**THIDIAZURON-INDUCED SOMATIC EMBRYOGENESIS AND SHOOT REGENERATION IN COTYLEDON EXPLANTS OF *MELIA VOLKENSII* GURKE.**

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**Abstract**

*Melia volkensii* Gurke is a drought-tolerant multipurpose tree species suitable for greening and hardwood timber production in the East African arid and semi-arid lands (ASALs). Conventional propagation is problematic due to seed extraction difficulties, seed dormancy and poor rooting of cuttings. We developed a simple, rapid and efficient protocol for *in vitro* regeneration of *M. volkensii* plants from cotyledon explants using Thidiazuron (TDZ). Mature cotyledons cultured on ½ MS medium supplemented with eight TDZ concentrations from 0 to 4 mg l-1 formed multiple green globular structures within 8 to 14 days, without an intervening callus phase. Regeneration was confined to the upper-facing, directly illuminated surfaces of the explants. Regeneration frequency was 88% in 0.05 mg l-1 TDZ and 100% in 0.125 – 4 mg l-1 TDZ. Explants in control media remained non-morphogenic. Scanning photomicrography and histological examination revealed multiple globular somatic embryos similar in shape and size to the zygotic embryos. Mature somatic embryos with shoot apices converted into leafy microshoots whilst still on induction media. Microshoots elongated when whole or half segments of cotyledons were subcultured to ½ MS supplemented with 0.1 mg l-1 Benzylaminopurine (BAP) alone or in combination with 0.01mg l-l Indole-3-acetic acid (IAA) or 10% coconut water (CW). The yield of transplantable shoots ranged from 5 to 18 per cotyledon. Medium containing BAP and IAA was superior for elongation, giving shoots of up to 10.1 cm in 35 days. Shoots rooted in ½ MS supplemented with either 0.1 mg l-1Indole-3-butyric acid (IBA) or 0.2 mg l-1 Napthoxyacetic acid (NAA), with NAA inducing up to 12 roots per shoot. This protocol could be applied in mass production of *M. volkensii* planting material for extension of plantations. It can also be applied in genetic modification of the species.

**Key words:** Propagation, *Melia volkensii*, drought-tolerant, multipurpose tree.

**Running title:** Shoot regeneration from *M. volkensii* cotyledons.

**Introduction**

Efficient plant regeneration via tissue and organ is a prerequisite for many biotechnological applications such as *in vitro* genetic improvement of crop plants, elimination of viruses from planting stock, micro-grafting, *in vitro* conservation and *in vitro* screening and selection for tolerance to various abiotic stresses (Benson 2002, Akin-Idowu et al. 2009, Loyola-Vergas and Ochoa-Aleyo 2012, Jamsheed et al. 2013). It is also vital for rapid clonal micropropagation of elite genotypes (Brown and Thorpe 1995), which could be vital in the rescue of medicinal, pesticidal and timber plant species threatened with extinction arising from the combined effects of over-exploitation and loss of habitat.

*Melia volkensii* Gurke (Meliaceae) is a drought-tolerant tree species endemic to the arid and semi-arid lands (ASALs) of East Africa (Orwa et al. 2009). It is a multipurpose tree suitable for use in dry land agro-forestry, apiary, greening of the ASALs, supply of emergency animal fodder during drought and as a source of medicinal drugs and botanical pesticides (Stewart and Blomley 1994, Orwa et al. 2009). Its use as an ornamental tree is alluded to by Rajab and Bentley (1992). The species has vast potential for use as a shade and ornamental tree in homesteads, in fence rows on farms and along roads and highways in the ASAL areas. Its most attractive features are valuable mahogany timber, drought-tolerance, fast growth and short maturity periods of 10 to 15 years, coppicing ability and resistance to termites.

*M. volkensii* natural populations have been depleted considerably as a result of over-exploitation for timber and habitat destruction (Runo et al. 2004, Hanaoka et al. 2012). This decline has been accelerated by difficulties in propagation from seed and cuttings (Stewart and Blomley 1994, Kidundo 1997, Indieka et al. 2007). Its seeds are trapped in a woody endocarp making extraction difficult. Germination is also hampered by mechanical and physiological dormancy, which are compounded by high post-germination mortality due to high susceptibility of seedlings to endogenous and exogenous fungal pathogens (Kimondo and Kiamba 2005).

In view of these constraints to seedling production, there is need for tissue culture protocols for rapid and efficient micropropagation of *M. volkensii*. There are only two previous reports of tissue culture in this species. The first report (Indieka et al. 2007) attained somatic embryogenesis in 60% of cotyledon explants using combinations of BAP and 2,4-D. The second report, by the present authors (Mulanda et al. 2012), attained high frequency (up to 96.67%) regeneration via callus-mediated somatic embryogenesis in mature zygotic embryos of *M. volkensii* using low-cost agrochemical Thidiazuron.

