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Morphological and RAPD-marker characterization of *Melia volkensii* (Gürke) *in vitro* plants regenerated via direct and indirect somatic embryogenesis

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Somaclonal variation induced *in vitro* during tissue culture can be a problem in clonal micropropagation of elite plants. This study investigated the extent of morphological and genetic similarity or dissimilarity between *Melia volkensii in vitro* plants (somatic seedlings) obtained via somatic embryogenesis and normal seedlings. Comparisons were made between *in vitro* plants regenerated directly from cotyledon explants, indirectly from zygotic embryos and normal seedlings of the same parent trees. Regeneration was achieved using half MS medium supplemented with 0.05 mg/l thidiazuron. Shoots were elongated in half MS with 0.1 mg/l BAP plus 0.01 mg/l IAA then rooted in half MS with 0.1 mg/l IBA and 0.1 mg/l NAA. Six morphometric and five meristic characters were used for the morphological characterization. PCR-RAPD markers were used for assessment of genetic similarity or distance. Multivariate analysis using principal coordinates, cluster analysis, analysis of similarities (Anosim) and similarity percentages analysis (SIMPER) revealed significant dissimilarities ($p< 0.0001$) in morphometric and meristic characters between the *in vitro* plants and normal seedlings. However, significant similarity ($p<0.01$) was observed in the RAPD-genic characters of the *in vitro* plants and normal seedlings. Out of six morphometric traits, taproot length, internode length and shoot height were the most important sources of dissimilarity, cumulatively accounting for 72.37% of overall morphometric dissimilarity. Number of lateral roots was the single most important source of meristic dissimilarity, with 77.02% contribution. Plants regenerated directly from cotyledons were more similar to the normal seedlings in morphological and RAPD-marker characters than those regenerated indirectly from zygotic embryos. This study paves the way for identification of trait-specific RAPD markers for further characterization through sequence-characterized amplified regions (SCARs).

Key words: Morphometric, meristic, PCR-RAPD, *Melia volkensii*, tissue culture, somaclonal variation.

INTRODUCTION

Melia volkensii Gürke (Meliaceae) is a drought-tolerant, fast-growing, hardwood multipurpose tree species endemic to the arid and semi-arid lands of Ethiopia, Kenya, Somalia and Tanzania (Orwa et al., 2009). The species is highly

valued for its suitability for dry land agroforestry and as a source of prized mahogany timber, termite resistant poles, animal fodder, bee forage, mulch, analgesics and botanical pesticides (Kokwaro, 1993; Stewart and Blomley, 1994; Shaalan et al., 2005; Orwa et al., 2009). *M. volkensii* outperforms other tree species found in arid and semi arid parts of Kenya, and has a higher profitability than *Eucalyptus camaldulensis* and *Grevillea robusta* in these environments (Wekesa et al., 2012). It has vast potential for arid afforestation and commercial tree farming in the region but this is hampered by difficulties in propagation via seed and vegetative means. The amenability of the species to tissue culture, first demonstrated by Indieka et al. (2007), can be exploited for mass propagation. However, tissue culture processes often induce somaclonal variations which can be problematic and require detailed characterization before the technique can be adopted for mass propagation of any plant species.

The term somaclonal variation refers to all the stable genetic, epigenetic and phenotypic variations exhibited by micropropagated plants (Larkin and Scowcroft, 1981). Some of the factors responsible for somaclonal variation include plant growth regulators, physiological and biochemical stresses imposed by the *in vitro* culture conditions, type of explant and mode of regeneration (Neelakanda and Wang, 2012). Tissue culture-induced somaclonal variation can produce off-types which are undesirable when the expected outcome is mass production of uniform genotypes (Bairu and Kane, 2011). However, it can also be a source of useful novel variations (Larkin and Scowcroft, 1981; Tang, 2005).

Somaclonal variations of genetic nature may arise from chromosomal alterations, DNA sequence changes, transposition or amplification (Neelakanda and Wang, 2012). Epigenetic variations are due to changes in gene expression arising from DNA methylation, histone modifications and small RNA-mediated regulation (Miguel and Marum, 2011; Neelakanda and Wang, 2012). DNA sequence changes can be detected by a variety of molecular markers such as random amplified polymorphic DNA (RAPD) and microsatellites or simple sequence repeats (SSR) (Jin et al., 2008), polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) (Kour et al., 2009) and amplified fragment length polymorphism (AFLP) (Li et al., 2007).

Morphological variation is common in tissue cultured plants (Bairu et al., 2011). The extent of morphological similarity or dissimilarity between tissue cultured plantlets (somatic seedlings) and normal seedlings can be characterized using morphometric and meristic traits. Morphometrics is the quantification of variation in body form or morphology of a plant or animal using measurable characteristics (Bookstein, 1991). On the other hand, meristics is the quantification of variation in body form using counts of organs and other parts of the body (Lawing et al., 2008). Morphometric traits tend to show continuous variation and are easily influenced by the environment. Conversely, meristic traits are usually fixed early in development and are less influenced by the environment.

Recent applications of multivariate analysis on morphometric and meristic data in the study of morphological variation in plants include Valenzuela et al. (2011) and Plazas et al. (2014). Similar approaches have been used for other tissue cultured plants such as tomato (Pratta et al., 2000), soybean (Radhakrishnan and Kumari, 2008) banana (Sheidai et al., 2008) and poplar tree (Gamburg and Voinikov, 2013).

Random amplified polymorphic DNA (RAPD) (Williams et al., 1990) is a simple and quick DNA fingerprinting technique that uses a single decamer primer of arbitrary nucleotide sequence and so does not require prior knowledge about the genomic DNA being amplified. It is ideal for species with scanty elucidation of their genomic DNA, such as *M. volkensii*. The polymorphism observed in the RAPD markers is due to nucleotide sequence variations formed in the random primer binding regions of template DNA as a result of nucleotide insertion, deletion or substitution (McGregor et al., 2000). Plant species for which RAPD markers have been applied to study tissue culture-induced somaclonal variation or stability include *Cinnamomum* spp. (Soulange et al., 2007), *Mucuna* spp. (Sathyanarayana et al.. 2008), olive (Peyvandi et al., 2009), banana (Sheidai et al., 2010; Abdellatif et al., 2012).

At present, there are only two reports on the use of genetic markers to study genetic variability in natural populations of *M. volkensii* (Runo et al., 2004; Hanaoka et. al., 2012). Runo et al. (2004) used RAPD markers to compare the population genetic structure of *M. volkensii* populations in eastern and coastal regions of Kenya. Hanaoka et al. (2012) used microsatellite markers to study genetic variation in three natural populations in Kenya.

