EFFICACY OF A LOW-COST AGROCHEMICAL THIDIAZURON IN INDUCTION OF REGENERATION FROM COTYLEDONS OF *MELIA VOLKENSII* GURKE

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Abstract
Mass propagation via tissue culture technology could rescue threatened indigenous medicinal, pesticidal and timber plants. However, its application remains underutilized locally due to high costs of tissue culture consumables. Local efforts towards reduction of costs have centered on use of cheaper carbon sources such as table sugar, substitution of Murashige and Skoog and Gamborg's salts with mixtures of fertilizers and substitution of conventional gelling agents agar, gelrite and phytogel with cassava starch. However, a gap exists on the identification of cheaper substitutes of conventional plant growth promoters yet these, particularly cytokinins, are important cost drivers in tissue culture. Half a gram of plant-cell-culture-tested Thidiazuron (TDZ) from Sigma-Aldrich costs 2,145 Euros (Ksh 235,500) while 1 g of Zeatin costs 4,220 Euros (Ksh 463,514). This study sought to determine whether a low-cost agrochemical TDZ that costed only 0.229 USD (Ksh 19.84) per gram and was recently reported by the present authors to support high frequency regeneration in mature zygotic embryos of *M. volkensii,* could elicit similar response in other explants. Cotyledons of mature seeds from wild trees in Kitui were cultured in ½ MS medium containing eight TDZ concentrations from 0 to 4 mg/l. High frequency (80 to 100 %) direct regeneration of shoots was observed within 14 days. TDZ treatments had a significant effect ($F_{test} p < 0.001$) on the regeneration response in relation to the non-morphogenic controls. Shoots were elongated on ½ MS + 0.1 mg/l 6-Benzylaminopurine in combination with either 0.01 mg/l 1AA or 10% coconut water and rooted in ½ MS + 0.1 mg/l Indole-3-butyric acid. Low-cost agrochemical TDZ could substitute the exorbitant plant-cell-culture-tested Thidiazuron in tissue culture of *M. volkensii*. We recommend testing of this agrochemical on more explants and species.

Key words: Regeneration, *Melia volkensii*, cotyledons, low-cost, thidiazuron

1.0 Introduction

Plant tissue culture forms the basis for *in vitro* genetic improvement of plants, elimination of viruses from planting stock, micro-grafting, *in vitro* gene banking, *in vitro* screening and selection for tolerance to abiotic stress. It can also allow high rates of clonal propagation of elite genotypes and the rescuing of threatened medicinal, pesticidal and timber plants. However, the high cost of conventional media components limits the wider adoption of plant tissue culture in these applications, especially in the developing countries (Demo *et al.,* 2008).

That high input costs are an impediment to commercialization of tissue culture technology can be illustrated by the increasing search for low-cost tissue culture inputs (Gitonga *et al* 2010; Kuria *et al.,*2008; Mbanaso 2008; Mulanda *et al.,* 2012; Ogero *et al.,* 2012a,b,c;). Successful use of agrochemicals may lead to establishment of innovative low-cost protocols for use in community-based tissue culture centres and could have significant impact on the reduction of the cost of tissue cultured plants.

The main growth regulators used in plant tissue culture are auxins and cytokinins. Cytokinins are the most expensive, with Sigma-Aldrich® plant cell culture tested Thidiazuron costing € 2,025 (Ksh. 215,662.5) for 0.5 grams while zeatin costs € 3,980 (Ksh. 423,870) per gram. In contrast, current prices of locally available agrochemical compounds containing plant growth regulators are in the range of Ksh. 750/= to5,000/= for packs of 100gor more for solids and 250ml or more for liquid formulations (Mulanda personal comm., 2013). The prices per gramof these agrochemical compounds are very minimal compared to the prices of conventional pure-grade plant-cell-culture-tested plant growth substances.

