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Full Length Research Paper

Micropropagation of marula, *Sclerocarya birrea* subsp. *caffra (Anarcadiaceae)* by axillary bud proliferation and random amplified polymorphic DNA (RAPD) analysis of plantlets

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The availability of a rapid vegetative amplification procedure of mass-selected superior trees greatly accelerates the development of a new tree species as a crop. This study outlined the protocol for *in vitro* propagation of marula nodal explants from marula seedlings. Surface sterilized explants were cultured on Murashige and Skoog media (MS) supplemented with 26 combinations of N^6-benzyladenine (BA) and kinetin (KN). Shoots were elongated on MS media supplemented with low BA and KN or BA and Gibberellin A3 (GA_3) concentrations. Elongated shoots were rooted on half strength MS media supplemented with indolebutyric acid (IBA) at differing concentrations. MS media supplemented with 4.8 µM BA and 2.4 µM KN resulted in average 2.5 shoots per responding explant. Moderate shoot elongation was achieved on MS media supplemented with 1.2 µM BA plus 1.0 µM KN. Maximum rooting was observed on half- strength MS media supplemented with 10 µM IBA. Marula plants were acclimatized and established in soil in the growth room at an average micropropagation rate of 0.56 per responding nodal explant. The developed protocol has potential for routine micropropagation of elite *Sclerocarya birrea* subsp. *caffra.* Randomly amplified polymorphic DNA (RAPD) analysis scoring 1845 markers showed intraclonal genetic stability between explant parent and micropropagated plants.

Key words: Anacardiaceae, axillary bud proliferation, marula, randomly amplified polymorphic DNA (RAPD), somaclonal variation.

INTRODUCTION

The marula tree, *Sclerocarya birrea* subsp. *caffra*, is an indigenous, drought tolerant multipurpose tree of the *Anacardiaceae* and is widely distributed in Africa (Palmer and Pitman, 1973; Mbuya et al., 1994; Simute et al., 1998). The marula tree has many indigenous uses as food, oil, dye, wood, timber and medicine. The tree is also important to a variety of animals for shade, feed and shelter. The high nutritional value of the fruit in terms of vitamin C (0.68 mg/g) and protein content (60%) presents a good source of nutrients and vitamins for people and animals (Venter and Venter, 1996). The high quality stable oil, about 56% of the nut, offers additional exploitation opportunities (Shone, 1979). In view of the novel flavor of the fruit, the highly stable oil, the derivation of many commercial products and the potential for rural job creation, *S. birrea* subsp. *Caffra* has been earmarked for crop development.

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Abbreviations: AC, Activated charcoal; **AFLP,** amplified fragment length polymorphism; **BA,** N^6-benzyladenine; **GA_3,** Gibberellin A3; **IBA,** indole-3-butyric acid; **KN,** kinetin; **MS,** Murashige and Skoog, **PVP;** polyvinylpyrrolidone; **RAPD,** randomly amplified polymorphic DNA; **ZN,** zeatin; **PGR,** plant growth regulator.

The marula tree can be propagated by seed or vegetatively. However, propagation by seed is unsatisfactory since a very heterogeneous progeny is obtained as a result of heterozygosity. Vegetative propagation plays an important role in agriculture because vegetatively propagated trees are homogeneous and thus ensure uniform quality and production, enabling standardization of growth, harvesting and processing. *In vitro* propagation offers a larger number of cloned materials to be produced in a shorter time starting from fewer plant materials. This is important especially for the rapid amplification of improved tree material.

Genetic stability of tissue culture material depends on the stability of donor material (D'Amato, 1986) and the propagation method (Saieed et al., 1994). Tissue culture procedures, especially when callus is observed and when culture stages are long, are reported to introduce somaclonal variation (Evans, 1989). Somaclonal variation is traditionally determined by chromosome staining, DNA measurement, cytogenetic and phenotypic evaluation (Freytag et al., 1989; Linacero and Vazquez, 1992; Leitch et al., 1993; Vuylsteke and Ortiz, 1996). Recently, amplified fragment length polymorphism (AFLP) and randomly amplified polymorphic DNA (RAPD) techniques have been used successfully to determine somaclonal variation among others, poplar (Rani et al., 1995), oak (Barrett et al., 1997), spruce (Fourre et al., 1997), asparagus (Raimondi et al., 2001), pecan (Vendrame et al., 1999) and oil palm (Rival et al., 1998).

