South African Journal of Botany xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

South African Journal of Botany

journal homepage: www.elsevier.com/locate/sajb

In vitro regeneration of *Guizotia abyssinica* Cass. and evaluation of genetic fidelity through RAPD markers

S. Baghel, Y.K. Bansal *

Plant Tissue Culture Laboratory, Dept. of Biological, Science, R. D. University, Jabalpur, M.P., India

ARTICLE INFO

Article history:
Received 8 June 2016
Received in revised form 1 December 2016
Accepted 8 January 2017
Available online xxxx

Edited by J. van Staden

Keywords:
Apical bud
Axillary bud
Leaf
Internode
RAPD analysis
Somaclonal variation
Energy crop

ABSTRACT

The objective of this study was to induce a rapid as well as prolific shoot regeneration protocol for micropropagation and RAPD analysis of *Guizotia abyssinica* Cass. which is an important herbaceous plant of immense industrial value *via* direct and indirect organogenesis from apical bud, axillary bud, leaf and internode explants. Best seed germination was obtained on cotton irrigated with liquid MS medium. Out of the four explants used, apical bud proved to be the best in terms of shoot regeneration and multiplication. Best shoot multiplication was obtained from apical bud, axillary bud and leaf explants on MS medium supplemented with 2.22 μM BAP + 2.85 μM IAA. Whereas supplementation of MS medium with 2.22 μM BAP + 28.55 μM IAA produced maximum number of shoots from internode explants. BAP (0.44 μM) in combination with Kn (0.46 μM) proved suitable for maximum mean shoot length. Moreover, culturing the regenerated shoots on half-strength liquid MS medium supplemented with NAA (2.68 μM) induced maximum rooting from elongated shoots (direct and indirect regeneration). The plantlets were established in plastic cups containing vermiculite, soil, sand and farm yard manure and then successfully transferred to field with 97.33% survival. Analysis of RAPD recognized 197 different amplification products and showed the presence of somaclonal variation in the plantlets arising from direct regeneration as well as from indirect regeneration. The protocol developed in this study is suitable for propagation of quality planting material for commercialization, germplasm conservation and for future genetic improvement studies.

1. Introduction

Guizotia abyssinica Cass. (Family: Asteraceae) commonly known as Niger, Ramtil or Jagni is a multipurpose, edible, oil yielding, annual herb and cultivated to a limited extent in Ethiopia, South Africa, East Africa, West Indies, Zimbabwe and India (Rajpurohit, 2011). The genus *Guizotia* comprises of six species *viz.*: *G. abyssinica* (L.f.) Cass, *G. scabra*, *G. arborescens*, *G. reptans*, *G. villosa and G. zavattarii*. However, *G. abyssinica* is the only cultivated species (Baago, 1974). Niger seeds contain about 35–40% (dry seed weight) edible oil with fatty acid composition of 75–80% linoleic acid, 7–8% palmitic, steric acids and 5–8% oleic acid (Dutta et al., 1994). Oil of Niger seeds is used to prepare various types of foods, paints, soaps and as an illuminant. The oil of the seeds is also used for the treatment of various diseases (Belayneh, 1991). Moreover *G. abyssinica* can be easily processed to replace partial or full petroleum based diesel fuel (Devi et al., 2006; Sarin et al., 2009). Thus the use of this plant for large scale biodiesel production is of great interest with regard to solving the energy shortage, reducing carbon emission and increasing the income of farmers in addition to its use in traditional medicines.

G. abyssinica has assumed it as potential biofuel crop because of the short reproductive period, low cost of seeds, high oil content, easy adaptation on all types of soil, requirement of moderate rainfall, cultivated successfully rotation with wheat or maize, suitable as fuel substitute without any alteration to the existing engines and above all yield levels reported to be 200–300 kg/ha although they can reach 500–600 kg/ha with good management (Getinet and Sharma, 1996; Sarin et al., 2009). To meet the large scale demand and ensure easy supply of this elite material, there is a need to establish mass multiplication technique. Despite being nutritionally rich and economically important, it has remained a neglected crop (Bhandari et al., 2009). Besides that, plant suffers from low yield due to self incompatibility, lodging, shattering, indeterminate growth habit, instability at higher temperature and susceptibility to diseases (Sarvesh et al., 1994; Getinet and Sharma, 1996; Murthy et al., 2003).