To date there are no reports on the characterization of the morphological or genetic variation in tissue cultured *M. volkensii* plants. The main objective of this study was to determine, for the first time, the extent of similarity or dissimilarity in morphological and RAPD-marker characteristics between Thidiazuron-regenerated *in vitro* plants and normal seedlings of the same parent trees. *In vitro* plants regenerated via direct and indirect somatic embryogenesis were assessed for morphological and RAPD-marker proximity to the normal seedling.

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Abbreviations: SCARs, Sequence-characterized amplified regions; **RAPD,** random amplified polymorphic DNA; **SSR,** simple sequence repeats; **PCR-RFLP,** polymerase chain reaction-restriction fragment length polymorphism; **AFLP,** amplified fragment length polymorphism; **PCOORDA,** principal coordinates analysis; **Anosim,** analysis of similarity; **SIMPER,** similarity percentage analysis.

Table 1. Morphometric and meristic characters used.

Character/unit	Description	Acronym
Morphometric measurements		
1. Shoot Height/mm	From shoot apex to base of the stem	SH
2. Internode length /mm	Mean length of first three internodes in lower half of the shoot	IL
3. Leaf Rachis Length /mm	Mean length of leaf rachis of three lowermost compound leaves	LR
4. Leaf mid-width/mm	Mean mid-leaf width of three lowermost compound leaves.	LD
5. Leaflet Length/mm	Mean length of leaflets of three lowermost leaves	LL
6. Length of Taproot /mm	From tip of taproot to base of stem	LTR
Meristic counts		
Character	**Description**	**Acronym**
1. Number of leaf nodes per shoot	Total number of leaf nodes present on the shoot	NL
2. Number of leaflets per rachis	Mean number of leaflets on each rachis, for three lowermost leaves	NLT
3. Number of lobes per leaflet	Mean number of leaf lobes or pinnules on leaflets of three lowermost leaves	NLB
4. Number of axillary bud sprouts	Total number of sprouted buds or lateral branches on the shoot	NAB
5. Number of lateral Roots	Total number of lateral roots arising from main root	NLR

MATERIALS AND METHODS

Plant materials

Seeds used as sources of explants for regeneration of *in vitro* plants and for germination into normal seedlings were obtained from the same cluster of five *M. volkensii* trees growing on a farm situated in Mavuria provenance in Mbeere, Embu county, eastern Kenya. The geo-reference coordinate of the seed collection site is 0° 46.379'S, 37° 39.308'E.

The two groups of *in vitro* plants used in the study were regenerated using the method described by Mulanda et al. (2014). One group was regenerated indirectly from calluses of mature zygotic embryos and the other directly from cotyledons. Induction medium was half Murashige and Skoog (1962) medium (MS) supplemented by 0.05 mg/l Thidiazuron (TDZ). Shoots were elongated in half MS with 0.1 mg/l 6-benzylaminopurine (BAP) and 0.01 mg/l indole-3-acetic acid (IAA), and rooted in half MS with 0.1 mg/l indole-3-butyric acid (IBA) plus 0.1 mg/l 1-naphthaleneacetic acid (NAA). Rooted plantlets with close phenotypic resemblance to normal seedlings were acclimatised and hardened for two weeks in the tissue culture lab in sterile vermiculite irrigated with half basal MS salts. They were then transferred to soil in pots, watered once with half basal MS and subsequently with sterile water. Hardening was attained through gradual and incremental opening of lids of culture bottles. The plants were maintained at 28±2°C, cool fluorescent daylight of 60 µmol photons m2 s-1 and 16 h photoperiod until they reached the 8 to 11 leaf stage.

Normal seedlings were obtained by germinating seeds under the same conditions. Initial germination of seeds was done in a substrate of sterile vermiculite and water. Prior to sowing, the seeds were scarified by nipping of the micropylar ends and slitting of the testa. This was followed by a 48 h pre-sowing soak in water at ambient temperature, with a change of the water after 24 h to get rid of phenolics. Two-leaf stage seedlings showing good vigour were transferred to soil and irrigated with sterile water till the 8 to 11 leaf stage. A total of 65 plants were used for the morphological characterisation, split as follows: 17 cotyledon regenerants, 18 zygotic embryo regenerants and 30 normal seedlings. A total of 130 plants were used for DNA extraction and RAPD analysis, split as follows: 20 Zygotic embryo non-clones, 20 cotyledon non-clones, 30 zygotic embryo clones, 30 cotyledon clones, and 30 normal seedlings. The disparity in number of plants used in morphological and RAPD characterization was caused by the smaller number of regenerants that rooted successfully and attained the 8 to 11 leaf stage set as prerequisite for the morphological study. Neither the rooting nor developmental stage constraints are applied to the RAPD study.

Morphometric and meristic characters used

Eleven morphological characters were used to characterize the extent of similarity or distance among and between the *in vitro* plants (regenerants) and normal seedlings. These characters were divided into two groups consisting of six morphometric measurements and five meristic counts (Table 1). Morphometric variables were measured to the nearest mm using a ruler. In order to minimize the effects of size-related differences and variations caused by the allometric nature of plant growth, the developmental stage of the somatic and normal seedlings was standardised at the 8 to 11 leaf-stage.

PCR-RAPD marker characterization

DNA extraction

The terminal portions of shoots of *in vitro* plants and normal seedlings were cut and 0.3 g fresh mass of the same used for DNA extraction. The tips were ground to a fine white powder in liquid nitrogen and 2 ml of extraction buffer added. Genomic DNA (g-DNA) was then extracted from the homogenates using the CTAB method of Doyle and Doyle (1987), with slight modifications. Extracted DNA was stored at -20°C in 40 µl of pH 8 Tris-EDTA (TE)

Table 2. Details of the decamer primers used for RAPD analysis.

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

buffer until required for gel runs or PCR. Presence of genomic DNA was confirmed by electrophoresis.

Genomic DNA confirmation and quantification

RNA was removed from the samples by adding 2 µl of RNAase cocktail (consisting of 500 units/ml of RNAase A and 20,000 Units/ml of RNAase T1 [Ambion®]) to 20 µl of nucleic acid-TE buffer mixture. Samples were incubated for 30 min at 37°C followed by 15 min at 65°C for inactivation of the RNAase. RNA removal was confirmed by electrophoresis. RNA-free DNA was quantified and assessed for purity using a Thermoscientific Nanodrop 2000 Spectrophotometer. Readings of DNA concentration in ng/µl, together with the A_{260}/ A_{280} and A_{260}/A_{230} absorbance ratios were obtained for assessment of purity as suggested by ThermoScientific (2014).

