cytokinin type, shoot tips (first and second generation) generally increased in height after 30 days of culture including the control treatments. Shoot height was significantly ($p<0.05$) influenced by cytokinin type and concentration. All three cytokinins tested did not induce shoot multiplication; only up to two shoots per explant and an average of 1.8 was obtained (Table 2). Kinetin generally produced well formed tall harvestable (>2 cm tall) shoots (Table 2), while zeatin induced production of nodal buds but formed non-harvestable shoots (poorly formed shoots). BAP also induced development of nodal buds at the highest concentration (2 mg l^{-1}) but generally formed shoots of fair quality as compared to zeatin (Table 2). All cytokinins formed compact callus at the base of explants. The callus score was influenced ($p<0.05$) by cytokinin type, where zeatin and BAP had higher callus scores than kinetin (Table 2).

Table 2. Effect of cytokinins alone on direct *in vitro* shoot multiplication using first generation shoot tip explants from glasshouse seedling stock plants after 30 days of culture.

Cytokinin type	Conc. (mg l^{-1})	No. of Shoots explant^{-1}	Height (cm)	Shoot quality score	Callus score
BAP	0.25	1.5**a***	2.1**b**	2.0**bc**	0.8**a**
	0.5	1.1**a**	1.5**ab**	2.0**bc**	0.9**a**
	1.0	1.5**a**	1.6**ab**	1.8**ab**	1.0**a**
	2.0	1.8**a**	1.5**ab**	1.4**ab**	1.0**a**
Kinetin	0.25	1.1**a**	3.2**c**	2.5**bc**	0.6**a**
	0.5	1.3**a**	2.4**b**	2.6**bc**	0.6**a**
	1.0	1.0**a**	2.1**b**	1.9**b**	0.8**a**
	2.0	1.2**a**	2.4**b**	2.8**c**	0.9**a**
Zeatin	0.25	1.0**a**	1.8**b**	1.3**ab**	1.0**a**
	0.5	1.0**a**	1.2**ab**	1.3**ab**	1.1**a**
	1.0	1.1**a**	1.3**ab**	1.6**ab**	1.5**a**
	2.0	1.1**a**	1.1**a**	1.0**a**	2.6**b**
Control	0	1**a**	2.2**b**	2.0**bc**	0**a**
CV%		**21.1**	**14.0**	**26.2**	**13.4**

*Values in a column followed by the same letter are not significantly different according to Newman-Keuls test ($p<0.05$). Values in the table are means of treatment in their original scale ($n=10$).

3.4 Effect of cytokinins combined with auxins on *in vitro* shoot multiplication

Similar to results achieved with cytokinins alone, callus formation, shoot height and quality of shoot were also significantly ($p<0.05$) influenced by cytokinin: auxin combinations and DAS. Up to two harvestable shoots (>2 cm) per explant were achieved in most cytokinin: auxin combinations except BAP: IAA. Generally kinetin: IAA combinations formed high quality harvestable shoots (Plate 6A), whereas zeatin: IAA formed poor quality shoots with nodal buds (Plate 6B). The BAP: NAA combinations induced formation of numerous non-harvestable shootlets (<3 mm tall), while zeatin: NAA combinations produced poor quality shoots in addition to high callus scores (Appendix 4). The BAP: IAA combinations generally induced shoot multiplication in both first and second-generation shoot tip explants (Plate 6C).

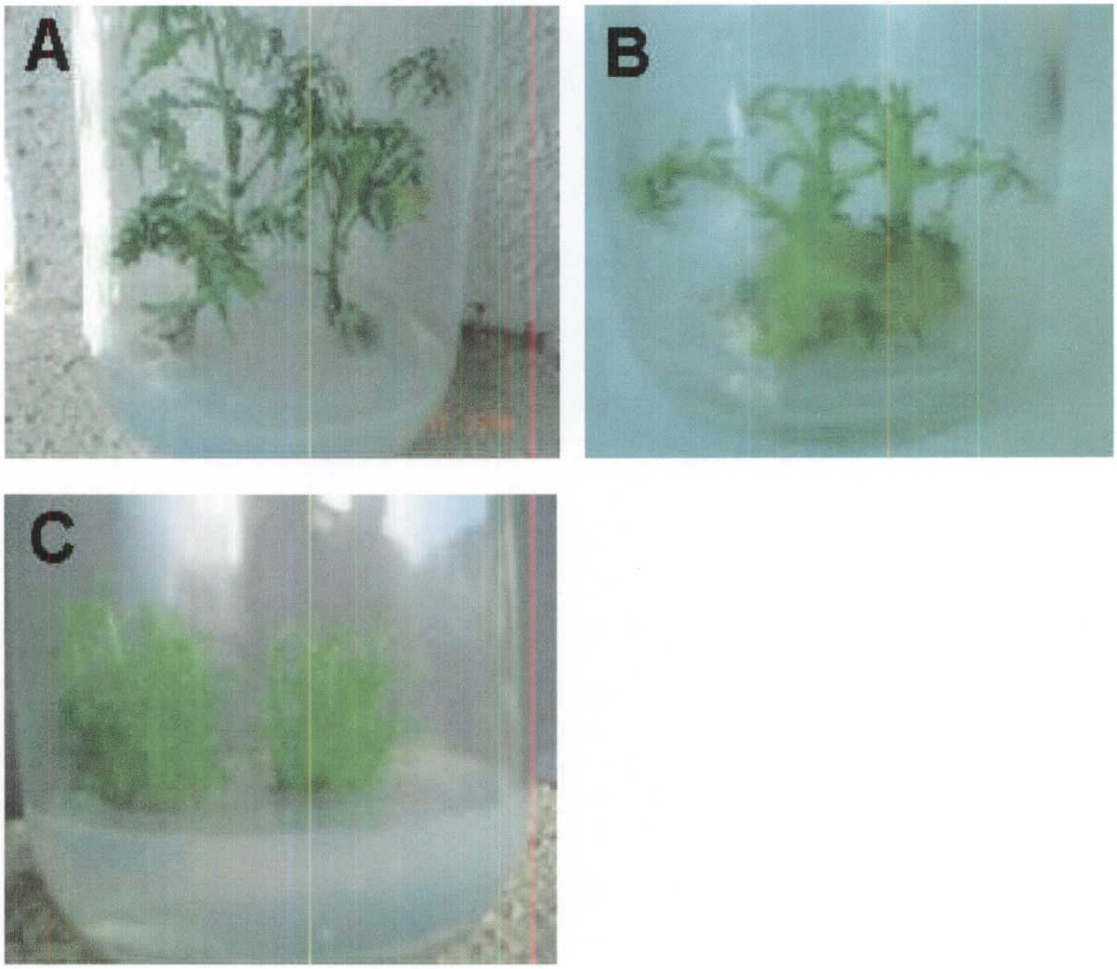

Plate 6. Effects of cytokinins combined with auxin (IAA) on shoots multiplication. (A) Kinetin: IAA (1.0: 0.5 mg l^{-1}) and (B) zeatin: IAA combinations (1.0: 0.2 mg l^{-1}) on shoot growth and quality after 30-day culture of first generation shoot tips. C) *In vitro* shoot multiplication of *Melia volkensii* on MS supplemented with BAP: IAA (0.5: 0.2 mg l^{-1}) at 30 days after subculture (DAS) of second-generation shoot tips

The number of multiplied shoots per explants was significantly (p<0.05) influenced by concentration combinations of BAP: IAA and DAS. Up to 44 % of the BAP: IAA cultures had more than 2.3 shoots per explant at 30 DAS (Table 3), with BAP: IAA at 0.5: 0.2 mg l^{-1} inducing up to nine shoots per explant. The highest mean number of shoots per explant (5.4 shoots) was achieved in BAP:IAA (0.5: 0.2 mg l^{-1}) (Table 3). However there were no significant (p<0.05) differences between BAP: IAA combinations at 1.0: 0.1 and 0.5: 0.2 mg l^{-1} on mean shoot height, number of shoots per explant, shoot quality and callus formation (Table 3).