The present study explored, for the first time, the use of Thidiazuron (TDZ) as sole plant growth regulator (PGR) for induction of somatic embryos and plant regeneration in mature cotyledons of *M. volkensii*. Thidiazuron is a urea derivative (N-phenyl- (N’ 1,2,3-thidiazol-5-yl)urea) originally developed as a cotton defoliant but was later found to have potent cytokinin activity (Mok et al. 1982, Huetteman and Preece 1993). TDZ has since then been used as a growth regulator in tissue cultures of many plant species (Guo et al. 2011). Its use simplifies the regeneration process by avoiding use of combinations of PGRs.

The aim of this study was to determine if use of TDZ could elicit a higher frequency of direct somatic embryos and plants from the cotyledon explants than that reported earlier by Indieka et al. (2007) using other plant growth regulators.

**Materials and Methods**

**Plant materials:** Mature fruits of *Melia volkensii* were collected in February 2013, from domesticated trees growing on farms in Mbathani-A village in Katulani District, Kitui County, South Eastern Kenya. The collection site was geo-referenced as follows: GPS coordinate 1o 28.528’S, 37o 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 and the stony endocarps dried in direct sun for 7 days. The endocarps were then stored at room temperature. Cotyledons extracted from the seeds were used as explants.

**Culture medium:** The culture medium consistedofhalf-strength Murashige and Skoog (MS) (1962) basal salts plus vitamins mixture sourced from 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-1 of low-cost agrochemical Thidiazuron from Kingtai Chemicals Ltd., China (Mulanda et al., 2012). Four types of shoot elongation medium were tested: hormone-free ½ MS mixture, ½ MS with 0.1 mg l-1 BAP alone or BAP combined with 0.01 mg l-1 IAA or 10% CW. Eight types of rooting medium were tested: Hormone-free ½ MS, ½ MS supplemented with 0.05, 0.1 or 0.2 mg l-1 IBA, ½ MS supplemented with 0.05, 0.1 or 0.2 mg l-1 NAA and ½ MS + 0.1 mg l-1 IBA and 0.1 mg l-1 NAA.

All induction and elongation media contained 20 g l-1 sucrose while rooting media contained 15g l-1 sucrose. All media were gelled with 8 g l-1 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 foil and autoclaved at 121o C for 15 minutes.

**Aseptic techniques.** Cotyledon explants were surface-sterilized by gentle shaking for 15 minutes 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.

**Research design:** The study used a completely randomized design with three replicates per experiment. Each replicate had six explants. The experiment was repeated four times.

**Culture conditions:** The cultures were incubated in an improvised growth chamber illuminated at 50 cm overhead distance by two 5-feet Phillips® cool daylight fluorescent tubes, each emitting a light intensity of 3.54 log lux at tube level as measured by a Phillip Harris light level sensometer. A 16 hour photoperiod was used. The mean maximum and minimum room temperatures during the culture period were 29.8 ± 0.8 and 25.5 ± 0.1 oC.

**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 in alcohol series (80, 90, 95 and 100%) and another three hours in absolute xylene series. Wax infiltration was done at 56o C for 8 hours using McCormick® paraplast tissue embedding wax. Tissues were processed using a Shandon Elliot® automatic tissue processor.

Processed tissues were embedded in paraplast blocks before trimming and sectioning at 5 to 8 µm thickness using a rotary microtome. Sections were mounted onto slides and stained using Johansen’s Safranin and Fast green staining method (Johansen 1940).

**Imaging and statistical analysis:** Topographicalimagingof 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 17.0 software. Percentage scores from count data were arcsine transformed using the equation *Y = arcsine √p*, where *p* = the proportion obtained by dividing the respective percentage value by 100, before carrying out the Anova test as suggested by Rangaswamy (2010). Separation of means was done using Tukey’s HSD test at *p ≤ 0.05*.

**Results and Discussion**

Cotyledon explants turned green and curved within5 to 7days in culture, with the surfaces of the explants facing the medium lifting upwards away from the medium. The greening and curving responses occurred in media supplemented with TDZ as well as in the TDZ-free controls, showing that these responses were not dependent on the presence TDZ. 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, showing that the presence of TDZ was essential for regeneration. This is consistent with findings of Murthy and Saxena (1998) who reported direct somatic embryogenesis from mature cotyledons of *Azadirachta indica* A. Juss. (Meliaceae) using MS medium with 0.3 – 10 mg l-1 TDZ and those of Vila et al. (2003) who reported direct somatic embryogenesis from immature zygotic embryos of *Melia azedarch* L. (Meliaceae) using MS medium with 0.1 to 3 mg l-1 TDZ.

Regeneration commenced in 7 to 10 days with explants developing numerous tiny green nodular structures. Within 10 to 16 days, these green nodules gave rise to well defined green globular somatic embryos ranging from 200 to 1500µm in diameter (Figures 1b and c). These globular somatic embryos formed directly from the surfaces of explants without an intervening callus phase, except for the low TDZ concentrations of 0.05 and 0.125 mg l-1 where slight callusing occurred at the proximal ends and on the margins of the cotyledons before the globular embryos emerged. The globular embryos had remarkable similarity in size and morphology to the zygotic embryos of mature seeds (Figures 1a, b,c). They were also strikingly similar to the scanning electromicographs of somatic embryos of *Melia azedarach* obtained by Vila et al. (2003).