Screening of RAPD primers

Eight random decamer primers sourced from Inqaba Biotech Industries (Pty) Ltd., South Africa, were screened for ability to amplify 10 samples of *M. volkensii* genomic DNA (5 g-DNA samples from wild type seedlings and 5 from tissue-cultured plants). The oligonucleotide sequences of seven of the primers (Mel-1 to Mel-7) were selected from lists of primers used in other RAPD-marker studies done on tree species belonging to three genera of the meliaceae family; *Azadirachta, Lansium* and *Xylocarpus* (Song et al., 2000; daSilva et al., 2013; Pawar et al., 2013). The eighth primer (Mel-8) is an arbitrary sequence chosen by the present authors. This approach was used because the only previous report of RAPD-markers in *M. volkensii* (Runo et al., 2004) did not reveal sequences of the primers used. Table 2 gives details of the primer sequences used in the present study and the related species where they were used.

PCR-RAPD amplification

The composition of the PCR reaction mixture and thermal cycler conditions were optimized by varying the type of Taq polymerase (Dream Taq® or Taq polymerase® from ThermoScientific, and MyTaq® from Bioline), concentration of Taq polymerase (0.75, 1.0, 1.25 and 2.5 units per 25 µl reaction), strength of PCR buffer (x1 or x1.5), concentration of primer (25 or 50 pmoles per 25µl reaction), annealing temperature (34, 35, 36, 37 and 38°C) and extension time (1 or 2 min). The optimum amplification reaction mixture per 25 µl reaction consisted of 5 µl of 5x Bioline MyTaq® reaction buffer, 0.5 µl Bioline MyTaq® DNA polymerase, 5 µl of 10 mM primer, 3.0 µl of g-DNA in TE buffer (after diluting the g-DNA-TE mixtures to half-strength using equal volume of PCR water) and 11.5 µl of nuclease-free PCR water (Sigma-Aldrich). Final concentrations of components in each 25 µl reaction were x1 MyTaq® buffer containing 1 mM dNTPs and 3 mM $MgCl_2$, 50 pmoles of primer, 2.5 units of MyTaq® DNA polymerase and 20 to 25 ng of gDNA. The polymerase chain reaction was carried out in a PTC-100 thermal cycler (Programmable Thermal Controller- MJ Research Inc., USA). Optimum PCR was attained with 5 min initial denaturation at 95°C, followed by 35 cycles consisting of 30 s denaturation at 94°C, 45 s annealing at 35°C, 60 s extension at 72°C and a single final extension of 5 min at 72°C.

Electrophoresis and visualization of gels

All electrophoresis runs were done for 45 min at 80 V (5.5 volts/cm) in a Pharmacia Biotech GNA horizontal tank. The g-DNA samples were run in 0.8% (m/v) agarose gel whilst PCR products were run in 1.0% (m/v) agarose gel, both in x1 TAE buffer. Gels contained 3 µl of 10 mM ethidium bromide as visualization stain. Wells were loaded with 7 µl DNA sample or PCR product and 3 µl of x6 bromophenol blue loading dye. A 1 kb ladder (Bioline® Hyperladder), with size markers from 200 to 10,037 base pairs, was used as molecular marker. The gels were visualized at 302 nm using a 2 UV Transilluminator and photographed using the integrated Multi Doc-it Digital Imaging System.

Data analysis

Morphometric and meristic data analysis

Morphological and meristic data were analyzed using the PAleontological STatistics (PAST) software version 2.17c (Hammer et al., 2001). Morphometric data was subjected to logarithmic transformation to minimise size-related differences, as suggested by Palmer and Strobeck (2003). No such transformation was performed for meristic data as meristic characters are usually independent of size of the organism, and tend to be fixed in the early stages of growth. The data were subjected to multivariate analysis as detailed below.

RAPD data scoring and analysis

Only clearly resolved bands were scored. The RAPD bands/markers were scored for each lane using 1 for presence and

Table 3. Variation in morphological characters (Mean ± standard error).

Population	SH	IL	LR	LL	LD	LTP
Morphometric measurements (mm)						
ZE regenerants	43.83±4.88	4.68±0.76	15.28±1.16	30.44±1.92	21.18±1.66	8.33±8.33
Cotyledon regenerants	51.00±3.58	5.54±0.56	14.04±1.85	30.65±1.96	22.60±1.15	27.41±8.17
Wild type	187.81±8.07	28.06±2.68	31.78±1.43	72.50±2.28	63.79±2.55	131.2±3.59
Meristic counts						
Population	**NL**	**NLT**	**NLB**	**NAB**	**NLR**	
ZE regenerants	10.06 ± 0.30	6.20 ± 0.33	7.01 ± 0.24	0.06 ± 0.06	0.00 ± 0.00	
Cotyledon regenerants	9.88 ± 0.29	5.77 ± 0.27	6.47 ± 0.21	0.06 ± 0.06	2.88 ± 1.04	
Wild type	8.41 ± 0.13	5.14 ± 0.18	7.64 ± 0.18	0.21 ± 0.12	24.11 ± 1.34	

SH= Shoot length; IL= Internode length; LR= Leaf rachis length; LD= Mid-of-leaf width; LL= Leaflet length; LTP= Length of taproot. NL = Number of leaf nodes per shoot; NLT= Number of leaflets per compound leaf; NLB= Number of lobes/pinnules per leaf; NAB= Number of axillary bud sprouts per shoot; NLR= Number of lateral roots per root.

0 for absence of a band. The resultant binary matrix of 1 and 0 scores was used for analysis. The matrix data was analyzed using the Popgene Population Genetic Analysis software Version 1.32 (Yeh et al., 2000). Six summary genic variation statistics were obtained; observed number of alleles (N_a), effective number of alleles (N_e), Nei's (1973) gene diversity index (H), Shannon's information index (I), number of polymorphic loci (N_p) and percentage of polymorphic loci (P_p). Also generated was a matrix of Nei's (1978) unbiased genetic similarity and genetic distance values between the groups.

Multivariate analyses

The log-transformed morphometric data, raw meristic data and the Popgene-generated genic variation values were subjected to the following multivariate analyses in PAST version 2.17c: Principal Coordinates analysis (PCOORDA) and Cluster analysis, one-way Analysis of Similarity (Anosim) and Similarity Percentage analysis (SIMPER) (Hammer et al., 2001). Principal axes values and Eigenvalues obtained were used to plot PCOORDA scatterplots and 95% confidence ellipses using the Gower measure of distance. This measure of distance is applicable to mixed type data (continuous and ordinal) (Hammer et al., 2001). Clustering was performed using the paired-group algorithm and Gower measure of distance, with 1000 bootstrap replications. Anosim and SIMPER were done using Bray-Curtis measure of similarity, which is the default measure. Pair-wise percentages of dissimilarity between groups were computed using Anosim, with 10,000 permutations. The Anosim test statistic R measures the overall dissimilarity between groups. R values closer to zero indicate no dissimilarity (significant similarity), those closer to 1 indicate significant dissimilarity between the groups. Individual and cumulative contributions of the characters to the overall average percentage dissimilarity were obtained using SIMPER.