Here, we report a micropropagation protocol for marula seedling explants *(Sclerocarya birrea* subsp. *caffra*) with preliminary evaluations on adult tree explants. The latter forms a first report where marula could be readily established in tissue culture from adult tree explants without a rejuvenation stage. The protocol may offer a commercial propagation method for superior tree material. We further used RAPD analysis to investigate somaclonal variation of micropropagated plants.

MATERIALS AND METHODS

Plant material

Marula seeds were germinated in sterile vermiculite and maintained at 25°C, 81 $\mu molm^{-2}s^{-1}$ and 12 h day/night cycle. Seedlings over 2 months old were used as a source of explants. Alternatively, young shoots from healthy adult trees were harvested in the field and either kept in water or fungicide (Funginex™: active ingredient Triforine 190 mg/l, Sentrachem) during transfer to the laboratory. Shoots from both sources were defoliated before surface sterilization.

Surface sterilization

Explants were washed in running tap water prior to surface sterilization. Surface sterilization of seedlings was by immersion for 1 min in 70% ethanol followed by 10 min in 30% bleach containing 0.1% (v:v) Tween 20. Explants were then rinsed five times in sterile distilled water. Adult tree material was surface-sterilized by submersion for 2 min in 70% ethanol followed by 20 min in 30% bleach containing 0.1% (v:v) Tween 20. Additional steps were evaluated: soaking in antibacterial dish washing liquid for 10 min followed by rinsing under running tap water before surface sterilization; soaking in fungicide for 2 h (Funginex™: active ingredient Triforine 190 mg/l, Sentrachem), rinsing prior to 10 minutes soaking in antibacterial dishwashing liquid, rinsing and surface sterilization; or dipping in sterile fungicide solution after surface sterilization before inoculation. Explants were cut into 1.5 cm sections before cultivation. Contamination was assessed 7 and 14 days after culture initiation. Live explants that were sterile at both 7 and 14 days were scored as the percentage of the total surface sterilized explants.

Culture conditions

The basal media contained Murashige and Skoog (MS, 1962) nutrients at full or half- strength salts, 3% sucrose, 0.6% agar and supplements of 0.08 or 0.1% PVP and 0.3% AC. The pH of the medium was adjusted to 5.8 prior to autoclaving. Plant growth regulator (PGR) were added to the media before autoclaving (20 minutes at 121°C). Cultures were incubated in the growth room at 25±1°C with a 16 h photoperiod of 81 $\mu mol\ m^{-2}\ s^{-1}$.

Optimization of culture media for multiplication and elongation

Surface sterilized nodal explants containing axillary buds were placed on MS medium containing different concentrations of cytokinins. Explant browning and subsequent death was observed in preliminary experiments. Incorporation of polyvinylpyrrolidone (PVP) (0.1%) in the medium with and without bi-daily transfers to fresh media during the first week of cultivation prevented browning and premature death of explants and enabled establishment in tissue culture. For shoot induction various concentrations of the cytokinins, benzyladenine (BA) at 1.2-6.0 µM and kinetin (KN) at 1.2-6.0 µM were evaluated. Initial duplicate experiments included all cytokinin combinations after which only those with above 40% budbreak and with more than one shoot per node were evaluated in additional repeat duplicate experiments. Data of all duplicate and repeat experiments were combined for statistical analysis.

Shoots from multiplication medium were transferred to elongation medium. MS basal medium at full strength inorganic salts supplemented with low concentrations of BA and KN (1.2 and 1.0 µM, respectively), gibberellin A3 (GA_3) (1 and 5 µM) and MS medium without PGR were evaluated.