There are no previous reports of TDZ-induced regeneration from *M. volkensii* cotyledons. However, Indieka et al. (2007) reported direct somatic embryogenesis from mature cotyledons of *M. volkensii* using combinations of BAP (0.5 - 4 mg l-1) and 0.2 mg l-1 2,4-D, with a maximal regeneration efficiency of 60 percent of explants and mean yield of 6.25 somatic embryos per explant. The present study attained a much higher regeneration efficiency with 100 % of explants forming dense clusters of somatic embryos in media containing TDZ at concentrations of 0.125 – 4 mgl-1.

In the present study, the regeneration occurred in a polarized pattern with the response confined to the upper-facing and directly illuminated surfaces of the explants. This pattern of regeneration was observed irrespective of whether the upper-facing surface was abaxial or adaxial (Figures 1b, c and d). The restriction of the regeneration response to the illuminated surfaces of the explants appears to be in conformity with the findings of Baweja et al. (1995) where higher light intensity was reported to promote somatic embryogenesis in hypocotyls of *Albizzia lebbeck* L.

In comparison to the control, all the TDZ concentrations tested had a significant effect ( Ftest, *p < 0.001*) on percentage of explants with somatic embryos (Table 1). The percentage of explants with conversion of somatic embryos to microshoots was also significantly higher ( Ftest, *p < 0.001*) in the TDZ-treated explants in comparison to the control, except for 4 mg l-1 TDZ (Table 1). There were also significant differences in conversion of somatic embryos to shoots between the various TDZ concentrations themselves (Table 1). Media with ≥ 0.5 mg l-1 TDZ showed qualitatively and quantitatively better somatic embryo induction but significantly lower conversion to shoots than media with lower (< 0.5 mg l-1) TDZ concentrations (Table 1) . Media with 0.25 mg l-1 TDZ was the best for shoot development. The embryos converted to shoots whilst still attached to the explant (Figure 3a, b and c). Occassional simultaneous rooting was observed as some embryos converted into microshoots.

Histological study revealed that the somatic embryos (S.Es) originated as nodular structures formed directly from epidermal and sub-epidermal cells. The nodular growths consisted of a distinct protoderm covering a dome-shaped mass of ground meristem cells. These meristematic cells were small, isodiametric, with densely staining cytoplasm (Figure 2a). The nodular structures gave rise to globular stages of somatic embryos, then heart-shaped and mature somatic embryos that could be easily separated from the mother explant (Figures 2b to e). Some torpedo stages were also observed (Figure 2e). This is in conformity with the findings of Indieka et al. (2007) in *M. volkensii* and those of Vila et al. (2003, 2010) in *M. azedarach*. Mature somatic embryos were bipolar, with a well developed shoot apical meristem and a root meristem at opposite poles (Figure 2f). Leaf primordia were evident at the plumular poles of the mature embryos. The embryos lacked vascular connection to the mother explant but most of them had well-defined procambium strands extending from the apical meristem to the radicular pole (Figures 2b and e).

The initiation of somatic embryos as green globular structures, their bipolar nature, lack of vascular connections to the mother explants, presence of procambial strands and ease of separation of the embryos from the explant have been reported in members of the Meliaceae family (Indieka et al. 2007, Murthy and Murthy 1998, Vila et al. 2003) and in other plant species (Quiroz-Figuera et al. 2006, Vega et al. 2009). Some heterogeneity was observed in the size and morphology of the somatic embryos in the present study, which calls for further study for characterization of the extent of the heterogeneity and its implications on the conversion of the embryos into plants. Similar heterogeneity of TDZ-induced somatic embryos was reported in *M. azedarach* by Vila et al. (2010).

Elongation of microshoots was achieved when whole or half segments of cotyledons having microshoots were subcultured to ½ MS supplemented with 0.1mg l-1 BAP, 0.1 mg l-1 BAP + 0.01 mg l-1 IAA or 0.1 mg l-1 BAP + 10% coconut water (CW). Media containing BAP in combination with the IAA caused the best elongation of the microshoots that had formed earlier on induction media, with no morphological abberations observed (Table 2). However, media with BAP combined with IAA or CW also allowed some somatic embryos that had failed to convert to microshoots on the induction media to do so, though the conversion frequency was generally low.

The elongation of the microshoots on medium containing 0.1 mg l-1 BAP alone or in combination with either coconut water or 0.01 mg l-1 IAA is in conformity with our previous findings (Mulanda et al. 2012) for *M. volkensii* shoots regenerated from mature zygotic embryos using TDZ, and with the findings of Shahin-uz-zaman et al. (2008) in *Azadirachta* *indica*. In the present study the shoots also continued to elongate after transfer to rooting medium containing 0.05 or 0.1 mg l-1 IBA. The mean shoot height after 30 days on the IBA supplemented media was 4.22 ± 0.51 and 3.45 ± 0.44 cm respectively on ½ MS + 0.05 mg l-1 IBA and ½ MS + 0.1 mg l-1 IBA. The mean number of leaf nodes per shoot were 7.94 ± 0.60 and 7.31 ± 0.44. However, there was no significant difference in either shoot height or number of leaf nodes between these two media when the independent samples t-test was applied to the data.