Table 3. Effect of BAP: IAA combinations on shoot multiplication using second-generation shoot tip explants at 30 days after sub-culture (DAS).

BAP:IAA ($mg\ l^{-1}$)	Shoot height (cm)	No of shoots $explant^{-1}$*	Shoot quality	Callus score
0:0	0.8a**	1.0a	1.0a	0a
0.5:0.1	2.8bc	2.3ab	1.6cd	1.8b
1.0:0.1	3.2dc	4.0bc	1.9d	1.6b
2.0:0.1	2.1b	1.0a	1.0a	1.9b
3.0:0.1	1.5ab	1.0a	1.0a	0a
0.5:0.2	2.6c	5.4c	1.6cd	1.5b
1.0:0.2	2.1cb	2.3ab	1.3bc	1.7b
2.0:0.2	1.4ab	1.0a	1.0a	1.7b
3.0:0.2	0.9a	1.0a	1.0a	0.4a
CV %	**8.0**	**19.0**	**25.4**	**17.3**

*Data was transformed using $[X' = \sqrt{(X + 0.5)}]$ prior to analysis. **Values in a column followed by the same letter are not significantly different according to Newman-Keuls test ($p<0.05$). Values in the table are means of treatment in their original scale ($n=10$).

Increasing DAS from 15 to 30 resulted in increased number of harvestable shoots per explant in BAP: IAA combinations, where shoot multiplication was induced (Fig. 2A). Generally lower BAP concentrations (≤1.0 mg l^{-1}) in combination with IAA at 0.1 and 0.2 mg l^{-1} induced shoot multiplication. Nonetheless further increase in BAP concentration combined with IAA (0.1 and 0.2 mg l^{-1}) reduced the number of shoots obtained per explant (Fig. 2A). A similar trend was also observed on shoot height (Fig. 2B) and shoot quality scores. Only 15 % of the field-collected shoot tip explants were successfully decontaminated. They were also multiplied using BAP: IAA combination (0.5: 0.2 mg l^{-1}) and an average of 3.5 shoots per explant were achieved.

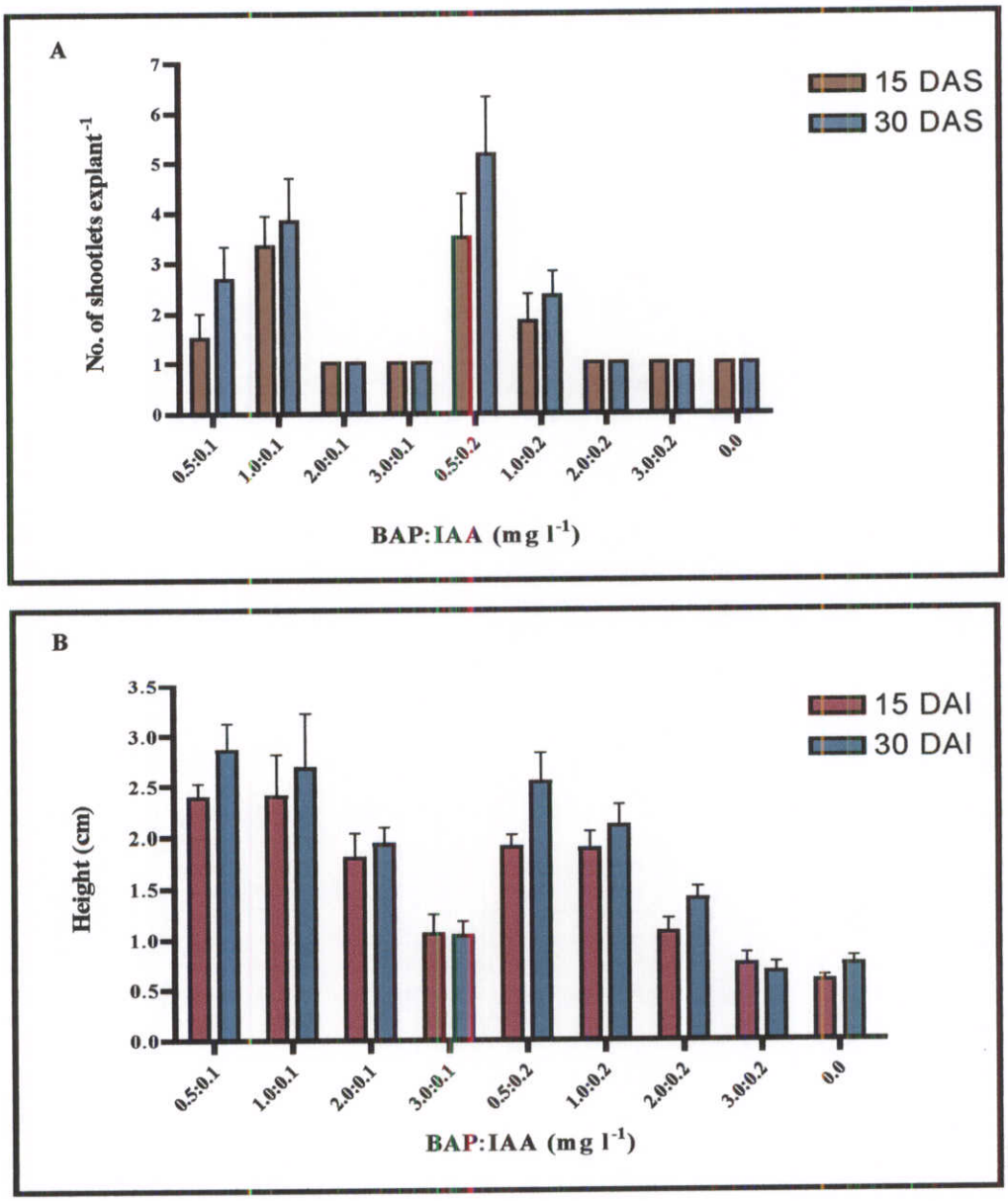

Figure 2. Effects of BAP: IAA combinations on direct shoot multiplication. (A) Shoot multiplication and (B) shoot height, using second-generation shoot tip explants at 15 and 30 days after subculture (DAS). Vertical bars represent standard error of means ($n = 10$).