Rooting of elongated shoots

Elongated shoots with 2 to 3 new leaves were isolated and transferred to MS medium without PGR for 2 weeks prior to transfer to rooting medium. For root induction, half-strength MS supplemented with 0 to 10 µM of the auxin IBA and either 0.3% activated charcoal (AC) or 0.08% PVP and 0.08% PVP with the bottom wrapped in aluminium foil for 7 days were evaluated.

Acclimatization of plantlets

Rooted plantlets were washed with sterile water to remove medium from roots and plantlets were transferred into baby food jars containing sterile vermiculite supplemented with half-strength MS. The caps were gradually opened to reduce relative humidity in the jars. After 2 weeks, plantlets were potted in garden soil and covered with beakers that were gradually lifted over a period of 4 weeks. Acclimatized plantlets were transferred to potting bags in the plastic

house and reported as a percentage of the total. Micropropagation rate was calculated as the multiplication of aseptic, bud break, shoot proliferation, elongation, rooting and acclimatization rates of the adopted micropropagation protocol.

RAPD analysis

Leaves from five seedlings and ten micropropagated plantlets were harvested twice. DNA was extracted from both leaf sets using the cetyltriammonium bromide (CTAB) method according to a modification by Doyle and Doyle (1987) of the method of Saghai-Maroof et al. (1984) and Rogers and Bendich (1985). The DNA was re-precipitated, dissolved in 1 mM TRIS and 0.1 mM EDTA and its concentration estimated by comparison with a known concentration of lambda *Hind*III molecular weight marker after gel electrophoresis and ethidium bromide visualization. Polymerase chain reactions were carried out in 20 µl volumes containing 5 and 10 ng template DNA of the two independent extractions, 0.2 µM dNTP, 0.3 µM primers Genosys 60-4, 60-6, 60-8, 60-9, 60-10, 70-6, and 70-9, 1x *TaKaRa Ex-Taq*™ buffer and 2.5 units *TaKaRa Ex-Taq*™ polymerase. Denaturation was at 94°C for 5 min, followed by 45 cycles of (94°C for 45 s, 36°C for 1 min, 72°C for 2 min) and a final extension at 72°C of 7 min, in a Perkin Elmer 9700 PCR machine. PCR products were size-fractionated by 1.5 % agarose gel electrophoresis and visualized by UV-illumination of ethidium bromide stained fragments. Only reproducible bands observed among the RAPD products obtained with 5 and 10 ng template DNA from two independent DNA extraction procedures were scored.

Statistical analysis

One-way and factorial ANOVA were conducted on results of duplicate and repeat experiments using STATISTICA version 6 software from Softstats^{TM}. Percent values of budbreak and elongation were log-transformed before analysis. Rooting percent values were normally distributed (Chi-Square = 0.92; P = 0.33) and mean percent values were compared between treatments using factorial ANOVA. Multiple comparison of treatment means was carried out using Tukey HSD post-hoc test (Winer et al., 1991) to determine significant differences between treatments at an error level of <5% ($p<0.05$).

RESULTS

Surface sterilization

Surface sterilization of nodal explants grown in the growth room routinely resulted in 80% aseptic culture establishment. Immersion for 2 minutes in 70% ethanol followed by 20 min in 30% bleach supplemented with 0.1% (v:v) Tween 20 was sufficient to decontaminate field-grown adult tree material harvested at the end of the rainy season. However, in October-November at the beginning of the rainy season, when new shoots sprout, this sterilization method resulted in all contaminated cultures. Among the modifications tested, a 2 h fungicide soak, followed by 10 min in antibacterial dishwashing detergent proved significantly more effective than others, although it remained significantly lower than the aseptic rate obtained for growth - room - grown seedling nodal explants. On average, an aseptic rate of 73% was obtained for field-grown adult tree nodal explants from young twigs.

Bud break and shoot proliferation

Axillary bud break was observed within 14 days of culture on medium with as well as without cytokinins in 20 to 72% of the explants (Table 1). Bud break of 72% was observed on MS media containing 4.8 µM BA plus 2.4 µM or 3.6 µM BA plus 3.6 µM KN. More than 50% bud break was observed on media containing an intermediate cytokinin concentration, MS 2.4 µM BA plus 6.0 µM KN, MS 3.6 µM BA plus 2.4 or 3.6 µM KN, MS 4.8 µM BA plus 1.2 to 4.8 µM KN. Lower and higher cytokinin concentrations resulted in lower bud break percentage rates.

