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

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Abstract

Melia volkensii Gürke is an ornamental, drought-tolerant multipurpose tree species suitable for greening and hardwood timber production in the East African arid, and semi-arid lands. 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 TDZ. Mature cotyledons cultured on half-strength 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 on control medium remained non-morphogenic. Morphological 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 variants of induction medium. Microshoots elongated when whole or half segments of cotyledons were subcultured to half-strength MS supplemented with 0.1 mg l^{-1} BAP alone or in combination with 0.01mg l^{-1} IAA or 10% coconut water (CW). The mean yield of transplantable shoots ranged from 4.67 to 11.25 per cotyledon. The most significant rooting (39.3%) and highest mean number of roots (5.64) of the shoots was achieved on half-strength MS medium supplemented with combination of 0.1 mg l^{-1} NAA and 0.1 mg l^{-1} IBA.

Key words: drought-tolerant, multipurpose tree, popagation,

INTRODUCTION

Melia volkensii Gürke (Meliaceae) is an ornamental tree suitable for use in the gardens, roads and highways in arid and semi-arid lands. It is also multi-purpose, fast-growing species used in agro-forestry lands as a melliferous plant, source of mahogany timber, medicinal drugs and botanical insecticides (Orwa et.al 2009). However, its 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 produc-

tion, 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 6-benzylaminopurine (BAP) and 2,4-Dichlorophenoxyacetic acid (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 (1-Phenyl-3-(1,2,3,-thiadiazol-5-yl)urea (Thidiazuron, TDZ). Thidiazuron is an urea derivative 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 1° 28.528'S, 37° 58.050'E and altitude 1,028 m. Collection was done within 200 m radius from this coordinate.

Extraction and disinfection of the explants

The processing of fruits and seed extraction was done using our earlier published method (Mulanda et al. 2012). Cotyledons extracted from the seeds were used as explants. Explants were disinfected using 10% v/v Jik and 2 drops of Teepol liquid detergent as reported in Mulanda et al. (2012). Inoculation of the culture medium was done in a laminar-flow chamber.

Culture medium

The induction culture medium consisted of half-strength Murashige and Skoog (1962) (MS) basal nutrients and vitamins mixture (sourced from Duchefa Biochemie B.V., Netherlands) supplemented with 0, 0.05, 0.125, 0.25, 0.5, 1.0, 2.0, and 4.0 mg l^{-1} TDZ (Kingtai Chemicals Ltd., China). Four variants of half-strength MS medium were tested for shoot elongation: Plant growth regulators (PGRs)-free medium, medium with 0.1 mg l^{-1} BAP alone or 0.1 mg l^{-1} BAP combined with either 0.01 mg l^{-1} IAA or 10% coconut water (CW). Eight variants of half-strength MS medium were tested for rooting: PGRs-free medium, medium supplemented with 0.05, 0.1 or 0.2 mg l^{-1} indole-3-butyric acid (IBA), medium supplemented with 0.05, 0.1 or 0.2 mg l^{-1} α-naphtaleneacetic acid (NAA) and medium with 0.1 mg l^{-1} IBA and 0.1 mg l^{-1} NAA combined.

The variants of the medium used for induction and elongation of shoots contained 20 g l^{-1} sucrose while those used for rooting contained 15 g l^{-1} sucrose. All medium variants were gelled with 8 g l^{-1} of agar (Thomas Baker, India). The pH of the medium was measured by Exstick® digital pH meter and adjusted to 5.80 + 0.1 using 1M HCl or 1M NaOH. Fifty ml of the medium were dispensed into honey jars of 400 ml capacity. These were covered with foil and autoclaved at 121°C for 15 min.

Variants of induction and elongation medium were inoculated with 6 explants per bottle, with 3 replicates and 4 repetitions for each concentration of TDZ. Each variant of rooting medium was inoculated with 4 shoots per bottle, with 6 replicates and three repeats. Regeneration and elongation of shoots were recorded after 30 days and 20 days respectively on variants of induction and elongation medium, the scores of rooting were recorded after 22 days in respective variants of rooting medium.