3.5 Rooting of *in vitro* multiplied shoots

Shoots inoculated into MS and ½ MS supplemented with NAA or IAA did not form any roots. However those inoculated into MS and ½ MS supplemented with IBA at 1.0-3.0 mg l^{-1} formed roots. Irrespective of MS strength and auxin type, callus was formed at the bases of rooted and non-rooted shoots except for controls. In all rooted shoots, callus formation preceded production of roots. In MS supplemented with IBA at 2.0 mg l^{-1} up to 40 % of the inoculated shoots formed roots, while IBA at 1.0 and 3.0 mg l^{-1} initiated rooting in only 20 % of the cultures. In ½ MS up to 30 % of the shoots formed roots with IBA at 3.0 mg l^{-1} and 20 % at 1.0 and 2.0 mg l^{-1}. In all MS formulations, IBA at 0.5 mg l^{-1} did not induce formation of roots.

The results in all the MS formulations were however not significantly ($p<0.05$) different. However, more roots of better quality were obtained in full strength MS impregnated with IBA at 2.0 mg l^{-1} (Plate 7A). Shoots from the field-collected explants responded poorly to rooting (10 %) and most of the shoots did not form roots. Nevertheless up to 20 % of the rooted shoots from glasshouse-sourced explants survived, when they were transplanted into moist sterile vermiculite packed in plastic cups covered with polythene bag (Plate 7B). They were also successfully hardened (Plate 7C).

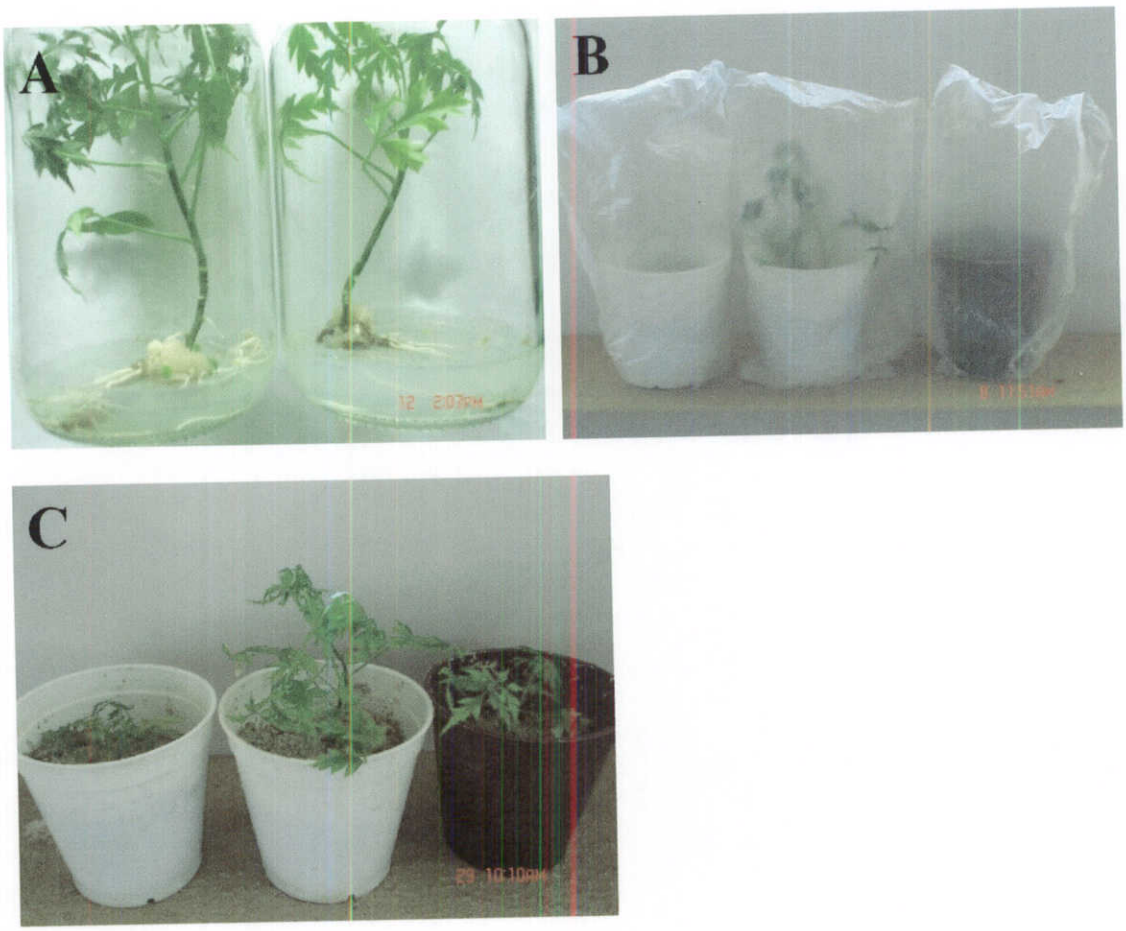

Plate 7. Rooting and hardening of *in vitro* multiplied shoots. (A) *In vitro* rooted shoots on MS supplemented with 2.0 mg l^{-1} IBA after 40-day culture; (B) Hardening and (C) weaning of *in vitro* regenerated *M. volkensii* plantlets.

3.6 Response of zygotic embryo explants on induction of somatic embryos

Germination of zygotic embryo explants was observed after 3 weeks of culture in MS media without PGRs (control) as well as those supplemented with BAP, NAA and 2,4-D alone at 0.5 mg l^{-1}. Similar results were also observed in BAP (0.5 mg l^{-1}) either combined with 2,4-D (0.2 mg l^{-1}) or NAA (0.2 and 0.5 mg l^{-1}). After 4 weeks in culture, embryo explants in all the concentrations of NAA and 2,4-D tested alone had formed loose friable callus. However, callus formation was higher in 2,4-D than NAA treatments. The embryo explants cultured in the medium consisting of BAP alone were furrowed and slightly callused. On the other hand, the zygotic explants in 2.0 and 4.0 mg l^{-1} BAP combined with either 2,4-D or NAA (0.2 and 0.5 mg l^{-1}) had more than 60 % of the explants callused with no visible signs of direct somatic embryos (Plate 8A) after 4 weeks in culture.

Callus score on the zygotic embryo explants was not significantly ($p<0.05$) influenced by the combination of BAP with NAA or 2,4-D. The embryo explants cultured in the control treatments germinated into small plantlets after 4 weeks in experiments using explants from seeds stored for <3 months. The zygotic explants from seeds stored for >12 months responded poorly to induction medium containing PGRs after 4 weeks in culture. They were slightly callused and brown in colour.

3.7 Response of cotyledonary explants on induction of somatic embryos

The cotyledonary explants cultured in treatments consisting of 2,4-D and NAA alone callused after 3 weeks, and had no visible signs of somatic embryos by the 4th week. However, callus induced in NAA was less friable than that in 2,4-D. In addition, more than 30 % of NAA cultures formed roots primarily at the proximal end of the explants. In most BAP concentrations tested alone, the explants were swollen and slightly callused on the cut surfaces after 3 weeks in culture. On the other hand, more than 50 % of the cotyledonary explants were swollen in all combinations of BAP and 2,4-D treatments. Direct somatic embryos were visible on the surface of swollen cotyledonary explants in all the BAP concentrations when combined with 0.2 mg l^{-1} 2,4-D (Plate 8B).