The highest proliferation rate was observed on MS medium supplemented with 4.8 µM BA and 2.4 µM KN (Table 1 and Figure 1). Two to four shoots, on average 2.5 shoots per responding bud or 1.8 shoots per sterile cultured explant, was significantly different (Tukey HSD post-hoc test, $p<0.05$) from all but three other cytokinin concentrations tested. This proliferation rate was not significantly different at $p< 0.05$ from that observed on MS 6.0 µM BA plus 1.2, 3.6 or 6 µM KN when shoots per responding bud and from that observed on MS 4.8 µM BA plus 3.6 µM KN and 6.0 µM BA plus 3.6 or 4.8 µM KN when shoots per explant were compared. However, on the latter media, much callus was observed and MS 4.8 µM BA plus 2.4 µM KN was chosen as the medium with the highest bud break and proliferation rate. Absence of cytokinin and low cytokinin concentrations generally resulted on average in only one shoot per responding bud. Higher cytokinin concentrations resulted on average in more than one but less than 2.5 shoots per responding bud. On high cytokinin media callus proliferation was excessive.The combination and concentration of 4.8 µM BA and 2.4 µM KN was adopted for nodal explants from field grown adult trees. A bud break of 100% and one shoot per responding bud or a rate of 0.73 shoots per cultured explant when loss due to contamination was considered for adult tree material (Figure 2). This differed significantly from that observed for seedling explants (Table 1, Tukey HSD post-hoc test, $p<0.05$).

Shoot elongation

After 4 weeks on shoot proliferation medium, elongated shoots with two nodes and measuring around 2 cm were transferred from MS supplemented with 4.8 µM BA and 2.4 µM KN to MS medium supplemented with zero or lower cytokinin concentration (1.2 µM BA plus 1.0 µM KN) or 1 and 5 µM GA_3 (Table 2). Inclusion of 1 and 5 µM GA_3 in the medium resulted in low elongation rates of 10

Table 1. Effect of BA and KN concentrations on bud break and shoot proliferation of marula explants.

Origin of nodal explants	BA, KN (µM)	Bud break (%)	Mean number of shoots per responding explant
Growth room grown seedlings	0.0, 0.0	30 (14)^{ab}	1.0 (0.0)^{a}
	1.2, 1.2	40 (14)^{ab}	1.5 (0.0)^{a}
	1.2, 2.4	30 (14)^{ab}	1.0 (0.7)^{a}
	1.2, 3.6	30 (14)^{ab}	1.0 (0.0)^{a}
	1.2, 4.8	30 (14)^{ab}	1.0 (0.0)^{a}
	1.2, 6.0	20 (0)^{a}	1.0 (0.0)^{a}
	2.4, 1.2	40 (0)^{ab}	1.0 (0.0)^{a}
	2.4, 2.4	40 (0)^{ab}	1.0 (0.0)^{a}
	2.4, 3.6	30 (14)^{ab}	1.0 (0.0)^{a}
	2.4, 4.8	40 (0)^{ab}	1.3 (0.4)^{a}
	2.4, 6.0*	61 (14)^{b}	1.2 (0.2)^{a}
	3.6, 1.2	40 (0)^{ab}	1.3 (0.4)^{a}
	3.6, 2.4	64 (17)^{b}	1.3 (0.2)^{a}
	3.6, 3.6	72 (12)^{b}	1.2 (0.2)^{a}
	3.6, 4.8*	30 (14)^{ab}	1.0 (0.0)^{a}
	3.6, 6.0*	40 (0)^{ab}	1.3 (0.4)^{a}
	4.8, 1.2	66 (21)^{b}	1.4 (0.3)^{a}
	4.8, 2.4	72 (27)^{bx}	2.5 (0.4)^{bx}
	4.8, 3.6*	69 (20)^{b}	1.6 (0.4)^{a}
	4.8, 4.8*	53 (12)^{ab}	1.4 (0.1)^{a}
	4.8, 6.0*	47 (12)^{ab}	1.4 (0.3)^{a}
	6.0, 1.2	40 (0)^{ab}	1.8 (0.4)^{ab}
	6.0, 2.4*	50 (14)^{ab}	1.3 (0.4)^{a}
	6.0, 3.6*	50 (14)^{ab}	1.8 (0.2$^{)ab}$
	6.0, 4.8*	35 (14)^{ab}	1.6 (0.1)^{a}
	6.0, 6.0*	40 (0)^{ab}	1.8 (0.4)^{ab}
Field grown adult trees	4.8, 2.4	100 (0)^{y}	1.0 (0.0)^{y}