Research efforts on the search for cheaper culture media have been concentrated on the identification of low cost gelling agents and carbon sources (sugar), as these, quantitatively, form the bulk of plant tissue culture media. Various starches, such as cassava and potato starch, and plant gums have been found to be cheaper substitutes for the more expensive agar and other conventional gelling agents such as phytagel (Kuria *et al.,* 2008; Mbanaso 2008; Santana*et al.,* 2009). Table sugar and molasses have also been found to adequately substitute conventional high-grade sucrose, as carbon and energy sources in plant culture medium (Demo *et*

al., 2008; Kaur *et al.*, 2005; Kodym and Zapata-Arias, 2001; Santana *et al.*, 2009; Tyagi *et al.*, 2007). Cassava plants grown on media supplemented with molasses were stronger, greener and more vigorous than those grown on high grade sucrose (Santana *et al.,* 2009).

The use of agrochemical products as low-cost sources of plant growth regulators in plant tissue culture media has also been reported. Santana *et al*. (2009) reported the use of Radixone 3AS® (a commercial root promoter containing the auxins IAA, IBA and NAA) and Activol®, a commercial preparation containing gibberellic acid (GA), as low cost sources of auxins and GA in tissue culture of cassava. Srinivasan *et al.* (2006) also reported the use of three urea-derived herbicides viz monuron, diuron and diphenyl urea for use as cytokinins in tissue cultures of alfalfa (*Medicago sativa*) and Coleus (*Coleus forskohlii*). Mulanda *et al.* (2012) reported high frequency shoot regeneration from zygotic embryos of *Melia volkensii* using agrochemical Thidiazuron. Gitonga *et al.* (2010) reported successful use of the agrochemical Anatone® as a low-cost source of the auxin NAA for rooting of banana plantlets.

In the search for cheaper nutrients, Escobar *et al.* (2005) reported the use of various types and concentrations of fertilizers as low-cost substitutes for the conventional Murashige and Skoog or MS (1962) and Gamborg *et al's* B5 (1968)macro and micro nutrient basal medium. Santana *et al.,* (2009) also demonstrated the ability of an agricultural fertilizer (Hydro Agri 12-11-18/3 MgO-EDTA) to adequately substitute MS macro and micronutrients in propagation of cassava.More recently, Ogero *et al.* (2012a and b) reported successful use of the fertilizer Easygro® as a substitute for Murashige and Skoog (MS) salts in tissue culture of sweet potato (*Ipomoea batatas* (L) Lam) and cassava (*Manihot esculenta* L). Ogero *et al.* (2012c) further reported the use of Stanes Iodised Microfood® produced locally by Osho Chemicals Industries as an alternative source of micronutrients.

The urea derivative N-phenyl- (N' 1,2,3-thidiazol-5-yl)urea, commonly referred to as Thidiazuron or simply TDZ, was originally developed as a cotton defoliant but was later found to have high cytokinin activity (Mok *et al.*, 1982). Since then, TDZ has been used as a growth regulator in tissue cultures of many crop plants (Guo *et al.*, 2011). The present study tested the potency of a low-cost agrochemical Thidiazuron, manufactured by Kingtai Chemicals Ltd China for use as a cotton and peanut defoliant, in regeneration of *M. volkensii* from mature cotyledons. This agrochemical costed only USD 160 (Ksh) and has remained potent since 2011 when it was procured. This study is thefirst report of TDZ-induced somatic embryogenesis from cotyledons of *M. volkensii* Gurke.

2.0 Materials and Methods

2.1 Plant materials

Mature fruits of *Melia volkensii* were collected in February 2013 from trees growing on farms in Mbathani-A location in Katulani District, Kitui County, South Eastern Kenya. The collection site was geo-referenced as follows: GPS coordinate 1° 28.528'S,37° 58.050'E and altitude 1,028 meters above sea level. Collection was done within 200 meter radius from this coordinate.Fruits were de-pulped to extract stony endocarps which were dried in direct sun for 7 days and then stored at room temperature. Seeds were obtained by cracking dried stony endocarps then used as sources of explants.