RESULTS AND DISCUSSION

Morphological variation

Significant morphological variation was observed between the regenerants and wild seedlings in morphometric and meristic traits. In the morphometric characters, the regenerants differed significantly from wild type seedlings in all the six traits used (Table 3). Significant stunting of shoots occurred in both zygotic embryo (indirect) regenerants and cotyledon (direct) regenerants. As shown in Table 3, mean shoot heights of the two types of regenerants (43.83 and 51.00 mm) were one-quarter and one-third, respectively, of the mean shoot height of wild type seedlings (187.81 mm). The respective mean internode lengths (4.68 and 5.54 mm) of the regenerants were one-sixth and one-third of the internode length of wild type seedlings (28.06 mm) (Table 3). The leaves of the two types of regenerants also had considerably shorter rachis (14.04 and 15.28 mm), leaf lengths (30.44 and 30.65 mm) and mid-leaf widths (21.18 and 22.60 mm), in comparison to the respective mean rachis length, leaf length and mid-leaf width (31.48, 72.50 and 63.79 mm) of the normal seedlings. However, the greatest difference in morphometric traits was seen in the mean length of taproots, with taproot length of wild seedlings (131.20 mm) being on average nearly 16 times longer than that of zygotic embryo regenerants (8.33 mm) and nearly 5 times longer than that of cotyledon regenerants (27.41 mm). These results are not unusual as similar stunting of shoots regenerated using TDZ has been reported in grapes (Gray and Benton, 1991) and in tree species such as *Cassia* (Parveen and Shahzad, 2010), *Myrica rubra* (Asghari et al., 2013) and *Calophyllum inophyllum* (Thengane et al., 2006). TDZ use has also been associated with failure of regenerated shoots to root well and general inhibition of rooting (Gray and Benton, 1991; Lu, 1993; Ranyaphia et al., 2011).

As for meristic characters, there was less morphological difference between the regenerants and wild type seedlings (Table 3). Out of the five meristic traits used, the mean numbers of four of them namely number of leaves per shoot, number of leaflets per rachis, number of leaf pinnules per leaflet and number of axillary bud

Table 4. Concentration and purity ratios of extracted DNA

Group	DNA concentration (ng/µl)	A260/A280 ratio	A260/A230 ratio
Cotyledon regenerants	915.40±86.59	1.81±0.04	1.33±0.28
Zygotic embryo regenerants	379.70±104.04	1.60±0.02	0.68±0.03
Wild type seedlings	1528.32±148.60	1.83±0.03	1.69±0.08

Values are means ± standard error.

Table 5. Differences in genic variation revealed by candidate primers with 10 samples of g-DNA.

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

Number of distinct RAPD band types/markers = 30. N_a= observed number of alleles; N_e=Effective number of alleles; H=Nei's gene diversity index; I=Shannon's information index; N_p= Number of polymorphic alleles; P_p= Percentage polymorphism (Means ± standard deviation).

sprouts were generally similar between regenerants and wild seedlings. Only one meristic trait, mean number of lateral roots per plant, was different with the zygotic embryo regenerants having complete absence of lateral roots, cotyledon regenerants having a mean of 2.88 lateral roots per plant and the wild seedlings having a mean of 27.41 lateral roots (Table 3). In general, there was significant inhibition of lateral root formation in the regenerants. Overall, inhibition of rooting was greater in *in vitro* plants regenerated indirectly from zygotic embryos than those regenerated directly from cotyledon explants, suggesting a possible role of the mode of regeneration in determination of rooting response. The poor root meristics of both types of regenerants can also be attributed to the reported inability of plants regenerated by TDZ to root well (Gray and Benton, 1991; Lu, 1993; Ranyaphia et al., 2011).

RAPD-produced genic variation characters

Both the yield and purity levels of the extracted DNA were good (Table 4). The A_{260}/A_{280} ratios for the g-DNA of cotyledon regenerants (1.81) and that of wild type seedlings (1.83) were close to the ideal ratio of 1.80 expected for pure DNA as suggested by ThermoScientific (2014). However, the A_{260}/A_{280} ratio for the zygotic embryo regenerants (1.60) was lower than ideal indicating some contamination with proteins, whose peak absorbance is usually at 280 nm. A_{260}/A_{230} ratios obtained for the three groups of plants ranged from 0.68 to 1.69 and were much lower than the ideal ratio of 2.0 to 2.2 suggested by ThermoScientific (2014). Low A_{260}/A_{230} ratios are often encountered in plants due to a considerable carrying-over of carbohydrates during extraction. This indicates that the ratio of CTAB to sodium chloride used in this study may have failed to precipitate all the carbohydrates.

All the eight primers tested amplified *M. volkensii* g-DNA and generated a grand total of 371 monomorphic and polymorphic RAPD bands/markers giving a mean tally of 46.38 bands per primer for the 10 g-DNA sample tested. Overall amplification frequency for the total of 80 PCR-RAPD primer-screening reactions carried out was 69 out of 80 (86.25%) (Table 5). The mean number of bands/loci per lane was 5.03±1.39. Mel-5 primer, with an oligonucleotide sequence of 5' TGC CGA GCT G 3', produced the highest number of highly resolved bands (9) in 50% of the amplified samples (Figure 1), the largest mean number of bands/loci per lane (7.11±2.52) and the second highest overall percentage of polymorphic loci (23.33) (Table 5). Consequently, Mel-5 was selected as the best primer among those tested for amplification of g-DNA of the regenerants and wild seedlings. Altogether, the eight screened primers produced a total of 30 polymorphic RAPD bands/markers from the 69 amplified DNA samples. This is consistent with the findings of Runo et al. (2004), where eight random primers generated 38 scorable polymorphic bands from 90 PCR-RAPD reactions using DNA obtained from *in situ* populations of *M. volkensii.* However, a detailed comparison of primer performance between that study and the present one was not possible as Runo et al. (2004) did not reveal