Surface sterilized nodal explants were placed on MS medium containing different concentrations and ratios of BA and KN in one to four duplicate experiments of five to eight explants per treatment (a total of 465 growth room grown seedling and 71 field grown adult tree explants). Bud break is shown as the percentage of responding nodes of the total number of nodes placed on the respective medium. The mean number of shoots per responding node was calculated. Standard deviations are shown between parentheses. *indicates excessive callus proliferation. Different letters (a, b, c...) in superscript along the column indicate significant difference (Factorial ANOVA, Tukey HSD post-hoc test, df= 55, $p<0.05$). Different letters (x, y, z…) in superscript along the column indicate significant difference between the explant source, that is, seedling and field explants at the same PGR treatment (One-way ANOVA, Tukey HSD post-hoc test, df= 12, $p< 0.05$).

and 4% of the transferred shoots, respectively. Transfer to zero or low cytokinin medium resulted in elongation of 34 and 42% of the transferred shoots, respectively; a response that was significantly different from the tested GA_3 containing media. MS medium supplemented with 1.2 µM BA plus 1.0 µM KN was adopted as the shoot elongation medium for marula shoots (Figure 3).

Rooting of marula shoots

After 4 weeks on the adopted shoot elongation medium, shoots were transferred to MS medium without PGR for 2 weeks before being transferred to root induction medium (Table 3). Shoots in media supplemented with PVP or PVP wrapped with aluminum foil rooted poorly, significantly differing from those on the AC supplemented media (Tukey HSD post-hoc test $p<0.05$). Plantlets on half-strength MS media supplemented with PVP exhibited stubby and short adventitious roots, which did not elongate upon prolonged incubation. Although IBA did not have significant impacts on rooting per se, rooted plantlets on half-strength MS medium containing 0.3% AC plus 0-5 µM IBA produced one to two long adventitious roots and those on half-strength MS medium containing 0.3% AC plus 10 µM IBA two to three long adventitious roots (Figure 4). Maximum rooting of 82% was observed on half-strength MS supplemented with 0.3% AC and 10 µM IBA. This medium was adopted in routine micropropagation. No callus was observed on

Figure 1. Micropropagation of marula explants using seedling material, propagation of marula explants on MS medium supplemented with 4.8 µM BA and 2.4 µM KN.

Figure 2. Micropropagation of marula explants using adult tree material, propagation of marula explants on MS medium supplemented with 4.8 µM BA and 2.4 µM KN.

Table 2. Effect of BA, KN and GA3 on shoot elongation.

PGR (µM)	Elongated shoots (%)
0.0	34 (3)a
BA (1.2), KN (1.0)	42 (3)a
GA3 (1.0)	10 (3)b
GA3 (5.0)	4 (0)b

After bud break and shoot multiplication, microshoots were transferred from MS supplemented with 4.8 µM BA and 2.4 µM KN to MS medium without PGR or supplemented either with low concentrations of BA and KN or GA3 for shoot elongation. Percentage shoot elongation was calculated as the number of shoots elongated out of the total number of shoots transferred. Results of two experiments of 25 explants per treatment are shown (a total of 200 microshoots). Standard deviations are shown in parentheses. Different letters (a, b, c...) in superscript indicate significant difference (One-way ANOVA, Tukey HSD post-hoc test, df= 4, $p< 0.05$).