After 4 weeks, 30-60 % of the cotyledonary explants in BAP (0.5, 1.0, 2.0 and 4.0 mg l^{-1}) combined with 2,4-D (0.2 mg l^{-1}) had formed direct somatic embryos ranging from 3-14 per explant (Table 4). Cotyledonary explants inoculated on control treatments only formed meristematic nodules on the cut surface after 4 weeks in culture (Plate 8C). The production of somatic embryos on cotyledonary explants was significantly (p<0.0001) influenced by the concentration and combination of BAP and 2,4-D. For instance, increasing the concentrations of BAP and 2,4-D reduced the number of somatic embryos initiated per explant (Table 4). Cotyledonary explants cultured in BAP and NAA combinations at 0.5 mg l^{-1} formed callus on the on the cut surface. The callus tissue consisted of whitish nodular-like protrusions, but without somatic embryos (Plate 8D). Further subculturing using the same media formulations did not induce formation of somatic embryos or shootlets.

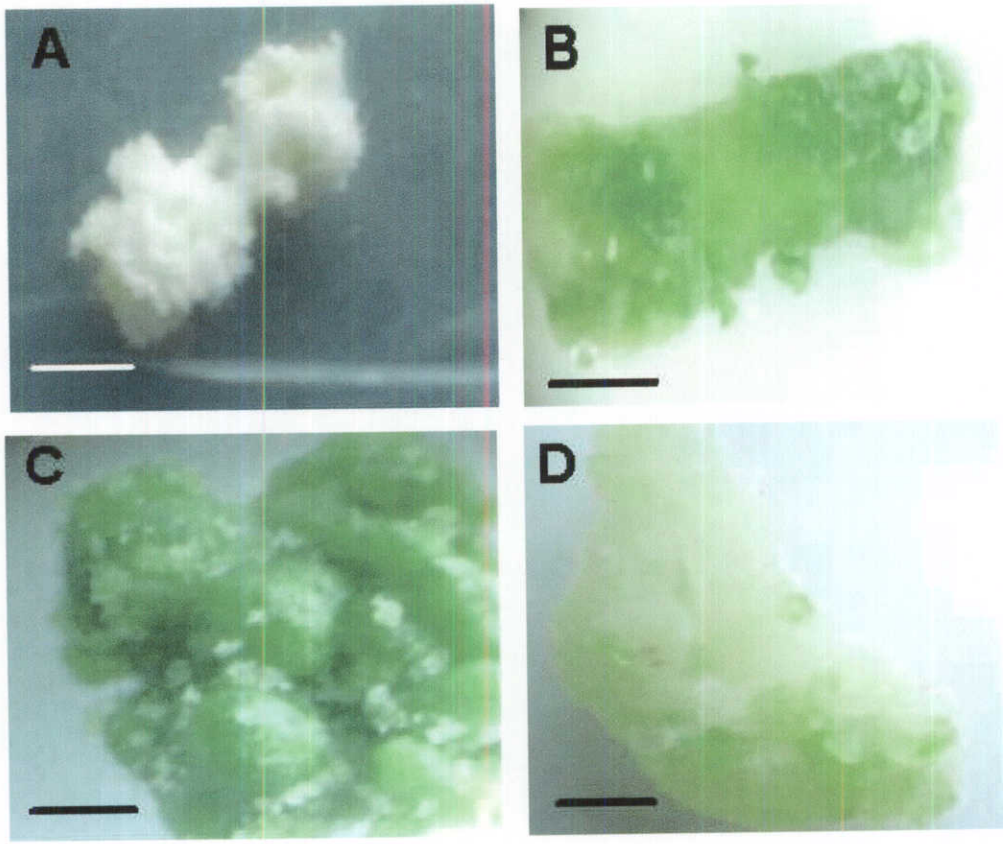

Plate 8. Response of cotyledon and zygotic embryo explnts to different embryo induction medium. (A) Callused zygotic embryo explants on MS supplemented with 2 mg l^{-1} BAP and 0.5 mg l^{-1} NAA after 4 weeks of incubation (bar =3mm); (B) Direct somatic embryos visible on swollen cotyledonary explants on MS supplemented with BAP (0.5 mg l^{-1}) and 2, 4-D (0.2 mg l^{-1}) after 3 weeks of incubation (bar =3.5 mm); (C) Meristematic nodules initiated on cotyledon explants on MS devoid of plant growth regulators (bar =1.5 mm); (D) Nodular-like protrusions on cotyledon inoculated on MS supplemented with BAP at 4 mg l^{-1} combined with NAA at 0.2 mg l^{-1} (bar =3.5 mm).

Table 4. The effect of BAP in combination with 2,4-D on direct induction of somatic embryos after 4 weeks culture of mature cotyledon explants from <3 months old seeds.

BAP: 2,4-D (mg/L)	**% Somatic embryo genesis**	**Range in No. of embryo explant^{-1}**	**Mean number of embryos explant^{-1}***
0:0	0	0	**0a****
0.5:0.2	60	5-14	6.2**c**
1.0:0.2	50	4-12	5.0**bc**
2.0:0.2	30	5-8	2.2**ab**
4.0:0.2	30	3-7	1.9**ab**
0.5:0.5	0	0	0**a**
1.0:0.5	0	0	0**a**
2.0:0.5	0	0	0**a**
4.0:0.5	0	0	0**a**
CV %			**18.5**

*Data were transformed [$X' = \sqrt{(X + 0.5)}$] prior to ANOVA ($n = 20$). **Values in the column followed by the same letter are not significantly different according to the Newman-Keuls test ($p<0.05$).

Clusters of somatic embryos were more frequently observed at the proximal than distal ends of the cotyledonary explants. Different stages of somatic embryos namely torpedo (Plate 9A) and cotyledonary (Plate 9B) were observed in the clusters formed. Somatic embryos were also formed on slightly callused regions of the cotyledonary explants, especially on the cut surfaces (Plate 9C). Most of the cotyledonary explants from seeds stored for >12 months did not respond to BAP and 2,4-D combinations, but turned yellow instead. Direct somatic embryogenesis was observed in only 20 % of the cotyledonary explants on the medium consisting of 1.0 mg l^{-1} BAP and 0.2 mg l^{-1} 2, 4-D.

The clusters of somatic embryos transferred onto semi solid MS and ½ MS did not germinate into plantlets. Instead they formed clusters of shootlets, which were further used as sources of explants for multiplication of *M. volkensii* (Plate 9D). Nevertheless during shoot multiplication, 70 % of the shoot tip cultures formed 4-7 shoots per explant on MS media augmented with BAP (0.5 mg l^{-1}) and IAA (0.2 mg l^{-1}) (Plate 9E). Root formation ranged from 5-33 % in all the IBA concentrations tested, with 2 mg l^{-1} producing the highest score. Although there was no general trend observed in most shoots, callusing preceded root formation (Plate 9F).