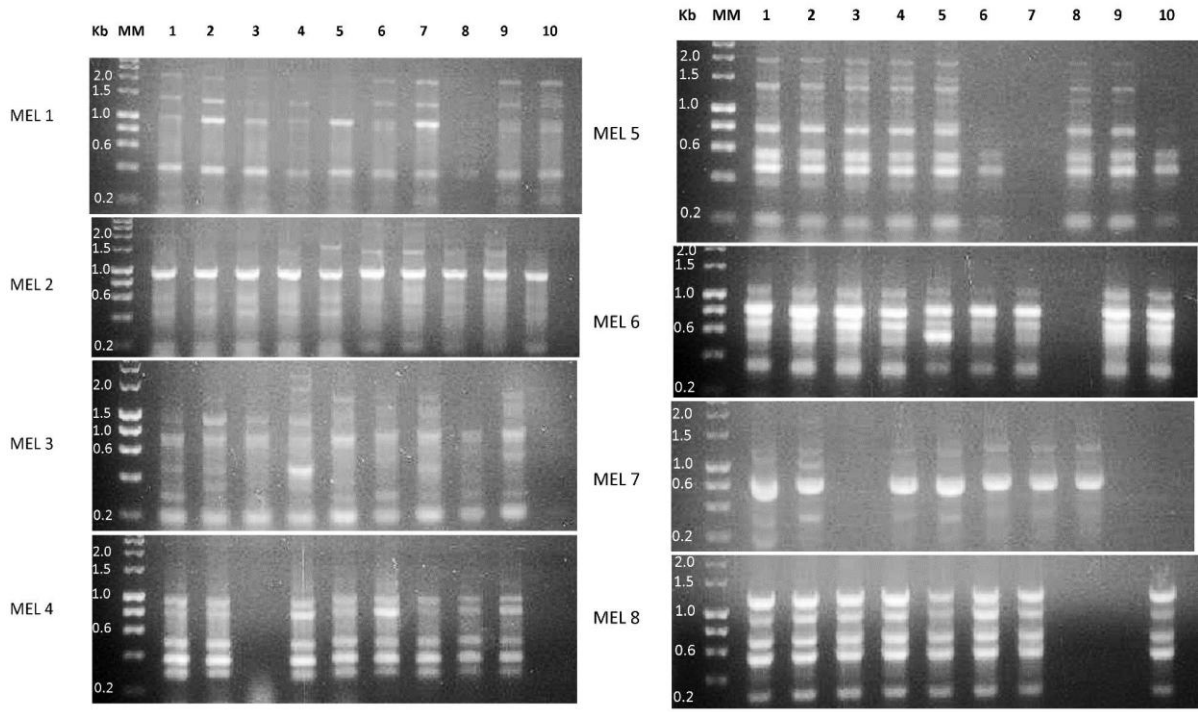

Figure 1. PCR-RAPD profiles of the eight candidate primers showing amplified and non-amplified lanes in 10 samples of g-DNA.

oligonucleotide sequences of primers used.

Amplification frequency for the selected primer (Mel-5) was 107 out a combined total of 130 PCR reactions (82.31%) for the regenerants and wild seedlings. From the 107 reactions, this primer generated a grand total of 759 monomorphic and polymorphic RAPD bands, giving a mean of 7.09 bands per lane. There were 19 distinct band sizes or loci ranging from 200 to 2000 base pairs with 17 of them being polymorphic. Two out of six genic characters namely observed number of alleles (N_a) and effective number of alleles (N_e), showed little difference between the regenerants and wild seedlings (Table 6). However, the range of Nei's gene diversity index (H) and Shannon's information index (I) was considerably greater in the regenerants (H= 0.09 – 0.18; I= 0.13 – 0.28) than the values obtained for wild type seedlings (H= 0.02; I= 0.03). In addtition, these two measures of diversity (H and I) were generally higher in the non-clone regenerants than in the cloned ones. Nei's gene diversity index was 9 to 9.5 and 4.5 to 6.5 times greater, respectively, in non-cloned and cloned regenerants than in wild type seedlings (Table 6). Shannon's information index was 9.33 and 4.36 times greater, respectively, in non-cloned and cloned regenerants than in wild type seedlings. The number of polymorphic loci and percentage of polymorphic loci each were 9 to 11 and 4 to 5 times greater, respect-tively, in non-cloned and cloned regenerants than in wild type seedlings (Table 6).

Nei's genetic variation index (H) describes variation within and between populations whereas Shannon's index (I) mainly describes variation within populations (Hennink and Zeven, 1991). A value of 0 occurs when populations are genetically identical whereas the value of 1 is for different species. The closeness to 0 obtained for both indices in the present study suggests a generally low level of genetic diversity within and between the regenerants and wild seedlings. However, since the regenerants gave higher values of Nei's gene diversity and Shannon's information indices than wild type seedlings, this indicates that the two regeneration systems used in the present study produced some genetic variations. The occurrence of such genetic variation during tissue culture is reported in other plant species (Larkin and Scowcroft, 1981; Neelakanda and Wang, 2012)

The values of Nei's gene diversity (H) index obtained for the regenerants in this study (0.09 to 0.19) compare favourably with those obtained by Runo et al. (2004) (c.a. 0.06 to 0.12) for widely dispersed natural populations of *M. volkensii* in Kenya. This indicates that genetic variability attributable to the regeneration systems used in this study may not significantly deviate from that which is found within and between natural populations of the species. The very low value of Nei's gene diversity index (0.02) seen in the wild type seedlings (Table 6) could be attributed to the fact that seeds used in raising the wild type seedlings for the RAPD analysis were from a cluster of parent trees standing on the same farm, which are probably closely related.

Table 6. RAPD-based genic characteristics obtained with selected primer for regenerants and wild seedlings

Population	Amplified/total DNA samples	N_a	N_e	H	I	N_p	P_p (%)
ZE non-clones	19/20	1.58±0.51	1.30±0.37	0.18±0.19	0.28±0.28	11	57.89
Cotyledon non-clones	17/20	1.47±0.51	1.33±0.41	0.19±0.22	0.27±0.31	9	47.37
ZE clones	22/30	1.26±0.45	1.25±0.42	0.13±0.22	0.18±0.31	5	26.32
Cotyledon clones	21/30	1.21±0.42	1.16±0.33	0.09±0.18	0.13±0.26	4	21.05
Wild type	28/30	1.05±0.23	1.03±0.12	0.02±0.07	0.03±0.12	1	5.26
Overall	107/130	1.90±0.03	1.45±0.04	0.26±0.02	0.39±0.03	30	31.58

Number of distinct RAPD bands/loci=19. ZE= Zygotic embryo. N_a= observed number of alleles; N_e=Effective number of alleles; H=Nei's gene diversity index; I=Shannon's information index; N_p= Number of polymorphic alleles; P_p= Percentage polymorphism (Means ± standard deviation).