Figure 3. Micropropagation of marula explants. Elongation of shoots from seedling explants on MS medium supplemented with 1.2 µM BA and 1.0 µM KN.

microshoots for any of the tested rooting media.

Acclimatization and field transfer

Rooted plantlets were acclimatized by gradually lowering the humidity (Figures 5 and 6). Acclimatization of plantlets was achieved with a survival rate of 90%. Potted

Table 3. Effect of IBA concentration, AC, PVP and wrapping of bottles on rooting (%) of elongated shoots.

IBA (µM)	AC	PVP	PVP dark	Average
0	79 (10)	50 (10)	36 (10)	55 (21)^{x}
1	71 (20)	54 (15)	57 (20)	61 (17)^{x}
3	61 (5)	43 (10)	46 (15)	50 (12)^{x}
5	61 (15)	68 (15)	43 (10)	57 (16)^{x}
10	82 (5)	61 (15)	57 (10)	67 (15)^{x}
Average	71 (13)^{a}	55 (13)^{b}	48 (13)^{b}	

AC, Activated charcoal; PVP, polyvinylpyrrolidone. Elongated shoots were transferred from MS supplemented with 1.2 µM BA and 1.0 µM KN to half-strength MS medium without PGR for 2 weeks before transfer to half-strength MS medium containing different concentrations of IBA, AC (0.3%) and PVP (0.08%). PVP bottles were also wrapped in aluminium foil for seven days (PVPdark). Rooting was scored as the percentage of transferred shoots that produced roots. Results of two experiments of 14 explants per treatment are shown (a total of 420 elongated shoots). Standard deviations are shown in parentheses. Different letters (a, b, c… and x, y, z…) in superscript indicate significant difference among the treatments and IBA concentrations, respectively (Factorial ANOVA, Tukey HSD post-hoc test, df = 15, $p<0.05$).

Figure 4. Micropropagation of marula explants. Rooting of elongated shoots derived from marula seedling explants on half strength MS supplemented with 10 µM IBA and 0.3% AC.

plants were transferred to planting bags and kept in plastic houses for several months up to 1 year before moving bags outside ahead of field planting.

Analysis of somaclonal variation by RAPD

Figure 7 shows a representative agarose gel of the RAPD products obtained with primer Genosys 60-8. Only bands reproduced at both DNA concentrations and extractions were scored. On average, 17.6 reproducible markers were scored per decamer primer. Of the 1845 bands scored, 900 (49%) were polymorphic among the seedling plants (Table 4). No reproducible polymorphic bands were observed between micropropagated shoots and their respective explant parents.

Figure 5. Acclimatization of micropropagated marula plants (hardening off).

Figure 6. Acclimatization of micropropagated marula plants (ready for transfer to the plastic house).

DISCUSSION

This is the first report on the micropropagation of marula, *S. birrea* subsp. *Caffra* by axillary bud proliferation; a wild drought tolerant fruit tree of Africa with great commercial potential. Similarities and differences were observed between procedures developed for marula and those reported for other members of the *Anacardiaceae*, pistachio and cashew (Onay, 2000; Das et al., 1996). One marula nodal explant from a growth room grown seedling resulted on average in a micropropagation success rate of 0.56 potted hardened marula plants in a