2.2 Culture Medium

The culture medium consistedofhalf- strength Murashige and Skoog [MS] (1962) basal salts plus vitamins mixture (Duchefa Biochemie B.V., Netherlands). The induction medium was ½ MS mixture supplemented with 0, 0.05, 0.125, 0.25, 0.5, 1.0, 2.0 and 4.0 mg/l of low-cost agrochemical Thidiazuron (Kingtai Chemicals Ltd., China). Five types of shoot elongation medium were tested: hormone-free ½ MS mixture, ½ MS with 0.1 mg/l BAP, and ½ MS supplemented with 0.1 mg/l BAP plus either 0.01 mg/l IAA, or 0.01 mg/l GA_3 or 10% CW. Three types of rooting medium were tested: Hormone-free ½ MS and ½ MS supplemented with either 0.05mg/l or 0.1 mg/l IBA.

All induction and elongation media contained 20g/l sucrose. Rooting media contained 15g/l sucrose. All media were gelled with 8 g/l of agar (Thomas Baker, India). The pH of the medium was adjusted to 5.80 + 0.1 using Exstick® digital pH meter. Fifty ml aliquots of the medium were dispensed into honey jars. These were covered with transparent autoclavable plastic tops and then autoclaved at 121° C for 15 minutes.

2.3 Aseptic Techniques

Cotyledon explants were surface-sterilized by gentle shaking in 10% v/v Jik commercial bleach (Reckitt Benckiser®, packed concentration 3.85% m/v sodium hypochlorite), with 2 drops of Teepol® liquid detergent added as surfactant and wetting agent. The explants were rinsed in four changes of sterile water to remove the sterilant. Inoculation of the culture medium was done in a laminar-flow chamber. Explants were picked and plated onto the sterile agar medium using forceps after flaming and cooled with sterile (autoclaved) water.

2.4 Research Design

The study used a completely randomized design with three replications having six explants per bottle.Three experiments were carried out, giving a total of 54 explants per TDZ treatment.

2.5 Culture Conditions

The cultures were incubated in a growth chamber illuminated at 50 cm overhead distance by two 5-feet Phillips® cool daylight fluorescent tubes emitting 3.54 log lux each, measured by Phillip Harris Light Level sensometer. The photoperiod used was 16 hours light: 8 hours dark while the mean maximum and minimum room temperatures were 29.8 ± 0.8 and 25.5 ± 0.1 ^{o}C.

2.6 Histological Studies

Cotyledon explants at different stages of regeneration were fixed for 24 hours in FAA fixative (200ml fixative: 100 ml of 95% ethanol, 70 ml distilled water, 20 ml of 37% formaldehyde and 10 ml of concentrated glacial acetic acid). Fixed cotyledons were dehydrated for three hours each in alcohol and xylene series. Wax infiltration was done at 56^{o} C for 8 hours using McCormick® paraplast tissue embedding wax. Processed tissues were embedded in paraplast blocks which were then trimmed before sectioning at 5 to 8 µm thickness using a rotary microtome. Sections were mounted onto slides and staining carried out using Johansen's Safranin and Fast green staining method (Johansen, 1940).

2.7 Imaging and Statistical Analysis

Topographical imaging of the early stages of regeneration was done using a Keyence (Z35) VHX digital scanning photomicroscope. Macroscopic images of shoot development and rooting were taken using a Sony digital camera (Model DSC-W390). Histological sections were observed using a Leica ICC 50 photomicroscope and images taken using its integrated LAS EZ digital imaging system. Data were analyzed by one-way analysis of variance using SPSS version 17 software. Separation of means was done using Tukey's HSD test at $p \leq 0.05$.

3.0 Results

Within5 to 7days in culture, greening and curving of the explants were observed with the lower surfaces of the explants lifting upwards, away from the surface of the medium. This occurred in TDZ-media as well as in the controls.However, explants in the TDZ-free medium remained non-morphogenic before turning brown and undergoing necrosis after 35 days in culture. Morphogenesis was only observed in TDZ-treated medium.

Regeneration commenced within 7 to 9 days with explants developing a pimpled appearance. The tiny nodules gave rise to well definedmultiple globular somatic embryos ranging from 200 to 800µm in diameter (Figures 1a and b) within 10 to 16 days. These globular somatic embryos formed directly from the surfaces of explants without an intervening callus phase, except for TDZ concentrations of 0.05 and 0.125 mg/l where slight callusing occurred at the proximal ends of the cotyledons before globular embryos emerged.