Principal coordinates (PCOORDA) analysis

Principal Coordinate Analysis (PCOORDA) for the morphological characters revealed a clear separation of the regenerants from the wild type (Figure 2a, b). The 95% confidence ellipses showed a greater difference between the regenerants and wild type seedlings in morphometric traits than in meristic traits. This was supported by the variance contributions of the principal axes and corresponding Eigenvalues, which explained 81.87% of the observed differences in morphometric traits but only explained 48.42% of the differences seen in meristic traits. For both types of morphological characters, the largest separation from the wild type was seen in regenerants obtained from zygotic embryos via callus-mediated (indirect) somatic embryogenesis. Plants regenerated from cotyledons via direct somatic embryogenesis were morphologically closer to the wild type as shown by larger overlap of ellipses. The PCOORDA plot for RAPD-based genic variation characters showed little or no genetic differentiation between the regenerants and the wild plants. There were very large overlaps of the 95% confidence ellipses of the regenerants and wild type seedlings (Figure 2c). Lack of clear separation of the regenerants and normal seedlings based on RAPD-genic variation characters could suggest that the morphological differences observed in this study may be epigenetic or physiological as suggested by Miguel and Marum (2011) and Bairu and Kane (2011).

Cluster analysis

Cluster analysis also showed a clear separation of the regenerants from wild type seedlings based on morphological characters but no significant separation in genic characters (Figures 3a, b). When clustered based on morphometric traits, there were two distinct clusters. Wild type seedlings formed the first distinct cluster with 84% bootstrap reliability. The second cluster grouped together the regenerants with a lower bootstrap value of 48%. There was a high level of within-cluster overlap among and between the two types of regenerants, showing high similarity and little differentiation of the regenerants in morphometric traits. Clustering using meristic traits also produced two significant clades, a smaller clade with 58% bootstrap reliability consisting of only three wild type seedlings and a much larger clade with 53% bootstrap reliability, in which most of the wild type seedlings and the two types of regenerants were clustered (Figure 3b). Within-cluster overlap was greater in meristic than morphometric traits for the wild type and the two types of regenerants. This indicates that wild type seedlings and regenerants had a higher level of within-group similarity in meristic traits than in morphometric characters.

Clustering based on genic variation characters revealed complete lack of genetic distance in 66 out of the total of 96 (68.75%) plants tested and presence of some genetic differentiation in the remainder (31.25%) (Figure 4). The no-distance clade consisted of 94.7% of all the wild type DNA samples tested, 71.4% of the entire DNA from cotyledon clones, 63.6% of the entire DNA from zygotic embryo clones, 52.6% of DNA from cotyledon non-clones and 42.9% of DNA from zygotic embryo non-clones. Consequently, based on this clustering, the largest genetic differentiation between regenerants and wild type seedlings occurred in zygotic embryo non-clones at 57.1% followed by cotyledon non-clones at 47.4%. Zygotic embryo clones had 36.4% genetic differentiation from wild type whilst the value for cotyledon clones was 28.4%, making cotyledon clones to be the closest in genetic similarity to the wild type seedlings.

The use of cluster analysis to complement either principal coordinate or principal component analyses of morphological or genetic traits in plants is well documented (Pratta et al., 2000; Song et al., 2000; Sheidai et al., 2008, 2010; Valenzuela et al., 2011; Abdellatif et al., 2012; Gamburg and Voinikov, 2013; Plazas et al., 2014). In the present study cluster analysis validated the PCOORDA plots. Regenerants were morphologically separate from, but genetically similar to, the normal seedlings. This seems to support the view that

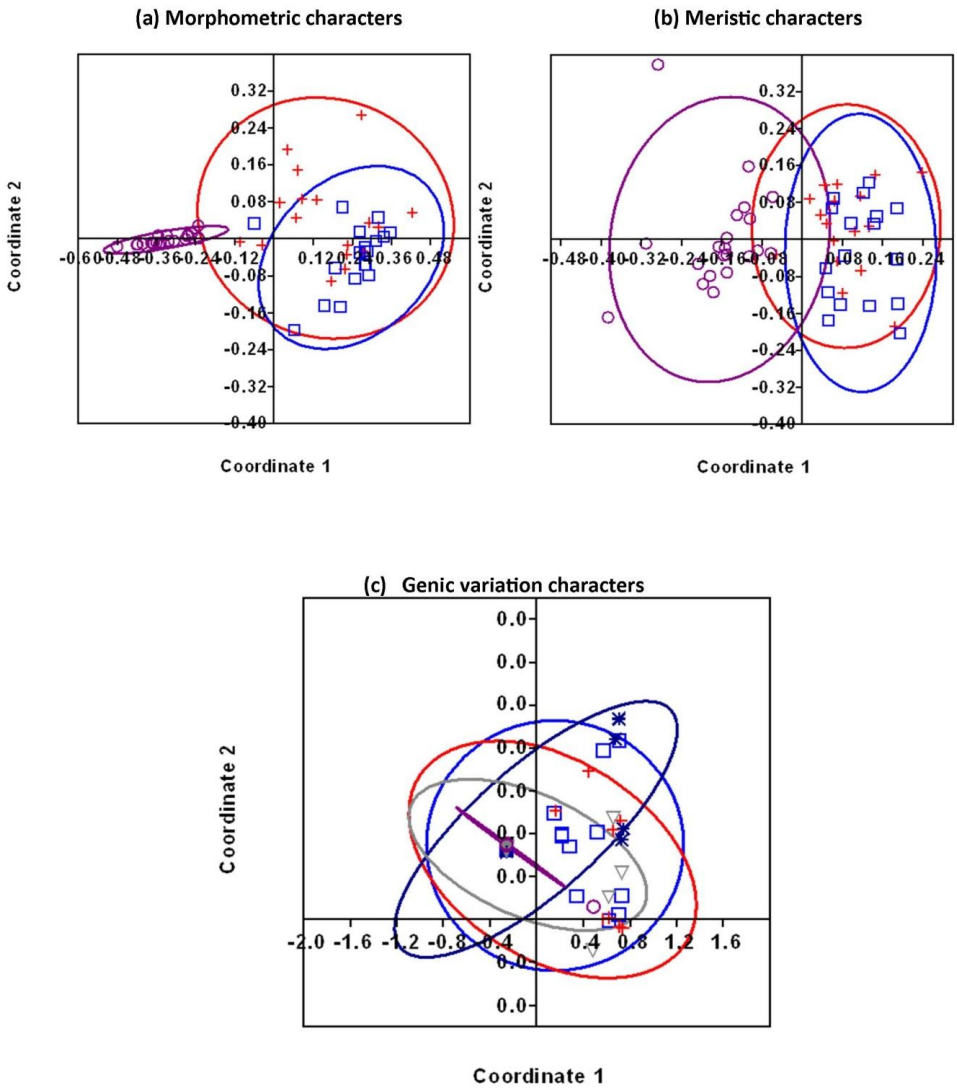

Figure 2. PCOORDA scatter plots and 95% confidence ellipses. **(a) and (b)** Zygotic embryo regenerants (□), cotyledon regenerants (+), wild type (○). **(c).** zygotic embryo non-clones (□), zygotic embryo clones (•), cotyledon non-clones (Δ), wild type (○).

morphological differences observed in this study may have arisen from epigenetic or physiological factors as described by Miguel and Marum (2011) and Bairu and Kane (2011).