Significant rooting (F test, p < 0.001) was observed only in media containing 0.2 mg l-1 NAA alone or 0.1 mg l-1 NAA in combination with 0.1 mg l-1 IBA ( Table 3). Although NAA was superior to IBA in root induction, rooting media containing 0.05 to 0.2 mg l-1 NAA were not as effective in stimulating shoot elongation as media with IBA. NAA-supplemented media also caused variable extents of leaf chlorosis in the shoots. These findings suggest that the restoration of apical growth and removal the stunting of shoots typically associated with plants regenerated using TDZ could be attained by exogenous application of an indole-type auxin.

In general IBA induced a single taproot per shoot (Table 3 and Figure 3d) while NAA induced multiple roots (Table 3). Rooted plants were successfully transferred to pots containing sterile vermiculite irrigated with basal ½ MS with 80% survival rate (Figure 4).

The frequency of rooting obtained in this study is similar to that reported by Indieka et al. (2007) for *M. volkensii*. Low rooting frequency is the only remaining barrier to the utilization of tissue culture for mass propagation of the species and there is need for further study for optimization of the same. Further work on the germination of isolated somatic embryos could be a means of avoiding these rooting difficulties.

The high frequency of regeneration attained in this study confirms our earlier report of high potency and efficacy of low-cost agrochemical Thidiazuron as a substitute for the exorbitant conventional TDZ in tissue culture of *Melia volkensii* (Mulanda et al. 2012). This agrochemical may be useful in commercial propagation of other tree species.

The findings of this study demonstrate the amenability of *M. volkensii* to mass propagation via tissue culture technology. This study also opens up possibilities for genetic transformation of the species.

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Table 1. Effect of Thidiazuron concentration on somatic embryogenesis and conversion of the somatic embryos to shoots in *M. volkensii* cotyledons after 30 days in induction media.

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
| TDZ  mg l-1 | Total explants | % explants with somatic embryos | % explants with microshoots | Number of shoots per explant |
| 0 | 72 | 0a | 0a | 0a |
| 0.05 | 72 | 88.88±2.27b | 88.88±2.27b | 4.67±0.42a |
| 0.125 | 72 | 100±0.00c | 76.39±7.98bc | 8.70±1.33b |
| 0.25 | 72 | 100±0.00c | 78.47±5.82bc | 11.25±2.80b |
| 0.5 | 72 | 100±0.00c | 76.38±3.49bc | 10.00±1.75b |
| 1.0 | 72 | 100±0.00c | 66.69±4.51bc | 7.80±1.17b |
| 2.0 | 72 | 100±0.00c | 59.72±3.49c | 9.44±1.52b |
| 4.0 | 72 | 100±0.00c | 20.83±5.73a | 5.44±0.97a |
| Total | 576 |  |  |  |

*Values are means of 3 replicates ± standard error of the mean.* *Values with the same superscript in a column do not differ significantly using Tukey’s HSD test at p≤ 0.05.*

Table 2. Effect of different PGR treatments on elongation of *M. volkensii* microshoots 20 days after transfer of cotyledon halves from induction media to elongation media.