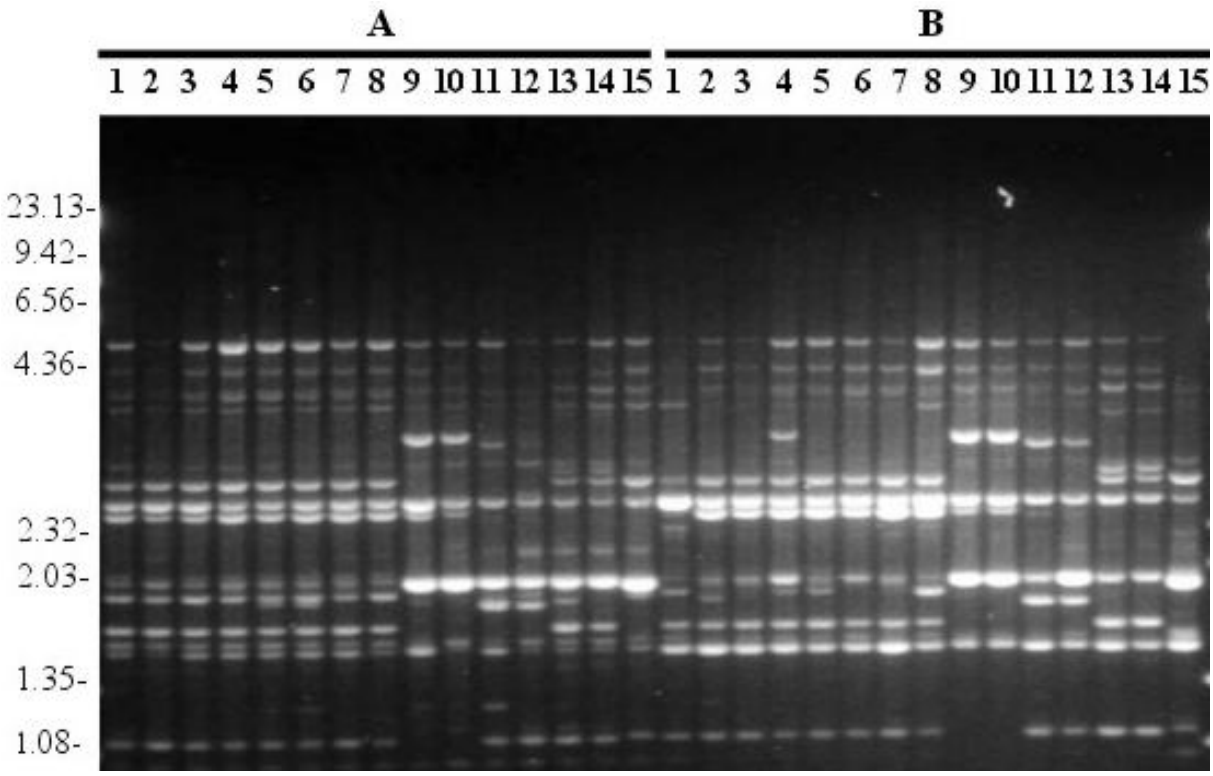

Figure 7. Agarose gel electrophoresis of a representative RAPD of parent and micropropagated marula plants. DNA 5 (A) and 10 ng (B) from independent isolations were subjected to RAPD analysis using decamer primer Genosys 60-8. Lanes 1, 9, 11, 13 and 15 represent DNA from seedling parents NS9, 2, 16, 18 and 6. Lanes 2-8, 10, 12 and 14 represent DNA of micropropagated plantlets from explant parents NS9, 2, 16 and 18, respectively. DNA molecular weight is indicated on the left in kbp.

Table 4. RAPD analysis of micropropagated and explant parent plants.

Parameter	Value
Number of decamer primers	7
Number of markers	123
Number of polymorphic markers	60
Average markers per primer	17.6 (2.3)
Average polymorphic marker per primer	8.6 (2.8)

Standard deviations are shown in parentheses.

period of 22 to 26 weeks, that is, 5 to 6 months. De Assis et al. (2012) observed 1 shoot per cashew nodal segment with axillary buds in 2 months culture period but they did not go beyond multiplication stage. Das et al. (1996) achieved a micropropagation rate of 0.5 to 1 potted cashew plant per leaf axil from one-month old seedling grown cashew in a calculated 15 weeks. Onay (2000) attained a micropropagation rate of 16 potted pistachio plants in 22 weeks per shoot tip induced on 10% of imbibed stems. However, when the amount of superior plant material is limited, propagation rates per tree node must be considered when comparing protocols. We were unsuccessful in attaining cycling of marula explants as excessive callusing on newly formed shoots was observed after repeat incubation on high cytokinin medium even when transferred from a low cytokinin concentration.

The relatively large variation in tissue culture response observed may be due to the heterogeneous nature of the starting material. Growth room grown marula seedlings were of different genetic background as evidenced by the interclonal RAPD polymorphism as well as of different ages (2 to 24 months).