Theregeneration occurred in a polarized pattern with response confined to the upper, directly illuminated surfaces of the explants irrespective of whether that surface was the abaxial or adaxialside of the explant (Figure 1a).Relative to the control, all the TDZ concentrations tested had a significant effect (F_{test}, $p < 0.001$) on percentage of explants with somatic embryos and percentage of explants with conversion of the somatic embryos to microshoots (Table 1). However, there was no significant difference in these responses between the various TDZ concentrations themselves.Media with TDZ of 0.5 mg/l and above were better at somatic embryo induction but less suitable for further shoot development than lower TDZ concentrations (Table 1) . Lower concentrations of the PGR allowed more conversion of somatic embryos into microshoots, with 0.25 mg/l TDZ medium being the best medium for shoot formation.The embryos converted to phenotypically normal shoots whilst still attached to the explant (Figures 3 and 4). Occassional rooting was observed as some embryos converted into microshoots (Figure 4).

Table 1: Effect of Thidiazuron concentration on somatic embryogenesis and shoot regeneration from M. volkensii cotyledons after 30 days in induction media

TDZ mg/l	Total explants	Percentages of explants (Means ± S.E.M) having: somatic embryos	microshoots (≤ 2mm)	macroshoots (≥ 5mm)
0	54	0[a]	0[a]	0[a]
0.05	54	100[b]	96.29 ± 1.85[b]	79.63 ± 3.70[b]
0.125	54	100[b]	92.57 ± 1.85[b]	75.93 ± 9.25[b]
0.25	54	100[b]	100[b]	82.40 ± 6.07[b]
0.5	54	100[b]	100[b]	74.07 ± 3.70[b]
1.0	54	100[b]	100[b]	75.92 ± 0.92[b]
2.0	54	100[b]	100[b]	63.89 ± 2.78[b]
4.0	54	100[b]	100[b]	20.18 ± 4.76[a]

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

Histological study revealed that the somatic embryos (S.Es) originated directly from epidermal and sub-epidermal cells. S.E induction commenced with nodular growths having a distinct protoderm covering a dome-shaped mass of ground meristem cells. These meristematic cells were sall, isodiametric, with densely staining cytoplasm and prominent nuclei (Figure 2c). The nodular structures gave rise to globular stages of somatic embryos, then heart-shaped and mature somatic embryos (Figures 2a, b and c).

Mature somatic embryos were bipolar, with a well developed shoot apical meristem and a root meristem at the opposite pole. Leaf primordia were evident at the plumular poles of the embryos. The embryos lacked vascular connection to the mother explant but most of them had well-defined procambium strands extending from the apical meristem to the radicular end (Figure 2a).

In an effort to elongate the microshoots and convert the remaining somatic embryos into shoots, half and one-third pieces of the cotyledons having mixtures of microshoots and somatic embryos were, after 30 days on induction medium, subcultured to ½ MS with four different PGR supplementations: 0 PGR, 0.1mg/l BAP, 0.1 mg/l BAP + 0.01 mg/l IAA and 0.1 mg/l BAP + 10% coconut water (CW). Media containing BAP alone or BAP combined with the IAA or CW supported good elongation of the microshoots formed prior to the subculture (Table 2; Figure 5 and 6). However, only media with BAP combined with IAA or CW allowed conversion of some more somatic embryos to shoots though the conversion frequency in terms of number of shoots per explant was generally low.

When shoots of 2.5 to 3cm height were excised from the cotyledon explants and planted into rooting media, they continued to elongate with some reaching up to 9.2 cm in 20 days on ½ MS + 0.1 mg/l IBA. The mean shoot heights after 30 days on the IBA supplemented media were 3.8 ± 0.4 and 3.2 ± 0.4 cm respectively on ½ MS + 0.05 mg/l IBA and ½ MS + 0.1 mg/l IBA. There was no significant difference in shoot elongation between these two media when the independent samples t-test was applied to the data.