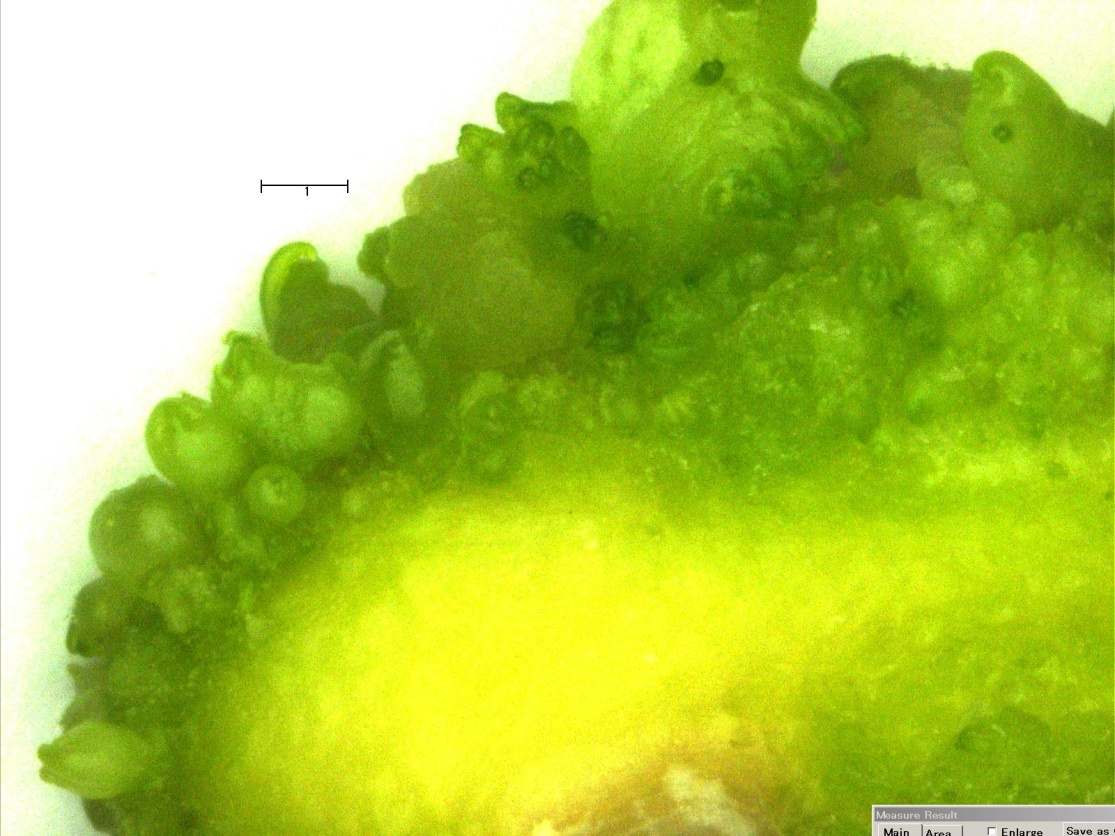
|  |  |  |
| --- | --- | --- |
| PGR combination | Days in this media | Mean shoot length/ mm (± S.E.M) |
| ½ MS + 0 PGR | 22 | 8.00 ± 1.08a |
| ½ MS + 0.1mg l-1 BAP  ½ MS + 0.1mg l-1 BAP + 10% CW  ½ MS + 0.1 mg l-1 BAP + 0.01mg l-1 IAA | 22  24  24 | 16.16 ± 1.94b  15.57 ± 1.85b  18.17 ± 1.58b |
|  |  |  |

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

Table 3. Effect of NAA and IBA on rooting of shoots after 22 days in rooting media.

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

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

**t**

**m**

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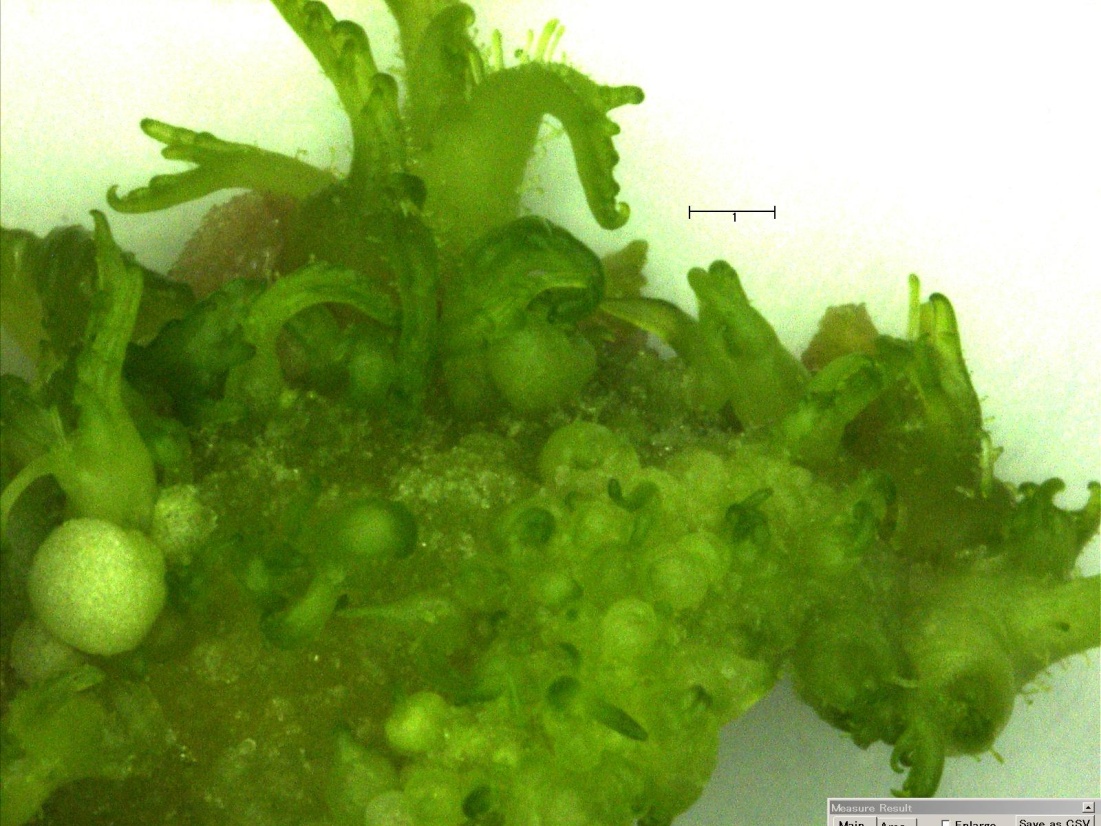
**cot**