Culture conditions

The cultures were incubated in a growth chamber with a light level of approximately 60 μM m^{-2} s^{-1} supplied by Phillips® cool daylight fluorescent tubes. A 16 h photoperiod was used. The mean room temperatures during the culture period was 29.8 ± 0.8°C.

Histological studies and imaging

Cotyledon explants at different stages of regeneration were fixed for 24 h in formalin-acetic acid-alcohol (FAA) fixative (200 ml 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 h in alcohol series (80, 90, 95, and 100%) and another three h in absolute xylene series. Wax infiltration was done at 56°C for 8 h 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).

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.

Research design and statistical analysis

The study used a completely randomized design. Data were analyzed using IBM SPSS statistics software version 17.0. The quadratic model of curvilinear regression was applied to data for percentage of explants with somatic embryos and shoots and significance was determined at $p \leq 0.05$ using the ANOVA test for regression. Data for shoot elongation and rooting in different PGR combinations was subjected to one-way ANOVA and the means were separated using Tukey's HSD test at $p \leq 0.05$. Percentage values from count data were arcsine transformed using the equation $Y = arcsine \sqrt{p}$, where p = the proportion obtained by dividing the respective percentage value by 100, before carrying out the ANOVA test as suggested by Rangaswamy (2010).

RESULTS AND DISCUSSION

Cotyledon explants turned green and curved within 5 to 7 days in culture, with the surfaces of the explants facing the medium lifting upwards away from the medium. The greening and curving responses occurred on variants of the medium supplemented with TDZ as well as in the TDZ-free controls, showing that these responses were not dependent on the presence of TDZ. Explants on the TDZ-free medium remained non-morphogenic before turning brown and undergoing necrosis after 30 days in culture. Morphogenesis was only observed on medium containing TDZ, 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. 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 azedarach* L. 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 (SEs) ranging from 200 to 1500 µm in diameter (Fig. 1B,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 (Fig. 1A,B,C). They were also strikingly similar to the photograph images of somatic embryos of *Melia azedarach* obtained by Vila et al. (2003).

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% of explants and mean yield of 6.25 somatic embryos per explant. The present study attained much higher regeneration efficiency with 100% of explants forming dense clusters of somatic embryos on medium containing TDZ at concentrations of 0.125 - 4 mg l^{-1} (Fig. 1, 2).

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 (Fig. 1B,C,D and 5A). The restriction of regeneration response to the illuminated surfaces of explants appears to be in conformity with the findings of Baweja et al. (1995) where presence of light was reported to promote somatic embryogenesis in hypocotyls of *Albizzia lebbeck* L.

The plot of percentage of explants with somatic embryos against TDZ concentration revealed a curvilinear relationship (Fig. 2). Regression analysis under quadratic model showed that TDZ concentration had a significant effect ($p < 0.05$) on the percentage of explants with somatic embryos (Fig. 2). The plot of percentage of explants with conversion of somatic embryos to microshoots was also curvilinear and quadratic, and regression was significant ($p < 0.05$, Fig. 3). The same trend and pattern was observed in the plot of number of shoots against TDZ concentration (Fig. 4).

Variants of the medium with ≥ 0.5 mg l^{-1} TDZ showed qualitatively and quantitatively better somatic embryo induction but lower conversion to shoots than medium variants with lower (< 0.5 mg l^{-1}) TDZ concentrations (Fig. 2, 3, 4). Medium with 0.25 mg l^{-1} TDZ was the best for shoot development. The embryos converted to shoots whilst still attached to the explant (Fig. 5A,B,C). Occassional simultaneous rooting was observed as some embryos converted into microshoots.