Analysis of similarity (Anosim) and similarity percentages (SIMPER)

Anosim results further validated the PCOORDA and cluster analysis results. A similar complementary role of the Anosim test to principal coordinate analysis is reported by Abouzied et al. (2013). Morphometric and meristic traits gave Anosim R-values of 0.778 ($p<0.0001$) and 0.688 ($p<0.0001$), respectively, indicating significant morphological dissimilarity (or distance) between the regenerants and wild seedlings (Table 7). However, RAPD-based genic variation characters gave an Anosim R-value of 0.047, showing no significant genetic dissimilarity (or significant similarity) between the regenerants and wild seedlings. The SIMPER analysis carried out for a pair-wise comparison of dissimilarities between groups revealed that morphological dissimilarities were generally greater between wild seedlings and regenerants than between the two types of regenerants themselves (Table 8).

Morphological dissimilarities were greatest between wild type seedlings and zygotic embryo (indirect) regenerants, at 27.10% for morphometric and 41.47% for meristic traits respectively. Dissimilarity percentages for genic variation characters averaged around 20% between wild type seedlings and non-clone regenerants on

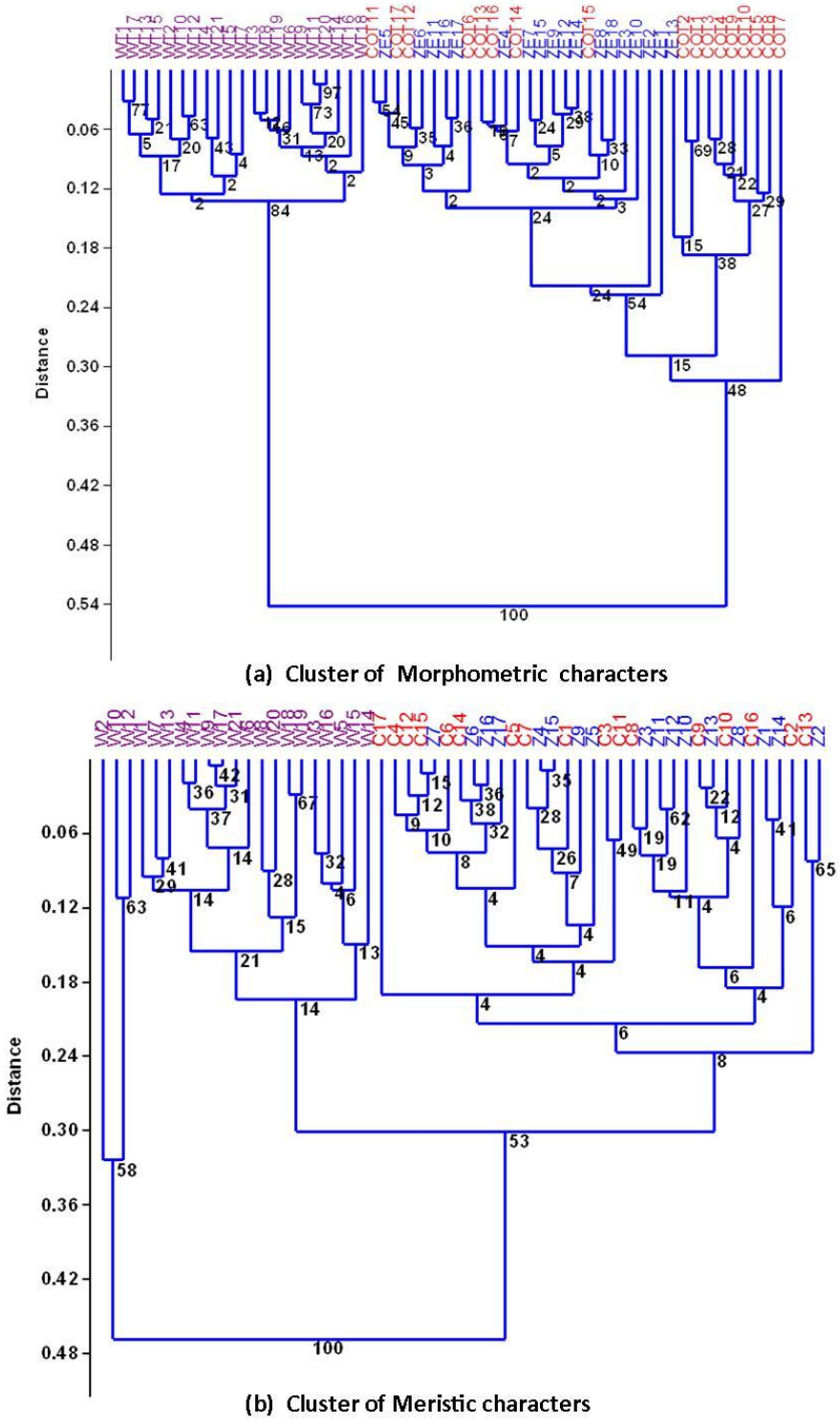

Figure 3. Paired-group clusters for (a) morphological (b) and meristic characters. Node values are bootstrap percentages for 1000 replications. W/WT= wild type seedlings; Cot/ C= cotyledon regenerants; ZE/Z= zygotic embryo regenerants.

one hand and 9.82 to 15.61% between wild type seedlings and cloned regenerants on the other hand. This indicates a high level of genetic similarity (84.39 to 90.18%) between the cloned *in vitro* plants and normal seedlings of the parent trees (Table 8) and less similarity between none-clones and normal seedlings. Out of the