Plant tissue culture technology has been extensively employed for crop improvement in several oil crops (Baskaran and Jayabalan, 2006). Commercial production of plants through micropropagation techniques has several advantages over traditional methods of propagation

Abbreviations: BAP, 6-Benzyladenine; Kn, 6-Furfurylaminopurine; IAA, Indole-3-acetic acid; IBA, Indole-3-butyric acid; NAA, α-Naphthalene acetic acid; RAPD, Random amplified polymorphic DNA.

* Corresponding author.
E-mail addresses: sapna.baghel153@gmail.com (S. Baghel), yogendrakbansal@rediffmail.com (Y.K. Bansal).

http://dx.doi.org/10.1016/j.sajb.2017.01.002
0254-6299/

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through seed, cutting, grafting, air layering, *etc.* It is the rapid propagation processes that can lead to the production of virus free plants (Garcia-Gonzales et al., 2010). *In vitro* micropropagation is an important tool for crop improvement in plant breeding and is a common application for induction of somaclonal variation. The significance of somaclonal variation in crop improvement depends upon establishing a genetic basis for variation (Nayak et al., 2003).

However, a major problem associated with *in vitro* culture is the possible occurrence of somaclonal variation among the subclones of potential lines (Larkin and Scowcroft, 1981). *In vitro* plants are usually susceptible to genetic changes due to cultural stress (Rani and Raina, 1998). Some investigators have noted that indirect regeneration of plantlets requires a longer induction. The growing medium is usually supplemented with cytokinins (Howell et al., 2003) and usually results in variability among the regenerating plantlets (Mondal et al., 2004; Pontaroli and Camadro, 2005; Bairu et al., 2006; Park et al., 2006; Jeong et al., 2009). Genetic changes may occur at cellular and (more frequently) at ploidy levels such as in chromosome structure (Radic et al., 2005) or at molecular levels with punctual mutations in DNA (Chen et al., 2012)

The cause of somaclonal variation in higher plants has been reported during different biochemical and molecular events including changes in DNA methylation pattern, activation of transposable elements and chromosome remodeling (Hirochika, 1993; Price et al., 2002). Several approaches such as karyotyping and isoenzyme profiling can be used to assess the genetic fidelity of the *in vitro* derived clones, but most of these methods have their own limitations. Karyotyping does not reveal the alterations in specific genes or small chromosomal rearrangements (Isabel et al., 1993) whereas isoenzyme markers are subject to ontogenic variations. Therefore, molecular markers have been exploited for the detection of somaclonal variation, including random amplified polymorphic DNA (RAPD) (Chen et al., 1998; Rival et al., 1998), methylation sensitive restriction fragment length polymorphism (RFLP) (Jaligot et al., 2000, 2002; Kubis et al., 2003) and microsatellite sequence variation (Alou et al., 2004). Polymerase chain reaction (PCR) techniques which use random amplified polymorphic DNA (RAPD) markers to detect the variations or genetic relationship among individuals between and within species (Carlson et al., 1991; Roy et al., 1992; Tripathi et al., 2007). RAPD markers have been successfully used to assess genetic stability and quality among micropropagated plants, thus, ensuring the quality of tissue cultured plantlets.

RAPD technique has several advantages such as the ease and rapidity in analysis, relatively low cost, availability of a large number of primers and the requirement of a very small amount of DNA for analysis (William et al., 1990). RAPD analysis using polymerase chain reaction (PCR) in association with short primers of arbitrary sequence has been demonstrated to be sensitive in detecting variation among individuals. RAPD-mediated DNA fingerprinting has been extensively used for detecting polymorphism among *in vitro* micropropagated crops such as *Prunus persica* (Hashmi et al., 1997); *Allium sativum* (Al-Zahim et al., 1999); *Colocasia esculenta* (Hussain and Tyagi, 2006); *Gypsophila paniculata* (Barakat and El-Sammak, 2011) and *Solanum melongena* (Mallaya and Ravishankar, 2013).

Few efforts have been made to propagate *G. abyssinica* using *in vitro* techniques. Multiple shoot formation *via* organogenesis was obtained from different explants *viz.* hypocotyls, cotyledons, apical bud and axillary bud has been already reported in the literature (Ganapathi and Nataraja (1993); Nikam and shitole (1993, 1997); Sarvesh et al. (1993); Bhandari et al. (2009); Disasa et al. (2011); Baghel and Bansal (2014). Somatic embryogenesis was also reported by Sarvesh et al. (1994) and Naik and Murthy (2010), the analysis of tissue culture derived plants for somoclonal variations is yet to be published. Therefore, the present work was undertaken to establish an efficient protocol for direct and indirect micropropagation and the subsequent RAPD analysis of the regenerated plantlets of *G. abyssinica*.