Histological study revealed that SEs originated as nodular structures (pro-embryonic masses) 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 (Fig. 6A). 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 initial explant (Fig. 6B-E). Some torpedo stages were also observed (Fig. 6E). This is in conformity with the findings of Indieka et al. (2007) in *M. volkensii* and 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 (Fig. 6F). Leaf primordia were evident at the plumular poles of the mature embryos. The embryos lacked vascular connection to the initial explant but most of them had well-defined procambium strands extending from the apical meristem to the radicular pole (Fig. 6B,E).

The initiation of somatic embryos as green globular structures, their bipolar nature, lack of vascular connections to the initial explants, presence of procambial strands, and ease of separation of the embryos from the explant have been reported for for *Azadirachta indica* (Murthy and Saxena 1998) *Melia azedarach* (Vila et al. 2003) *Melia volkensii* (Indieka et al. 2007), coffee and grapevine (Quiroz-Figuera et al. 2006) as well as in rice (Vega et al. 2009). Some heterogeneity was observed in the size and morphology of somatic embryos in the present study, which calls for further study for characterization of the extent of heterogeneity and its implications on the conversion of embryos into plants. Similar heterogeneity of TDZ-induced somatic embryos was reported in *M. azedarach* by Vila et al. (2010).
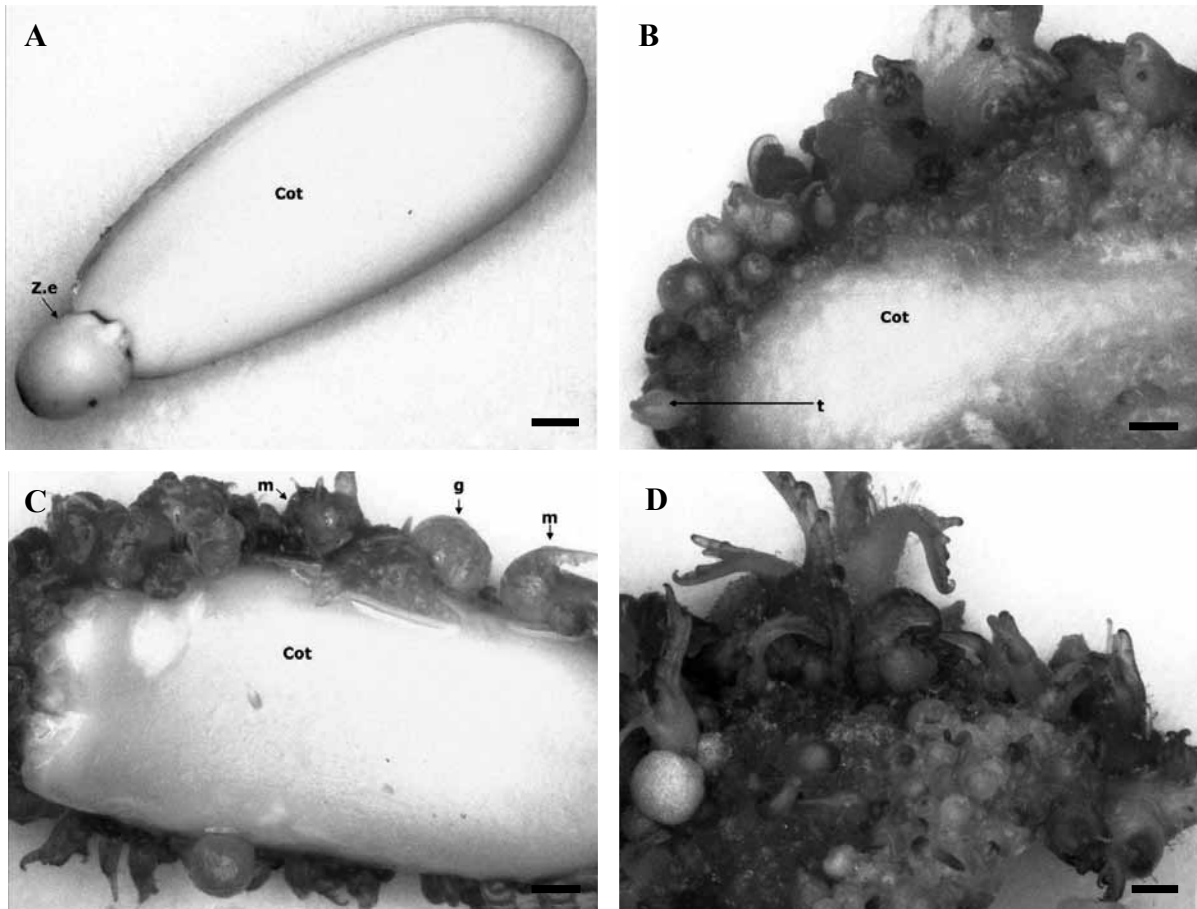
Fig. 1. A) Mature zygotic embryo (z.e) of *M. volkensii* attached to the cotyledon (cot); included to show the close similarity in the globular nature and size of the zygotic embryo to the somatic embryos is shown in Figs. 1 B-D. B to D) Cotyledon explants in various stages of somatic embryogenesis after 20 days of culture on half-strength 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 cotyledon was turned upside down to show the polarized nature of the regeneration response, which occurred only on the upper-facing, directly illuminated surfaces of explants. Scale bars = 1 mm.