No rooting was observed on IBA-free ½ MS and on ½ MS + 0.05mg/l IBA. Shoots on the IBA-free medium showed slight apical elongation with no swelling or callusing at the bases of the shoots. However, all the shoots on ½ MS + 0.05 and 0.1 mg/l IBA formed slight calluses at the bases of shoots with callus diameters of up to 1.2cm on media containing 0.05mg/l IBA. Some rooting was observed on media with 0.1 mg/l IBA but the frequency of rooting was low, ranging between 25% to 40% (Figure 7). However, good taproots were formed which later produced many lateral roots. Rooted plants were successfully transferred to sterile vermiculite irrigated with basal ½ MS. Further hardening and acclimatisation is in progress before transfer to the green house.

Figure 1a

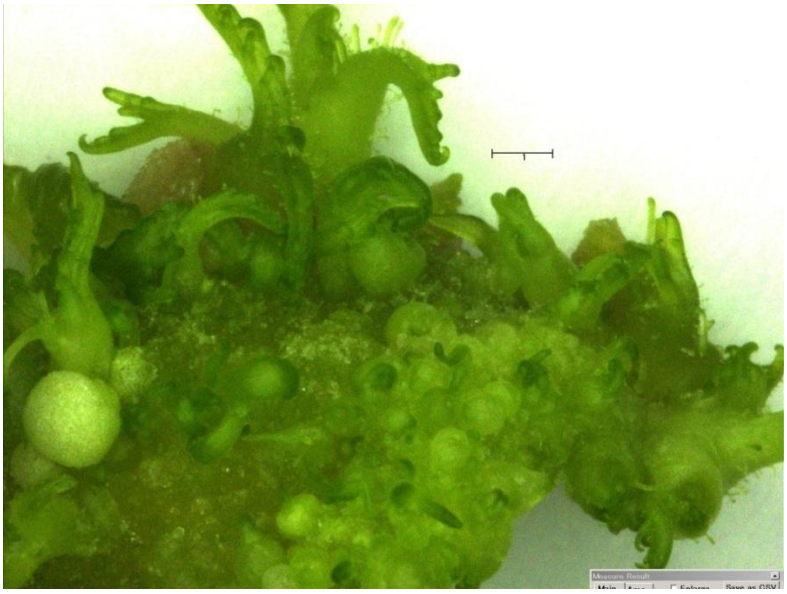

Figure 1b

Figure1:Scanning photomicrographs of M. volkensii cotyledons showing multiple globular somatic embryos regenerated from cotyledon explants. ***Fig.1a*** *shows regeneration confined to the illuminated surface of the explant after 28 days on ½ MS + 0.5 mg/l induction TDZ.* ***1b*** *is a close-up view of the regenerated surface showing globular somatic embryos with some in various stages of conversion into microshoots.Scale bars = 1mm*