**m**

**cot**

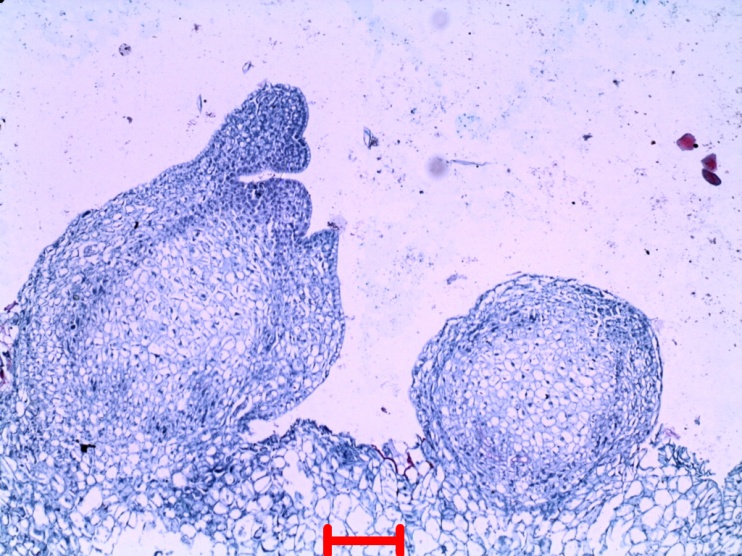
**t**

(a) (b)

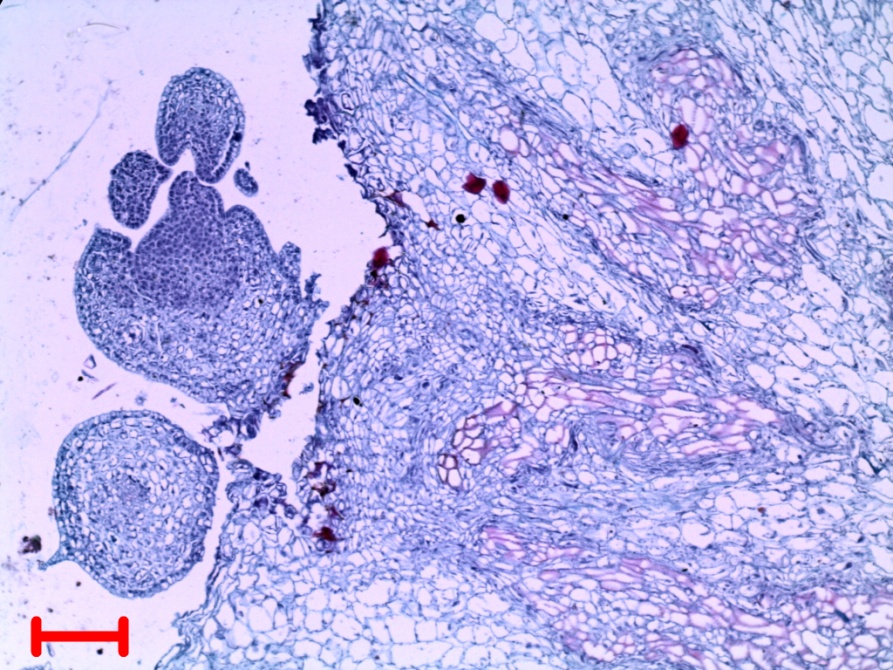
(c) (d)