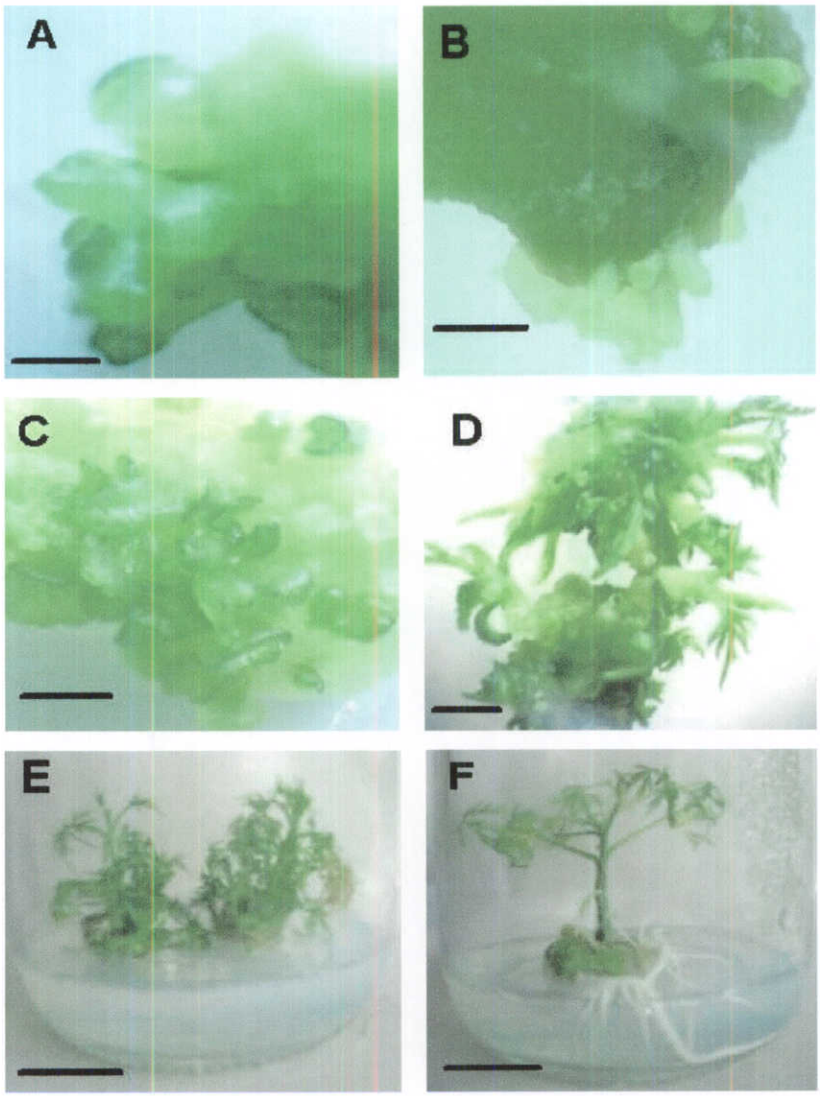

Plate 9. Regeneration of *Melia volkensii* through direct somatic embryogenesis. (A) Torpedo and (B) Cotyledonary stages of somatic embryos initiated on cotyledonary explants after 4 weeks of culture; (C) Somatic embryos initiated on slightly callus explant (bar = 1 mm); (D) Shootlets produced when embryos clusters were subcultured on Plant growth regulator free MS after 4 weeks of culture (bar =10 mm), (E) shoot multiplication using shoot tip explants after 6 weeks of incubation (bar =12 mm), (F) Rooted shoot on MS supplemented with 2 mg l^{-1} IBA after 4 weeks of incubation (bar =12 mm).

CHAPTER FOUR: DISCUSSION, CONCLUSION AND RECOMMENDATIONS

4.1 Macro-propagation

Rejuvenation of mature *M. volkensii* trees by coppicing and root pruning was less problematic. This is because the species coppices well and easily forms root suckers when the roots are injured (Stewart and Blomley, 1994; Tedd, 1997). In this study up to 33 % rooting of rejuvenated leafy stem cuttings was achieved. This is much higher than <10 % obtained in previous studies with mature stem cutting (Milimo, 1989a; Mulatya, 2000). Rejuvenation and use of leafy stem cuttings has been reported to greatly improve rooting of cuttings from difficult to root tropical tree species such as *Triplochiton scleroxylon* (Longman and Wilson, 1993). These results therefore confirmed the importance of rejuvenation in improving rooting of stem cuttings from mature sources and difficult to root tree species.

The enhanced survival of cuttings due to fungicide pretreatment and surface sterilization, underscored their significance in rooting of *M. volkensii* leafy cuttings: especially under the warm and moist conditions of mist propagators that are ideal for fungal infestation. Nearly similar procedures have successfully been applied to control fungi in micro- and macro-propagation of *Acer pseudoplatanus*, *Castanea sativa* (Jinks, 1995) and *Ficus thonningii* (Danthu *et al.*, 2002) tree species. The results obtained with *M. volkensii*, confirms that pretreatment and surface sterilization cannot substitute the procedures generally applied in tree nurseries when cuttings are used as propagules: such as selection of healthy looking and disease free cutting sources.

The stem cutting materials (suckers and coppices) were collected during rainy season, at the time when they were experiencing active growth. This may also have influenced the improved rooting success achieved in this study. The results of this study may be comparable to those of *Robinia pseudoacacia* and *Grewia optiva* leafy cutting (Swamy *et al.*, 2002), in which improved rooting was achieved when cutting materials were collected during the active growth season. Nonetheless, relatively higher concentrations of PGRs in particular IBA (4-10 g l^{-1}) induced development of adventitious roots on the leafy stem cuttings. A similar range of concentrations has also been reported to initiate roots in *Khaya ivorensis*, a member of Meliaceae family (Longman and Wilson, 1993). In contrast, low levels (mg l^{-1}) of IBA have also been successfully used to root juvenile or seedling stem cuttings of *M. volkensii* (Milimo, 1989a) and other tropical tree species such as *Prunus africana* (Tchoundjeu *et al.*, 2002) and *Olea europium* (Negash, 2003). In comparison to IBA concentrations used in this study, seedling stem cuttings particularly those of *M. volkensii* seem to be more responsive to relatively lower concentrations (mg l^{-1}) of PGRs (IBA). The differences in rooting potentials between the juvenile and mature stem cuttings may be attributed to physiological state of the cuttings in reference to the endogenous levels of the PGRs (Puri and Khar, 1992).

Although auxins have been proved to promote rooting of cuttings, results obtained in this study clearly demonstrated that IBA was preferred over IAA and NAA and the optimum concentration was 8 g l^{-1}. Preference by cuttings for IBA over other auxin types has also been reported in *Azadirachta indica* (Meliaceae) and *Pongomia pinnata* (Palanisamy *et al.*, 1998). Generally, IBA has been widely used for rooting a wide range of tropical and

non-tropical trees (Copes and Mandel, 2000; Negash 2003). Hence results obtained in this study confirmed the benefits and importance of IBA in induction of roots in *M. volkensii*. On the other hand the variation in number and length of roots per cutting within treatments may have been due to the amount of IBA applied per cutting, since in the quick dip method used no fixed volume of PGRs solution was applied per cutting. The effect of IBA concentration on root number and length has also been reported on *Khaya ivorensis* (Longman and Wilson, 1993). However genotypic differences, leaf area, cutting orientation with respect to position on sucker or coppice stem and root initiation time may also have contributed the variations noted, since rooting can only be maximized when the optimal combinations of factors have been achieved (Tchoundjeu *et al.*, 2002).