Elongation of microshoots was achieved when whole or half segments of cotyledons having microshoots were subcultured on half-strength 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% CW. Medium containing BAP in combination with IAA caused the
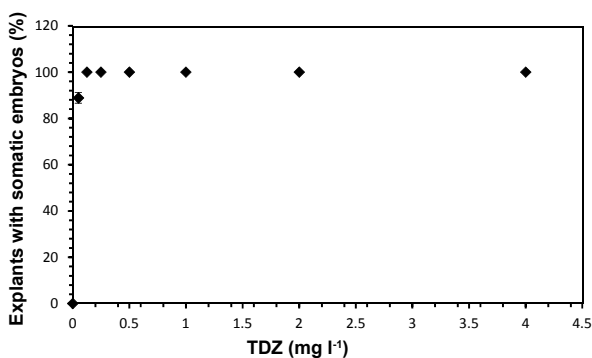

Fig. 2. Effect of TDZ on somatic embryo induction in cotyledon explants.
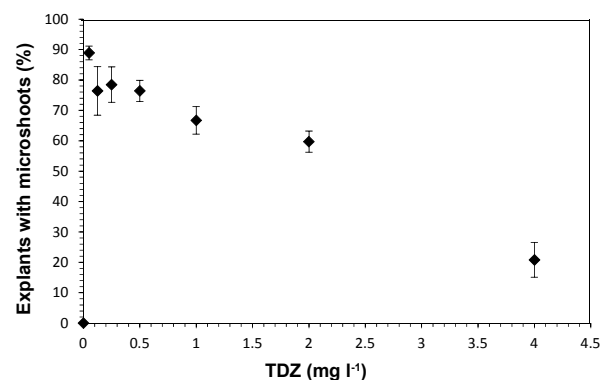$R^2 = 0.223$, $F_{regression} = 4.168$ (d.f. 2.29), $p = 0.026$.

Fig. 3. Effect of TDZ on conversion of somatic embryos to shoots in cotyledon explants.
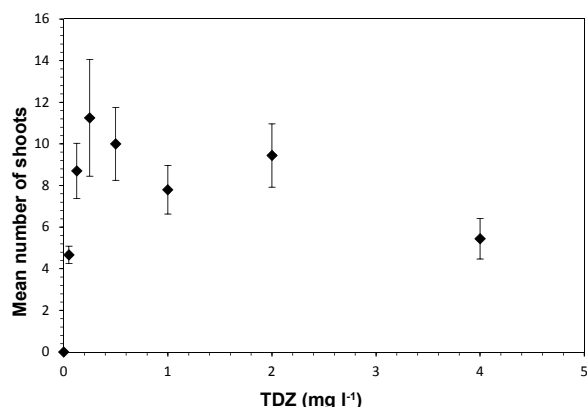$R^2 = 0.258$, $F_{regression} = 5.045$ (d.f 2.29), $p = 0.013$.