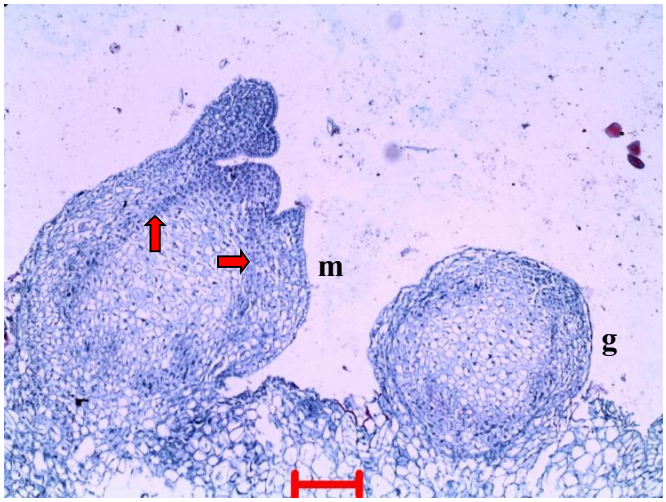

Fig.2a

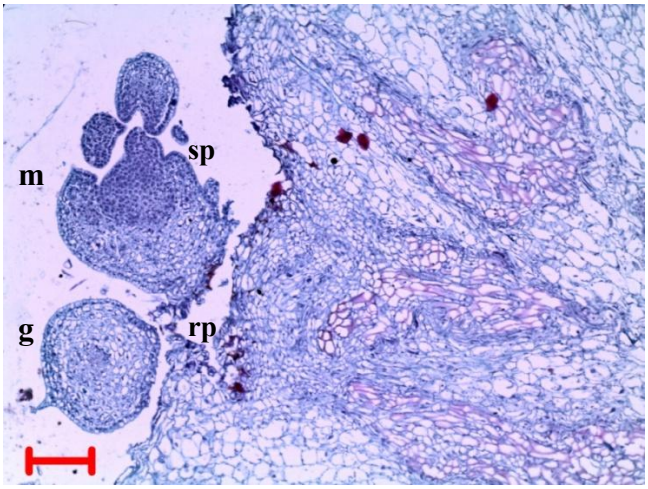

Fig.2b

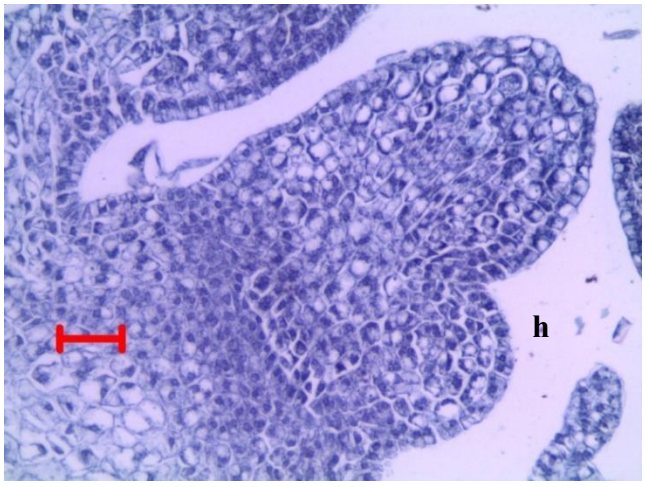

Fig.2c

Figure 2: Histology of cotyledon explants after 27 days on 0.05 mg/l TDZ, showing globular(g) and heart-shaped (h) stages of somatic embryogenesis as well as mature somatic embryos (m) Somatic embryogenesis was confirmed by absence of vascular connections between the embryos and the explant. Mature somatic embryos showed well defined procambial strands (Arrows) and clear polarity with distinct shoot pole (sp) and root pole (rp). Scale bars = 140 µm (Figure 2a and b) and 30µm (Figure 2c).

Figure 3: Cotyledon explants after 21 days on ½ MS + 1 mg/l TDZ induction medium. Three explants have well defined multiple shoots while the other three are still in early stages of regeneration

Figure 4: Cotyledon explants after 21 days on ½ MS + 0.5 mg/l TDZ induction medium showing occasional rooting as embryos convert into shoots

Table 2: Effect of PGRs on the conversion of somatic embryos into shoots after 22 days from date of transfer of cotyledon halves and thirds to the conversion media

PGR in the medium/ mg l^{-1}	% Cotyledon halves with S.E. conversion	Mean Shoot length/ mm	Significant observations
0	46.99 ± 4.32[a]	8.00 ± 1.08[a]	Shoots stunted but normal
0.1 BAP	83.54 ± 8.13[b]	14.82 ± 1.88[b]	Some thick stems
0.1 BAP + 10% CW	87.50 ± 8.46 [b]	14.13 ± 2.04[b]	Shoots with thick stems
0.1 BAP + 0.01 IAA	96.87 ± 3.13[b]	16.94 ± 2.07[b]	Shoots phenotypically normal

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

Figure 5:Cotyledon segments showing conversion of some embryos to shoots after 11 days of first subculture to ½ MS + 0.1 mg/l BAP + 0.01 mg/l IAA. This was after 30 days on induction media consisting of ½ MS + 1 mg/l TDZ. Cumulative age = 41days