The water logged rooting media was responsible for loss of cuttings at 45 DAI due to rotten bases. A number of comparative studies between different rooting media have indicated that species preference for rooting medium was influenced by the water holding capacity (Hartman *et al.*, 1990; Tchoundjeu *et al.*, 2002). Hence death of *M. volkensii* cuttings can be attributed to noxia due to inhibited oxygen diffusion as a result waterlogged sand rooting substrate; water was sprayed thrice per day to maintain high humidity within the propagator. Similar effect of water logging has been reported on *Cordia alliodora* cuttings inserted in sawdust as rooting substrate (Mesén *et al.*, 1997). Presence of callus without development of roots in the lower auxin concentration treatments, control and distilled water treatments suggested that callus was not a precursor for root initiation. Similar observations have also been reported on leafy cuttings of *Castanea sativa* (Jinks, 1995). The swelling and furrowing of cutting bases

observed in rooted and non-rooted cuttings treated with higher auxin concentrations have also been reported by Hartman *et al.* (1997).

4.2 *In vitro* shoot multiplication

The inability of cytokinins alone to induce shoot multiplication in *M. volkensii* contrasted results obtained on *Azadirachta indica* using Driver and Kuniyuki medium (Quraishi *et al.*, 2004) and *Ceratonia siliqua* on MS (Romano *et al.*, 2002). Nonetheless the results obtained in this study clearly demonstrated that BAP combined with IAA induced direct shoot multiplication of both rejuvenated and glasshouse explants. However there are other reports indicating that some members of Meliaceae family namely, *A. indica* and *Melia azedarach* were successfully multiplied using two cytokinins, in particular BAP and kinetin combined (Eeswara *et al.*, 1988; Vila *et al.*, 2004). Two optimal combinations of BAP: IAA (0.5: 0.2 and 1.0: 0.1 mg l^{-1}), were identified as capable of inducing shoot multiplication. These results are nearly similar to those obtained with juvenile leaf explants of *A. indica,* where BAP: IAA (2.0: 0.1 mg l^{-1}) induced shoot multiplication (Salvi *et al.*, 2001).

Shoot multiplication by inducing production of axillary shoots and subsequent subculture of axillary shoot tips, was successfully applied in this study. A similar approach has successfully been used in *Aristolochia indica* (Manjula *et al.*, 1997). Though, the synergistic effect of cytokinins and auxins in inducing shoot multiplication has been demonstrated by results achieved in this study on BAP and IAA. Nonetheless, failure to induce shoot multiplication in other cytokinin and auxin combinations tested, clearly

suggested the synergistic effect was also influenced by preference for PGRs type. In addition, failure of BAP and kinetin alone and in combination with NAA to induce shoot multiplication on *M. volkensii* contrasts the results obtained on *Acacia sinuata* (Vangadesan *et al.*, 2002) and *Balanites aegyptiaca* (Ndoye *et al.*, 2003), respectively. However shoot quality results obtained on *M. volkensii* were consistent with those obtained on *Trifolium resupinum* (Uranbey *et al.*, 2005).

Increasing BAP concentration (>1.0 mg l^{-1}) combined with IAA was inhibitory to shoot multiplication. The inhibitory effects of relatively higher BAP concentrations have also been observed on *A. indica* (Quirashi *et al.*, 2004). Unlike *M. volkensii*, Tiwari *et al.* (2002) reported that relatively high levels of BAP were inhibitory to growth of axillary buds on Teak (*Tectonis grandis*) explants. However addition of relatively low levels of auxins and cytokinins (0.1-0.9 mg l^{-1}) has also been shown to induce shoot multiplication in other tree species namely *Gmelina arborea* (Tiwari *et al.*, 1997) and *Sapium sebiferum* (Siril and Dhar, 1997). Nonetheless shoot multiplication due to interaction of cytokinins and auxins would vary from species to species with respect to type of PGRs and concentrations used as demonstrated in this study and in comparison with other studies.

The *in vitro* rooting results clearly showed that IBA was preferred over IAA and NAA. Induction of roots using IBA (1.0-3.0 mg l^{-1}) is consistent with results reported on *A. indica* (Salvi *et al.*, 2001; Quraishi *et al.*, 2004) and *M. azedarach* (Vila *et al.*, 2004) all members of Meliaceae family. Other tree species in which IBA also successfully induced *in vitro* rooting includes *C. siliqua* (Romano *et al.*, 2002) and *A. sinuate* (Vengadesan *et*

al., 2002). However, IAA and NAA have also been successfully used to induce roots in *Murraya koenigii* (curry leaf tree) (Babu *et al.*, 2000) and *Myrica esculenta* (Bhatt and Dharr, 2004). Successful initiation of roots *in vitro* using IBA has mainly been associated to its catabolism resistance. Unlike IBA, IAA rapidly undergoes photo-degradation forming IAA oxidase, which inhibits formation of roots in *in vitro* cultures (Swamy *et al.*, 2004). Rooting results obtained with IBA on *M. volkensii* contrasts those of *B. aeagyptiaca,* where relatively higher concentrations of IBA (10-20 mg l^{-1}) successfully induce roots (Ndoye *et al.*, 2003).

4.3 Somatic embryogenesis

Failure to induce direct somatic embryogenesis using mature zygotic embryo explants of *M. volkensii* contrasts the results reported on immature zygotic embryos of *Melia azedarach* (Meliaceae) using 0.1-3.0 mg l^{-1} thidiazuron (Vila *et al.*, 2003). In addition failure of BAP alone to induce somatic embryogenesis, but only in combination with 2,4-D, contrasts results from other workers who have managed to induce direct somatic embryogenesis on *Azadirachta indica* (Meliaceae) mature cotyledonary explants using 0.3-10.0 mg l^{-1} thidiazuron (Murthy and Saxena, 1998). Success in induction of direct somatic embryos on *M. azedarach* using thidizuron alone as opposed to BAP alone in this study may be attributed to differences in potency between the two cytokinins.

The reduced induction of somatic embryos due to increased concentrations of BAP and 2,4-D is an indication that relatively high levels of PGRs was detrimental to direct somatic embryogenesis. A similar pattern was observed by Wachira and Ogada (1995) on *Camellia sinensis* (Theaceae) in MS supplemented with kinetin, BAP and IBA. Nonetheless the frequency of somatic embryo clusters formed on the proximal end of explants, suggested that somatic embryogenesis was influenced by orientation. Vila *et al.* (2003) also reported that somatic embryos were frequently initiated on hypocotyls and cotyledonary axils of immature zygotic embryo explants of *M. azedarach*. Moreover, induction of somatic embryogenesis with respect to orientation has also been reported on *Panax ginseng* leaf explants and was attributed to the differences in cell structure on the abaxial and adaxial sides (Choi *et al.*, 1997). The induction of direct somatic embryos and failure to develop into plantlets on MS devoid of PGRs contradicts the results obtained on *M. azedarach* (Vila *et al.*, 2003). Interestingly, the results in this study were comparable to those reported by Puchooa (2004) for *Litchi chinensis*, a member of Sapindaceae family.