Fig. 4. Effect of TDZ on number of shoots per cotyle-don explants.
$R^2 = 0.129$, $F_{regression} = 5.496$ (d.f 2.29), $p = 0.006$.

Table 1. Effect of different PGR treatments on elongation of *M. volkensii* microshoots 20 days after transfer of cotyledon halves on elongation medium.

PGR combination in half-strength MS medium	Days on medium	Mean shoot length (± SE) (mm)
0 PGR	22	8.00 ± 1.08 a
0.1 mg l⁻¹ BAP	22	16.16 ± 1.94 b
0.1 mg l⁻¹ BAP + 10% CW	24	15.57 ± 1.85 b
0.1 mg l⁻¹ BAP + 0.01 mg l⁻¹ IAA	24	18.17 ± 1.58 b

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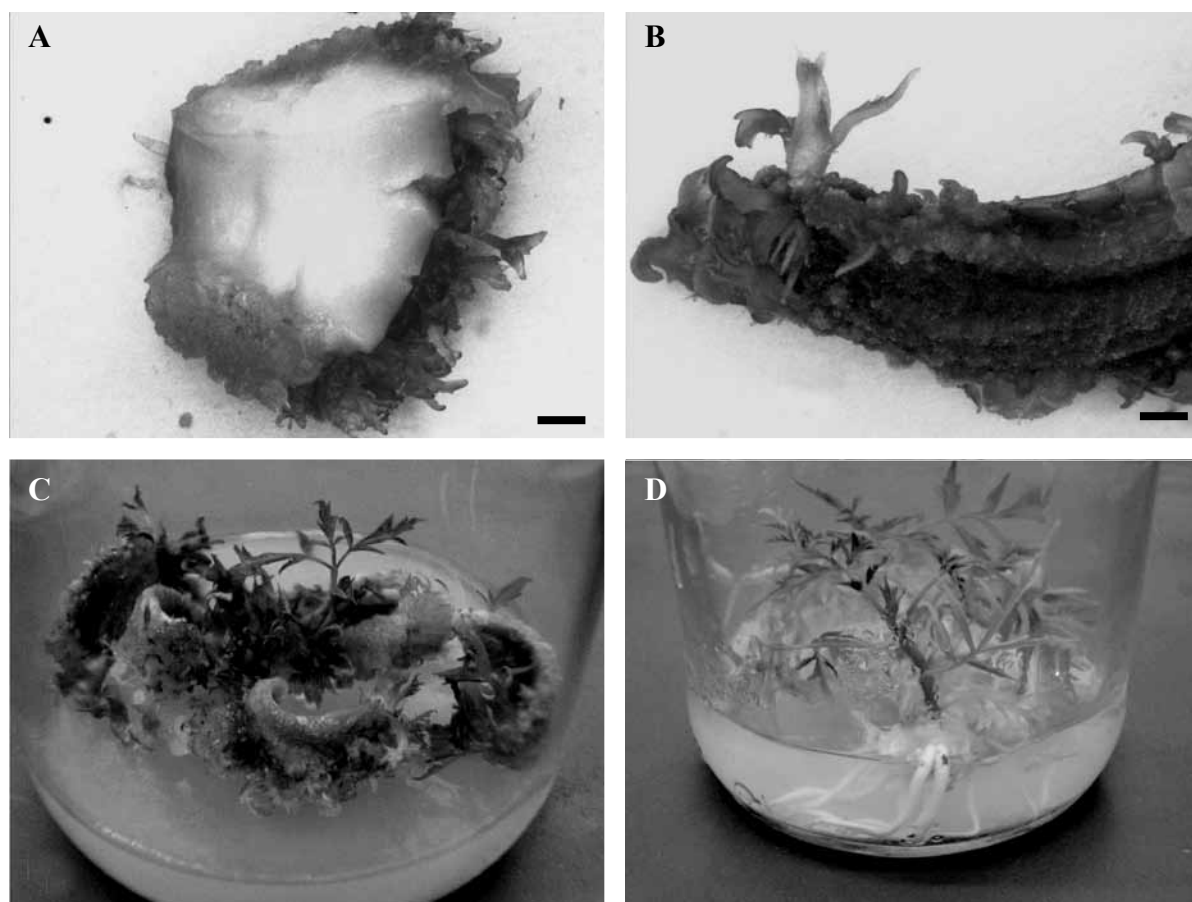