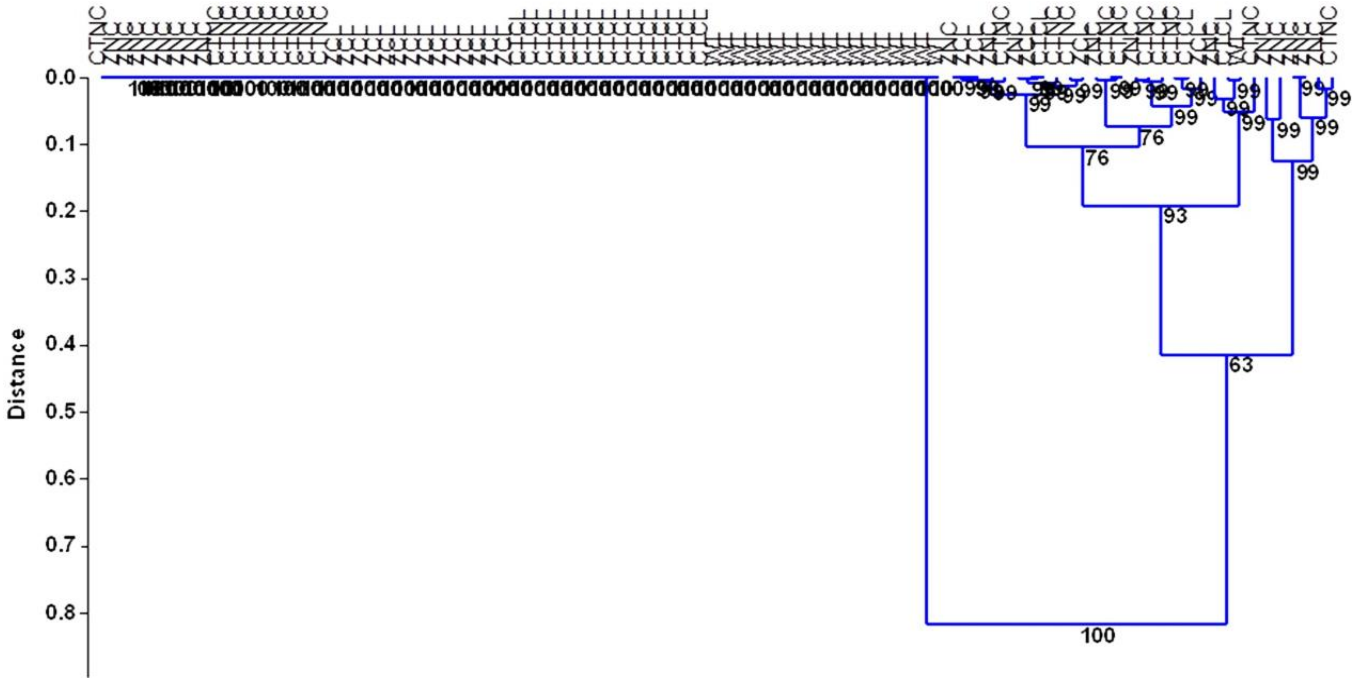

Figure 4. Paired-group clusters for genic characters. Node values are bootstrap percentages for 1000 replications. (CTNC= cotyledon non-clones, CTCL=cotyledon clones, ZNC= zygotic embryo non-clones, ZCL= zygotic embryo clones, WT= wild type seedlings).

Table 7. One-way Anosim results for morphological and RAPD-marker data.

Parameter	Morphometric characters	Meristic characters	RAPD-marker genic variation characters
Mean Rank within	365.8	397.8	2147
Mean Rank between	964.5	908.9	2253
R value	0.778	0.688	0.047
p(same)	0.000099	0.000099	0.0077
Inference	Significant dissimilarity between groups	Significant dissimilarity between groups	Significant similarity between groups

overall average morphometric dissimilarity observed, length of taproot alone accounted for 41.52% (Table 9). This was followed by internode length (17.25%) and shoot height (13.60%). These three traits accounted for 72.37% of the overall dissimilarity in morphometric characters. As for the meristic traits, the mean number of lateral roots was the single most important source of dissimilarity, accounting for 77% of the dissimilarity, followed by the mean number of leaves and leaflets at 7.82 and 7.17%, respectively. These three traits accounted for 91.99% of the overall dissimilarity in meristic characters. Observed number of alleles was the single most important source of dissimilarity among the genic variation characters, accounting for 37.65% of the dissimilarity. This was followed by effective number of alleles and Shannon's information index at 26.22 and 21.45%, respectively. These three variables accounted for 85.32% of the overall dissimilarity in genic characters. On its own, Nei's gene diversity index accounted for only 14.68% of the overall average dissimilarity. This shows that based on Nei's gene diversity index alone, the genetic similarity between the regenerants and wild type seedlings can be estimated at 85.32%.

Conclusions and recommendations

In vitro plants of *M. volkensii* regenerated via both direct and indirect TDZ-induced somatic embryogenesis differed significantly from seedlings of the same mother trees in morphological characters. However, RAPD markers did not reveal significant genetic difference. The regeneration systems used in the study can therefore be considered to be capable of maintaining high genetic fidelity and proximity

Table 8. Pair-wise SIMPER dissimilarity percentages for morphological and genic characters

Morphometric characters (%)			
	Cotyledon regenerants	**ZE regenerants**	**Wild type**
Cotyledon regenerants	0		
ZE regenerants	12.92	0	
Wild type	20.92	27.1	0

Meristic characters (%)			
	Cotyledon regenerants	**ZE regenerants**	**Wild type**
Cotyledon regenerants	0		
ZE regenerants	13.44	0	
Wild type	36.15	41.47	0

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

Table 9. SIMPER ranking of individual and cumulative contributions of characters to overall average dissimilarity between the groups.

Character	**Average dissimilarity**	**Contribution %**	**Cumulative contribution %**
Morphometric characters			
1 Length of tap root	8.64	41.52	41.49
2 Internode length	3.59	17.25	58.77
3 Shoot height	2.83	13.60	72.37
4 Mid-of-leaf diameter	2.15	10.33	82.70
5 Leaf length	1.82	8.75	91.45
6 Leaf rachis length	1.78	8.55	100
7 Overall average dissimilarity	20.81	-	-
Meristic characters			
Character	**Average dissimilarity**	**Contribution %**	**Cumulative contribution %**
1 Number of lateral roots	24.26	77.02	77.02
2 Mean number of leaf nodes per shoot	2.46	7.81	84.83
3 Mean number of leaflets per leaf	2.26	7.17	92.00
4 Mean number of pinnules/lobes on leaflets	2.12	6.73	98.73
5 Number of axillary bud sprouts on shoot	0.40	1.27	100
6 Overall average dissimilarity	31.50	-	-
Genic variation characters			
Character	**Average dissimilarity**	**Contribution %**	**Cumulative contribution %**
1 Observed number of alleles	6.85	37.66	37.66
2 Effective number of alleles	4.77	26.22	63.88
3 Shannon's information index	3.90	21.44	85.32
4 Nei's gene diversity	2.67	14.68	100
5 Overall average dissimilarity	18.19	-	-

to the parent trees. These are the first such findings for *M. volkensii.* Observed morphological differences may be due to epigenetic or physiological causes. Further molecular characterisation using other markers such as microsatellite (SSR) and amplified fragment length polymorphism (AFLP) may be required for confirmation of the apparent genetic similarity between the regenerants and normal seedlings. Use of methylation-sensitive PCR is recommended for detection of epigenetic changes. We also recommend the identification of RAPD markers that may be uniquely associated with particular morphological traits and the subsequent development of sequence-characterised amplified regions (SCARs).

Conflict of interest

The authors declare no conflict of interest.

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