Further subculture of callus consisting of whitish nodular-like protrusions formed in BAP and NAA (0.5 mg l^{-1}) combination did not induce somatic embryos or shootlets. Callus with similar protrusions to those found in this study has previously been reported on *Cola nitida* (Sterculiaceae) on MS supplemented with 0.8 mg l^{-1} kinetin combined with 2,4-D (0.2 and 0.6 mg l^{-1}). Conversely, further subculture of *C. nitida* resulted in formation of somatic embryos (Obembe *et al.* 1999). In this study, meristematic nodules were initiated on MS devoid of PGRs but did not form shoots or somatic embryos. Nevertheless,

initiation of meristematic nodules on MS supplemented with BAP and subsequent formation of shoots has been reported in *Charybdis* species (Wawrosch *et al.*, 2005). Meristematic nodules and cellular aggregates have been reported to have high regeneration rates in Poplar species and their use could easily facilitate automation of *in vitro* multiplication protocols (McCown *et al.*, 1988). Micro-propagation of *M. volkensii* by meristematic nodule is thus possible and can be an interesting alternative to direct and indirect somatic embryogenesis.

Low somatic embryogenesis (up to 20 %) obtained on cotyledonary explants from seeds stored for >12 months may be attributed to longer storage period. Furthermore, the results of the cotyledonary explants from seeds stored for >12 month also suggest that longer storage required relatively higher levels of BAP (1.0 mg l^{-1}) as compared to <3 months old cotyledonary explants (0.5 mg l^{-1}) to induce direct somatic embryogenesis. However, more studies are required to evaluate the effect of storage duration and conditions of *M. volkensii* seeds on induction of direct somatic embryos. The induction of callus on both cotyledonary and zygotic embryos irrespective of auxin type indicated that *M. volkensii* may not be auxin specific in terms of callus induction. These results contrast those obtained with mature cotyledonary explants *Parkia biglobosa* (Leguminoseae) where only 2,4-D, but not NAA induced callus formation (Amoo and Ayasire, 2005).

In general, the initiation of direct somatic embryos on cotyledonary explants was found to be optimum with BAP (0.5 and 1.0 mg l^{-1}) in combination with 2,4-D (0.2 mg l^{-1}). These results confirm that interaction of PGRs (mainly cytokinin and auxins) may be essential for induction of somatic embryos in woody species as outlined by Komamine *et al.* (1992). The results also indicated that 2,4-D was essential for the induction of somatic embroys as also outlined by Nanda and Rout (2003). Shoot multiplication and rooting from explants obtained through direct somatic embryogenesis clearly demonstrate the potential of using this technique to mass propagate *M. volkensii*. Murayama *et al.* (2003) regenerated Sawara cypress (*Chamaecyparis pisifera*) by a rather similar approach.

The successful induction of direct somatic embryos using mature cotyledon explants also confirmed the usefulness of mature tissues in somatic embryogenesis studies. However, there is also need to test immature cotyledonary and zygotic embryo explants of *M. volkensii* since it has been shown to be successful with *M. azedarach*, a closely related tree species (Vila *et al.*, 2003); even though there are also reports on failure of immature cotyledon explants of *Azadirachta indica* (Meliaceae) to establish in culture (Wei *et al.*, 1997). Similarly, trials with thidiazuron may also avoid the use of combinations of PGRs on somatic embryogenesis with *M. volkensii* explants, as has been demonstrated with other related species (Murthy and Saxena, 1998)

4.4 Conclusions

Rejuvenation, treatment with fungicide and PGRs (IBA) improved rooting success of *M. volkensii* stem cuttings. Rooting percentage (33 %) achieved in this study was higher compared to previous studies using mature stem cuttings (<10 %). Furthermore the results on macro-propagation clearly demonstrated that 2-3 node leafy stem cuttings are suitable for propagation of *M. volkensii* and IBA at 8 g l^{-1} was optimum for induction of adventitious roots.

Micro-propagation results also demonstrated that both glasshouse and field sourced shoot tip explants were amenable to direct *in vitro* shoot multiplication. However the response of seedling explants to multiplication medium was significant compared to field-sourced explants. The major problem faced with field-sourced explants was decontamination and relatively low rooting success (<10 %). Nonetheless BAP: IAA (0.5: 0.2 mg l^{-1}) combination was optimum for induction of direct *in vitro* shoot multiplication. On the other hand results obtained indicate that regeneration of *M. volkensii* through direct somatic embryogenesis using mature cotyledonary explants is possible. Furthermore the results also showed that initiation of direct somatic embryos using cotyledonary explants was optimum in BAP (0.5 and 1.0 mg l^{-1}) combined with 2,4-D (0.2 mg l^{-1}).

In general the results obtained in this study have verified that *M. volkensii* is amenable to propagation by rejuvenated leafy stem cuttings, direct *in vitro* shoot multiplication and somatic embryogenesis.

4.5 Recommendations

Based on the results obtained in this study, further work on macro-propagation should focus on the effects of misting regimes, rooting medium, auxin application methods, leaf area and genotype on rooting of *M. volkensii* rejuvenated leafy stem cuttings. There is also the need to develop reliable sterilization protocols for field-collected explants for *in vitro* shoot multiplication purpose. Further studies are therefore required to identify the appropriate antibiotic and optimum decontamination regime for *M. volkensii*. Due to poor response of shoots from field-collected explants to *in vitro* rooting, other options such as *ex vitro* rooting should also be explored as an alternative. Finally further studies on direct somatic embryogenesis should investigate the effect of other more potent cytokinins such as thidiazuron and, use of immature zygotic embryos and cotyledonary explants. This may avoid use of combinations of PGRs during initiation of direct somatic embryos, as has been demonstrated with other members of Meliaceae family (Murthy and Saxena, 1998; Vila *et al.*, 2003). The possibility of maturing individual somatic embryos into plantlets should also be explored as this has also been achieved in other members of Meliaceae family.

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APPENDICES

Appendix 1. Preparation of PGRs used for rooting of leafy stem cuttings (Hartman *et al.*, 1997).

Final concentration	IBA concentration	
(ppm)	mg l^{-1}	g l^{-1}
0	0	0
500	500	0.5
1000	1000	1
5000	5000	5
10000	10000	10

N.B.

- To make 10,000 ppm or (10, 000mg /l) or 1 % stock solution of IBA, dissolve 10 g of IBA in 15 to 20 ml of alcohol (ethyl, isopropyl or methyl) and then top to 1000 ml (1 litre) with 50 % alcohol.
- To make 1 litre (1000 ml) of 1000 ppm IBA solution from the 1 % stock solution, take 100 ml of the stock solution and add to 900ml of the 50 % alcohol. Similar calculations are done for the rest of the IBA concentrations required.
- For IBA with Potassium salt (K-IBA) follow similar methods the only difference is to use sterile or de-ionized water as solvent to avoid precipitation. i.e. K make s the IBA water soluble.