Fig. 5. A) Segment of cotyledon explant showing high frequency shoot regeneration after 16 days on half-strength MS + 0.5 mg l⁻¹ TDZ, B) Well-defined microshoot formed after 20 days on half-strength MS + 0.05 mg l⁻¹ TDZ (For A and B, scale bar = 1 mm), C) Macroscopic view of cotyledon explants showing shoot regeneration after 24 days on half-strength MS + 0.5 mg l⁻¹ TDZ induction medium, D) Rooting after 22 days on half-strength MS + 0.1 mg l⁻¹ NAA.

best elongation of the microshoots formed earlier on variants of induction medium, with no morphological abberations observed (Table 1). However, variants of the medium containing 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 percentage 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
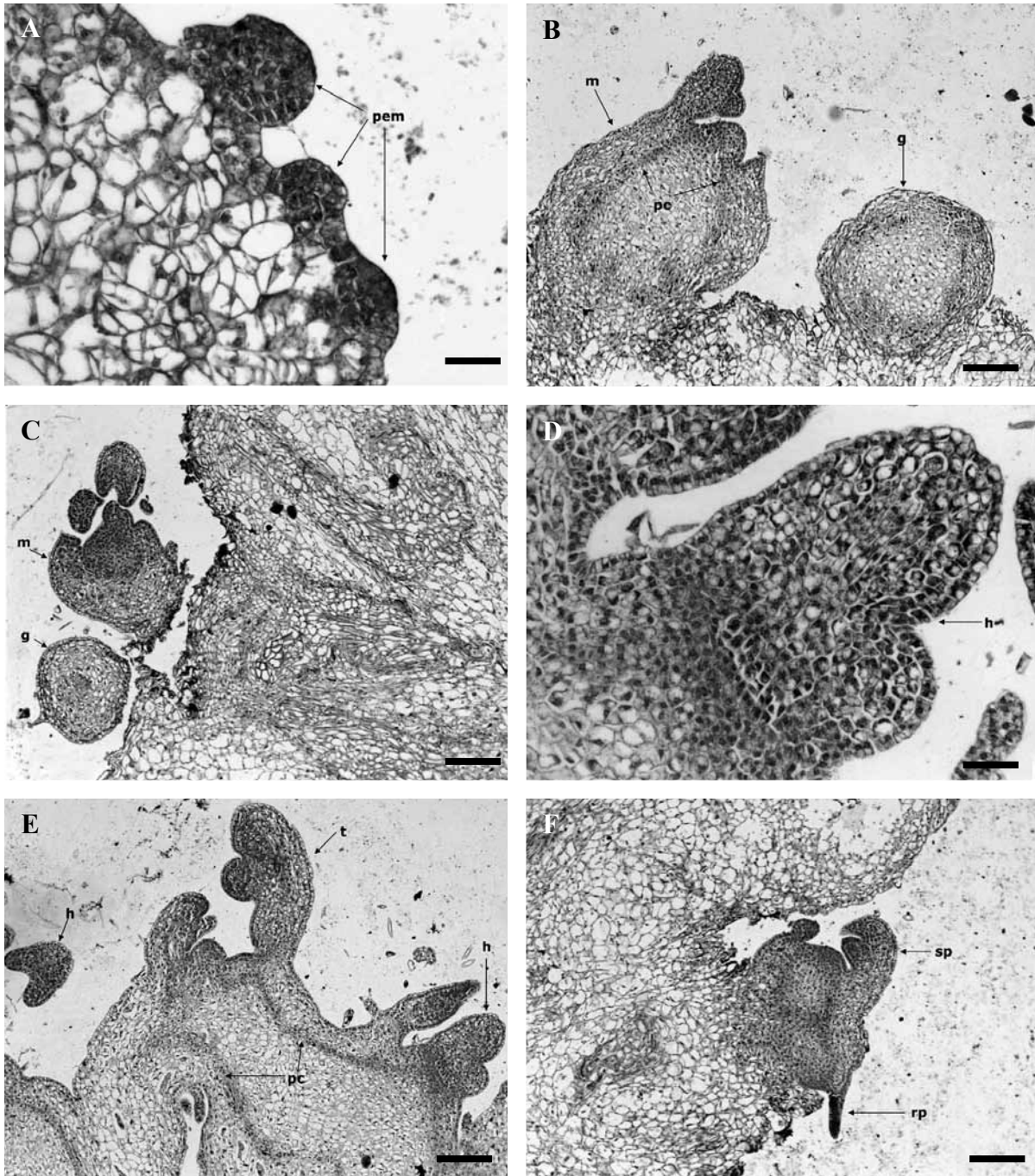
Fig. 6. A) Histology of cotyledon explants showing pro-embryonic masses (pem) arising from isodiametric, densely-staining epidermal and sub-epidermal cells, B-E) globular (g), heart-shaped (h) and torpedo (t) stages of somatic embryogenesis as well as mature somatic embryos (m). D, E) Mature somatic embryos showing well defined procambial strands (pc), F) Embryo showing clear polarity with a distinct shoot pole (sp) and root pole (rp). Scale bars = 30 µm (Fig. 6A and D) and 140µm (Fig. 6B,C,E,F).