Figure 6:Cotyledon explants showing good shoot elongation after32 days of first subculture to ½ MS with 0.1mg/l BAP+ 0.01mg/l IAA. This was after 30 days on induction media consisting of ½ MS + 1 mg/l TDZ. Cumulative age =62 days

Figure 7: Rooted shoot after 14 days on ½ MS + 0.1mg/l IBA.Cumulative age =94 days

4.0 Discussion and Conclusions

The greening and curving of the explants seen in the present study was independent of the presence TDZ as these responses also occurred on TDZ-free media. However, the presence of TDZ was essential for

regeneration as explants in control media remained non-morphogenic before browning and becoming necrotic. This is consistent with findings of other studies. Murthy and Saxena (1998) reported direct somatic embryogenesis from mature cotyledons of *Azadirachta indica* A. Juss. (Meliaceae) using MS medium with 0.3 – 10 mg/l TDZ. Vila *et al.* (2003) reported direct somatic embryogenesis from immature zygotic embryos of *Melia azedarch* L. (Meliaceae) using MS medium with 0.1 to 3mg/l TDZ.

In the only other reported study of somatic embryogenesis from mature cotyledons of *M. volkensii*, Indieka *et al.* (2007) used combinations of BAP (0.5 - 4 mg/l) and 0.2 mg/l 2,4-D, with a maximal regeneration efficiency of 60 percent of explants and mean S.E yield of 6.25 per explant. The present study attained a much higher efficiency with 100 % of explants forming hundreds of somatic embryos. The restriction of the regeneration response to the illuminated surfaces of the explants is in agreement with the findings of Baweja *et al*. (1995) where higher light intensity was reported to promote somatic embryogenesis in hypocotyls of *Albizzia lebbeck* L.

The histological observation showing somatic embryos as originating from epidermal and sub-epidermal cells is in conformity with the findings of Indieka *et al.* (2007)in *M volkensii* and those of Vila *et al.* (2003) in *M. azedarach*. The initiation of somatic embryos as globular structures, the bipolar nature of mature embryos, lack of vascular connections with mother explant, presence of procambial strands and ease of separation of the embryos from the explant have been reported the Meliaceae family (Indieka *et al.,* 2007; Murthy and Saxena, 1998; Vila *et al.,* 2003) and in other plant species (Quiroz-Figuera *et al*., 2006; Vega *et al*., 2009).

Elongation of the microshoots on medium containing 0.1 mg/l BAP alone or in combination with either coconut water or 0.01 mg/l IAA is in conformity with our previous finding on *M.volkensii* shoots regenerated from mature zygotic embryos using TDZ (Mulanda *et al*., 2012) and with the findings of Shahin-uz-zaman *et al*. (2008) in *Azadirachtaindica*. The fact that shoots continued to elongate very well on rooting medium containing 0.05 or 0.1 mg/l IBA appears to suggest that the stuntingtypically seen in shoots regenerated with TDZ could be overcome by exogenous application of auxin to restore apical growth.

The frequency of rooting obtained in this study is still low for mass propagation of the species and further work is required for optimization. However, a similarly low frequency of rooting of regenerated shoots in *M. volkensii*was reported by Indieka *et al*. (2007).The apparent difficulty in rooting of the shoots seen *in vitro* has also been encountered in studies of *in vivo* rooting of stem cuttings (Stewart and Blomley, 1994).Further work is in progress to induce germination of isolated somatic embryos as a means increasing plantlet recovery and avoiding rooting difficulties. Use ofshort pulse immersion of cut ends of shoots in auxin solutions before planting of the shoots in auxin-free media is also being investigated as a strategy for improved rooting.

This study supplements local and international efforts aimed at making tissue culture simpler and more affordable. This could open up possibilities for creation of simple tissue culture labs at the farm and community level. The high frequency of somatic embryogenesis attained in this study demonstrates the high potency and efficacy of the low-cost agrochemical Thidiazuron as a substitute for conventional TDZ in tissue culture of *Melia volkensii*. This agrochemical may be useful in other species. We recommend trials on other species and explants.

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