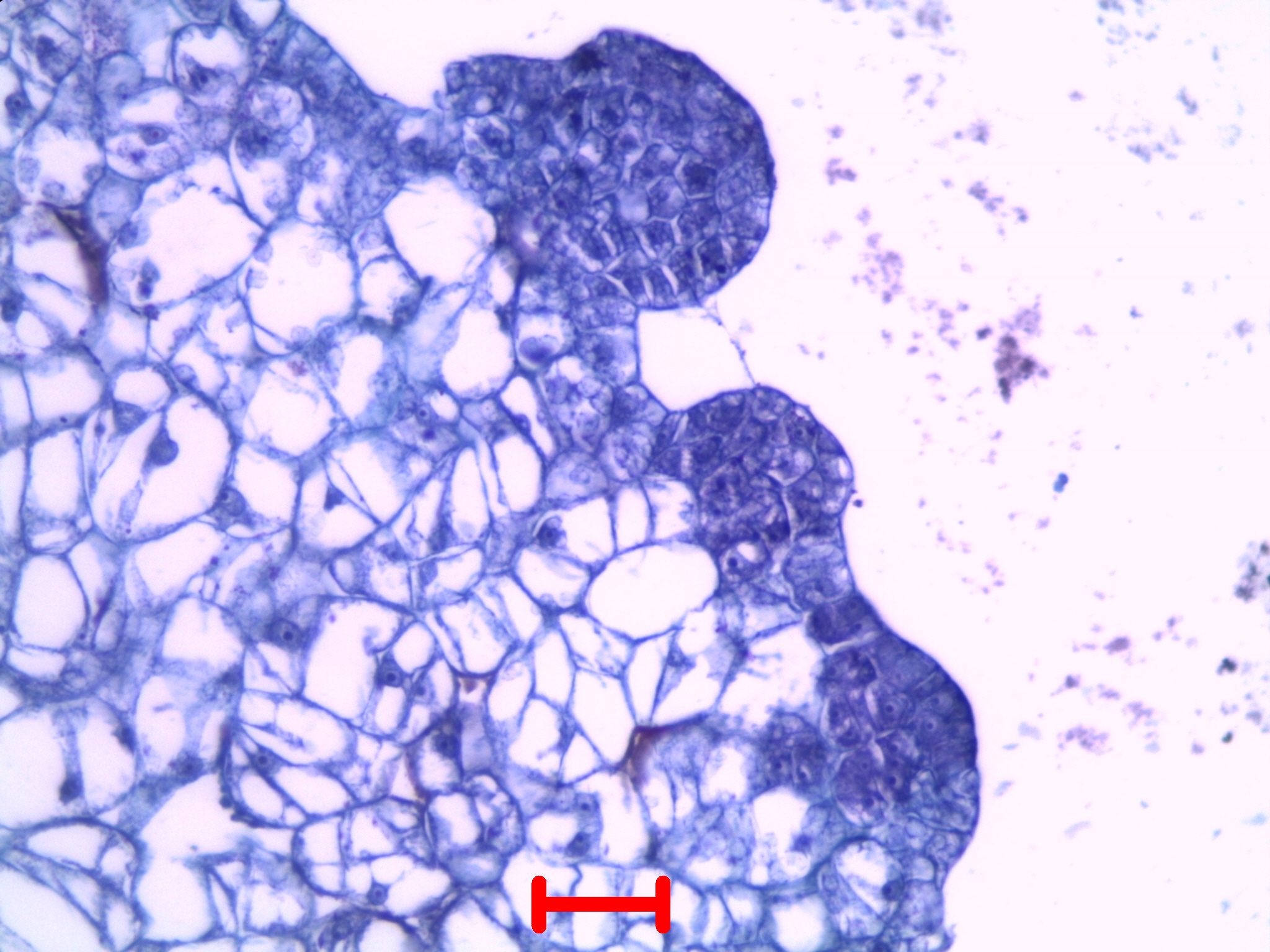
**Fig. 1.** Scanning photomicrographs (x 25): (**a**) Mature zygotic embryo (**z.e**) of *M. volkensii* attached to the cotyledon (**cot**); included to show the close similarity in the globular nature and size of the zygotic embryo to the somatic embryos in shown in Figures 1 (b) to (d). (**b to d**) Cotyledon explants in various stages of somatic embryogenesis after 20 days of culture on ½ MS + 0.125 mg l-1 TDZ, showing globular (**g**) and torpedo (**t**) stages and mature somatic embryos in early stages of conversion into microshoots (**m**). In Fig **1c** the cotyledonwas turned upside downto show the polarized nature of the regeneration response, which occurred only on the upper-facing, directly illuminated surfaces of explants. Scale bars = 1mm.

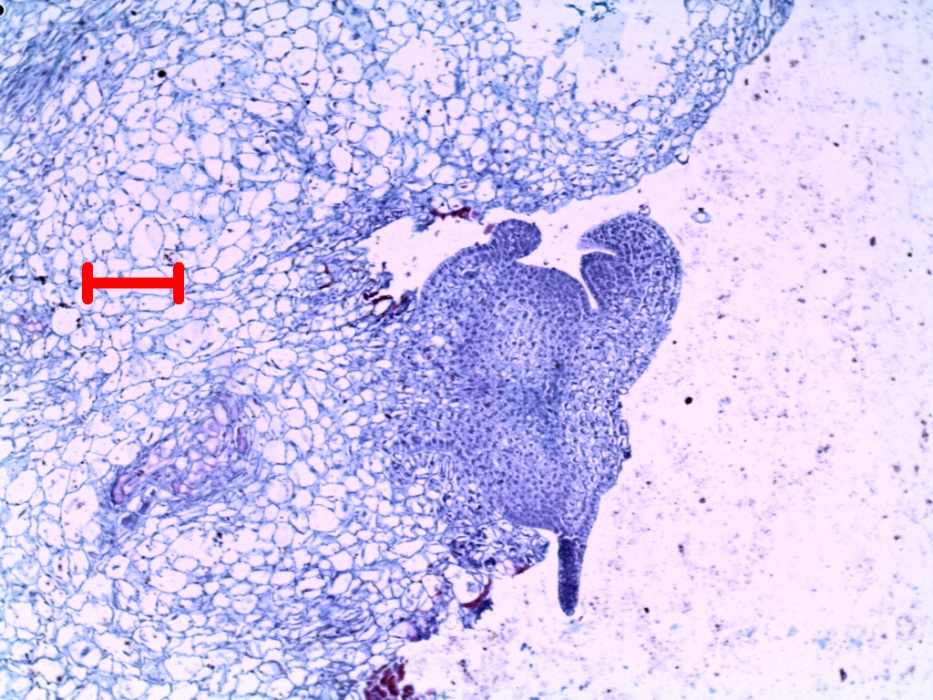












**h**

**t**

**h**

**pem**

**sp**

**rp**

**h**

**g**

**m**

**pc**

**pc**

(e)