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*. Significant rooting (F test, $p < 0.001$) was observed only on medium containing 0.2 mg l^{-1} NAA alone or 0.1 mg l^{-1} NAA in combination with 0.1 mg l^{-1} IBA (Table 2). NAA was superior to IBA in root induction. NAA-supplemented variants of the medium also caused variable extents of leaf chlorosis in the shoots (Fig. 5D). In general, IBA induced a single roots per shoot while NAA induced multiple roots (Table 2, Fig. 5D).

Table 2. Effect of NAA and IBA on rooting of shoots after 22 days on rooting medium.

PGR (mg l⁻¹) in half-strength MS medium	Days to start of rooting	Rooting (%) (Mean ± SE)	Number of roots (Mean ± SE)
0	12	5.71 ± 3.69 a	1.00 ± 0.00 a
0.05 NAA	-	0.00 ± 0.00 a	0.00 ± 0.00 a
0.1 NAA	8	8.88 ± 5.87 a	5.40 ± 1.29 b
0.2 NAA	8	32.40 ± 8.90 b	4.65 ± 0.67 b
0.05 IBA	-	0.00 ± 0.00 a	0.00 ± 0.00 a
0.1 IBA	10	6.67 ± 4.22 a	1.00 ± 0.00 a
0.2 IBA	-	0.00 ± 0.00 a	0.00 ± 0.00 a
0.1 NAA + 0.1 IBA	9	39.31 ± 6.14 b	5.64 ± 0.86 b

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

The percentage of rooting obtained in this study is similar to that reported by Indieka et al. (2007) for *M. volkensii*. Low rooting is the only remaining barrier to the utilization of tissue culture for mass propagation of the species. The high rate of regeneration attained in this study confirms our earlier report (Mulanda et al. 2012), based on mature zygotic embryos, of high potency and efficacy of Thidiazuron.

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