Much effort in propagation for the important cash crops, mango, cashew and pistachio; and emerging new crops such as marula is evidenced from reports on somatic

embryogenesis from cotyledon explants (Litz et al., 1984; Philip, 1984; Anathakrishnan et al., 2002; Cardoza and D'Souza, 2002), somatic embryogenesis from leaf explants (Moyo et al., 2009) and adventitious shoot formation from seedling explants (Barghchi and Alderson, 1983, 1985; Martinelli, 1988; Lievens et al., 1989; Leva and Falcone, 1990; D'Silva and D'Souza, 1991; Bogetti et al., 1999; Moyo et al., 2011). These explants however form inappropriate starting materials for commercial propagation, as offspring of heterozygous parent trees are neither genetically homogeneous nor identical to their selected superior parent. In our experience with marula, once the major bottlenecks of contamination and browning were overcome, the time of harvest proved most important to establish explants from adult trees. Bogetti et al. (1999) achieved 14% bud break of nodes from 5-year old glass house grown cashew trees but did not report on further progression of explants from adult trees through tissue culture. Shoot tip necrosis was observed in *in vitro* generated pistachio plantlets (Barghchi and Alderson, 1983, 1985, 1996). Bud break of field-grown adult marula tree explants was observed at a rate of 100% in contrast to 72% from seedling (two to 24 months old) derived explants. Only 1 shoot per node was formed whereas seedling-derived material which had a much smaller mass than tree-derived material formed 2 to 4 shoots per responding node on the same medium. Opportunities for further optimization and development of a commercial marula micropropagation procedure for adult trees may exist in the further optimization of PGR concentrations specifically for tree explants.

Rani et al. (1995) reported that RAPD markers are useful tools in genetic identification of micropropagated plants. Any of the seven decamer primers used was able to distinguish between the five different marula seedlings (Table 4 and Figure 7). The substantially higher interclonal polymorphism rate of 49% in marula compared to 20% in oak (Barrett et al., 1997) and 19% in oil palm (Rival et al., 1998) may be in part due to the absence of selection and breeding of marula and as well as its dioecious nature.

The RAPD data for marula suggested that all micropropagated plantlets even although some had been subject to extreme temperatures while in tissue culture (due to unplanned electricity outages), were genetically uniform within the limits of the RAPD method. Out of the 1845 bands of 123 markers scored, none were polymorphic within the cloned plantlet sets. For the seven clones NS9-1 to 7 and explant parent plant NS9, 85 markers were monomorphic suggesting an intraclonal polymorphism frequency below 0.1%.

Although RAPD and AFLP have been successfully used to determine somaclonal variation in different micropropagated plants and their parent plant such as*Triticum* (Brown et al., 1993), beet (Munthali et al., 1996) and *Arabidopsis* (Polanco and Ruiz, 2002)., in other cases these molecular techniques failed to detect genetic differences underlying obvious phenotypic changes due to somaclonal variation (Goto et al., 1998; Rival et al., 1998). Fourre et al. (1997), Wilhelm (2000) and Bouman and De Klerk (2001) observed differences in ploidy levels between clones and their explant parents that remained undetected when screening a large number of RAPD and AFLP markers.

Although somaclonal variation is considered a problem in breeding and mass propagation, mutation during mitosis is a natural process. In senescing cork tissue, one mutation in 1520 nucleotides was found after sequencing nuclear DNA of old and young cork tissues (Pla et al., 2000). AFLP analysis of *Arabidopsis* somaclones showed that the values of nucleotide diversity estimated of each group of regenerated plants were two to three orders of magnitudes smaller than natural variation described for natural ecotypes of *Arabidopsis* (Polanco and Ruiz, 2002). At what frequency phenotypic differences are generated during marula micropropagation needs further examination of traits in the field over several years.

Conclusion

Marula was propagated *in vitro* by seedling nodal explants with axillary buds; plantlets were successfully acclimatized. Micropropagated plantlets were genetically identical to their seedling parent plants within the limits of RAPD.

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