(d)

(c)

(b)

(a)

(f)

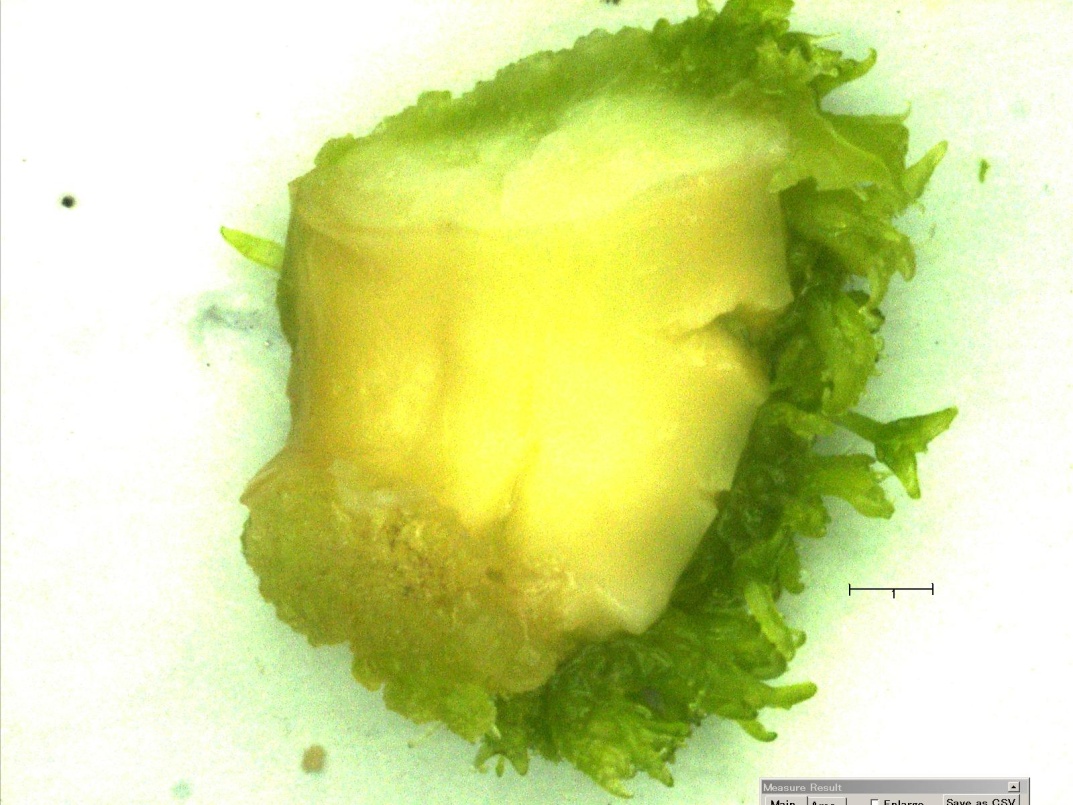
**m**

**g**

**g**

**g**

**Fig. 2.** Histology of cotyledon explants showing pro-embryonic masses (**pem**) arising from isodiametric, densely-staining epidermal and sub-epidermal cells, globular (**g**) heart-shaped (**h**) and torpedo (**t**) stages of somatic embryogenesis as well as mature somatic embryos (**m**) Mature somatic embryos showed well defined procambial strands (**pc**) and clear polarity with a distinct shoot pole (**sp**) and root pole (**rp**). Scale bars = 30 µm (Fig.2a and d) and 140µm (Fig.2b, c, e, f).

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**(a) (b)**

****

**(c) (d)**

**Figure 3.** **(a)** Scanning photomicrograph of a segment of cotyledon explant showing high frequency shoot regeneration after 16 days on ½ MS + 0.5 mg l-1 TDZ. **(b)** Scanning photomicrograph of a well-defined microshoot formed after 20 days on ½ MS + 0.05 mg l-1 TDZ [For (**a**) and (**b**), scale bar = 1mm]. (**c**) Macroscopic view of cotyledon explants showing shoot regeneration after 24 days on ½ MS + 0.5 mg l-1 TDZ induction medium. **(d)** Rooting after 14 days on ½ MS + 0.1 mg l-1 IBA.



**Figure 4**. *M. volkensii* plant after 29 days of successful establishment in a potting mixture of vermiculite watered with basal ½ MS solution. Scale bar = 5cm.