NB. IBA dissolved in water can be used to pre-treat the cuttings before detaching them from the tree stock to improve rooting of the cuttings.

Appendix 2. Preparation of Broughton and Dillworth balanced plant nutrient solution (Somasegaran and Hoben, 1985).

Stock solutions	Element	M	Form	MW	g/l	M
1	Ca	1000	$CaCl_2.2H_2O$	147.03	294.1	2
2	P	500	KH_2PO_4	136.09	136.1	1.0
3	Fe	10	Fe-citrate	355.04	6.7	0.02
	Mg	250	$MgSO_4.7H_2O$	246.5	123.3	0.5
	K	250	K_2SO_4	174.06	87.0	0.5
	Mn	1	$MnSO_4.H_2O$	169.02	0.338	0.002
4	B	2	H_3BO_3	61.84	0.247	0.004
	Zn	0.5	$ZnSO_4.7H_2O$	287.56	0.288	0.001
	Cu	0.2	$CuSO_4.5H_2O$	249.69	0.100	0.0004
	Co	0.1	$CoSO_4.7H_2O$	281.12	0.056	0.0002
	Mo	0.1	$Na_2MoO_2.2H_2O$	241.98	0.048	0.0002

For each 10 litres of full strength plant nutrient solution, take 5.0 ml each of stock solution 1 to 4, then add to 5.0 litres of water and dilute to 10 litres. Use 1 N NaOH to adjust the pH to 6.6-6.8. For plus N nutrient solution add 0.05 % of KNO3 to give an N concentration of 70 ppm

Appendix 3. Effect of IAA and NAA concentrations on rooting of 3-month old rejuvenated leafy stem cuttings at 60 days after insertion in sand rooting substrate

Plant growth regulator type	**Parameter**	**Plant growth regulator concentration (g l^{-1})**					
		0	**2**	**4**	**6**	**8**	**10**
IAA	No. of roots $cutting^{-1}$	0	0	1.7	0	0	0
	Root length (cm) $cutting^{-1}$	0	0	1.5	0	0	0
	% Rooting	0	0	5.0	0	0	0
NAA	No. of roots $cutting^{-1}$	0	0	0	0.3	0	0
	Root length (cm) $cutting^{-1}$	0	0	0	4.1	0	0
	% Rooting	0	0	0	5.0	0	0

Values in the table are means of data in their original scale

Appendix 4. Effect of cytokinins combined with auxins on direct *in vitro* shoot multiplication

(a) Effect of BAP: NAA on height, shoot quality, callus score and number of shoots per explant.

BAP:NAA	No. of shoots explant^{-1}	Height (cm)	Shoot quality score	Callus score
0:0	1.0	2.08	1.2	0
0.5: 0.1	1.44	3.21	1.55	3.0
1.0:0.1	1.28	2.21	1.55	1.87
2.0:0.1	1.0	3.03	1.0	2.0
3.0;0.1	1.49	2.41	2.0	2.99
0.5: 0.2	1.14	2.45	1.28	1.85
1.0:0.2	1.0	2.28	1.0	2.28
2.0:0.2	1.0	2.67	1.44	2.22
3.0:0.2	1.0	1.45	1.0	2.7
LSD(0.05)	**0.26**	**0.36**	**0.36**	**0.31**
CV %	**25.6**	**16.6**	**29.7**	**18.6**

Values in the table are means of data in their original scale

(b) Effect of kinetin: NAA on height, shoot quality, callus score and number of shoots per explant.

Kinetin:NAA	No. of shoots explant^{-1}	Height (cm)	Shoot quality score	Callus score
0:0	1.0	2.6	2.7	0
0.5: 0.1	1.0	2.2	2.2	0.6
1.0:0.1	1.0	2.61	1.9	0.75
2.0:0.1	1.0	3.36	2.8	1.67
3.0;0.1	1.143	3.2	2.0	2.42
0.5: 0.2	1.0	3.8	2.1	2.28
1.0:0.2	1.0	3.3	2.5	2.7
2.0:0.2	1.0	3.4	2.0	3.0
3.0:0.2	1.0	3.4	2.4	3.0
LSD(0.05)	**0.18**	**0.65**	**0.67**	**0.53**
CV %	**17.3**	**22.4**	**28.6**	**52**

Values in the table are means of data in their original scale

(c) Effect of zeatin: NAA on height, shoot quality, callus score and number of shoots per explant.

Zeatin:NAA	No. of shoots explant^{-1}	Height (cm)	Shoot quality score	Callus score
0:0	1.0	1.6	1.2	0
0.5: 0.1	1.0	1.2	1.0	1.6
1.0:0.1	1.0	1.51	0.9	1.75
2.0:0.1	1.0	1.36	0.8	1.67
3.0;0.1	0.9	1.2	1.0	3.42
0.5: 0.2	1.0	2.1	0.8	3.28
1.0:0.2	1.0	2.0	1.0	1.7
2.0:0.2	1.1	1.4	0.9	3.0
3.0;0.2	1.0	2.4	0.4	3.5
LSD(0.05)	**0.21**	**0.85**	**0.67**	**0.58**
CV %	**19.3**	**22.4**	**25.6**	**48.0**

Values in the table are means of data in their original scale

(d) Effect of zeatin: IAA on height, shoots quality, callus score and number of shoots per explant.

Zeatin:IAA	No. of shoots explant^{-1}	Height (cm)	Shoot quality score	Callus score
0:0	1.0	3.48	1.87	0.5
0.5: 0.1	1.0	3.25	1.87	1.12
1.0:0.1	1.0	3.50	2.42	2.14
2.0:0.1	1.0	3.45	2.0	2.77
3.0;0.1	1.0	3.62	2.25	2.25
0.5: 0.2	1.3	3.28	2.01.44	2.28
1.0:0.2	1.0	3.12	1.80	1.66
2.0:0.2	1.0	2.46	1.14	1.75
3.0;0.2	1.4	2.77	1.7	2.57
LSD(0.05)	**0.21**	**0.75**	**0.44**	**0.47**
CV %	**22.8**	**23.7**	**28.3**	**26.8**

Values in the table are means of data in their original scale

(e) Effect of kinetin: IAA on height, shoot quality, callus score and number of shoots per explant.

Kinetin:IAA	No. of shoots explant^{-1}	Height (cm)	Shoot quality score	Callus score
0:0	1.16	4.97	2.83	1.40
0.5: 0.1	1.2	4.28	2.8	1.60
1.0:0.1	1.0	3.58	2.20	1.25
2.0:0.1	1.0	2.55	2.02.0	1.66
3.0;0.1	1.0	2.87	1.4	1.8
0.5: 0.2	1.0	2.78	2.0	2.33
1.0:0.2	1.0	3.38	2.6	2.8
2.0:0.2	1.16	3.84	1.6	2.6
3.0;0.2	2.2	2.62	2.2	1.4
LSD(0.05)	**0.51**	**1.14**	**0.71**	**0.58**
CV %	**36.8**	**30.4**	**29.6**	**32.4**

Values in the table are means of data in their original scale