2. Materials and methods

2.1. Plant material for in vitro regeneration

Seeds of *G. abyssinica* var. JNC 6 were obtained in the month of July 2013 from Jawaharlal Nehru Krishi Vishwavidyalaya (JNKVV) Jabalpur, (M.P.) India.

2.2. Surface sterilization of seeds of G. abyssinica

Healthy and well formed seeds were soaked in water for 24 h and washed thoroughly under running tap water for 30 min and kept in 1% (*w*/*v*) Bavistin (Carbendenzim Powder, BASF Ltd., India) for 10 min and then treated with wetting agent labolene (1%) and then rinsed in running water (1 h). They were then surface sterilized using (*w*/*v*) 0.1% $HgCl_2$ for 6–8 min followed by three rinses with sterile distilled water. Seeds were then inoculated on a filter paper, a sterilized moist cotton with liquid MS (Murashige and Skoog, 1962) medium and the solidified medium with 0.8% (*w*/*v*) agar. Each experiment was performed in triplicate and germination percentage was checked for each medium.

2.3. Explant preparation

Seed germination started with in 7–8 days. Four types of explants *viz.* apical, axillary buds (1.0 cm each), leaf (1.0 cm) and internode (0.8–1.0 cm) were isolated from 20 to 25 day old seedlings.

2.4. Culture media conditions, establishment and shoot regeneration

In the first set of experiments, explants were cultured on shoot regeneration (SRI) medium with varying concentrations of growth hormones or PGR free basal MS medium (control) for multiplication and shoot elongation. For direct and indirect shoot regeneration apical bud, axillary bud, leaf and internode explants were inoculated onto shoot regeneration medium (SRI) composed of MS (basal) supplemented various combination of cytokinin like BAP (0.44–22.2 μM) with Kn (0.46–23.2 μM) and BAP in combination with various auxins *viz.* IAA, IBA, NAA, *etc.* Explants were cultured on medium (SRI) for three subculture cycles of 20–25 days each. All media contained 3% (*w*/*v*) sucrose and 0.8% (*w*/*v*) agar. The pH of the medium was adjusted to 5.6–5.8 (before adding agar) with 1 N NaOH and 1 N HCl before dispensing into culture tubes (15 × 150 mm) and autoclaving at 121 °C for 15 min.

All the cultures were maintained at a temperature of 25 ± 1 °C and under a photoperiodic cycle of 16/8 h provided by Philips (India) cool white fluorescent tubes [approx 1500 lx]. The cultures were transferred to fresh culture medium every 4 week interval. The number and length of the shoots per explant were recorded after 4 weeks.

2.5. In vitro rooting

In the second set of experiments, well elongated shoots with fully expanded leaves were cultured onto half-strength MS medium (liquid) supplemented with 3% (*w*/*v*) sucrose with or without auxins like NAA, IAA, IBA (0.1–5 mg/l each) for root induction.

2.6. Hardening and acclimatization

In vitro regenerated plantlets (3–4 months old and 4–5 cm in length) possessing well developed shoot and root system were washed with tap water to remove adhering agar without damaging the delicate root system and treated with 1% (*w*/*v*) bavistin for 5 min.

For hardening and acclimatization of *in vitro* raised plantlets treatments were carried out to three different planting substrates. In the first method, the plantlets were transferred to plastic cups containing sterilized sand, soil and farm yard manure (1:1:1). In the second

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method the plantlets were transferred to vermiculite alone while in the third method the plantlets were transferred to vermiculite, soil, sand and farm yard manure (1:1:1:1) mixture.

To maintain humidity, hardened plantlets were completely covered with plastic bags and irrigated regularly with distilled water. After 2 weeks the plastic covers were perforated with small holes. The bags were removed intermittently (5–10 min) to aid adaptation of *in vitro* plantlets to normal environment conditions. Subsequently exposure time was gradually increased in the following weeks and after 7–8 weeks plastic covers were removed completely. After 8 weeks of hardening the plantlets were transferred to the field.

2.6.1. Histological study

The newly *in vitro* regenerated plant tissues of different morphogenetic stages (2–4 weeks) were fixed in Formalin-Aceto-Alcohols (FAA) solution (90 ml of 70% ethyl alcohol, 5 ml of glacial acetic acid and 5 ml of formalin) (Berlyn and Miksche, 1976; Tisserat and DeMason, 1985) for 18 h, washed for 30 min with tap water, dehydrated by transferring through an ethanol–xylol series and then embedded in paraffin. Tissues were sectioned at 10 μm thickness with microtome, mounted on glass slides, and stained with 0.5% (*w*/*v*) safranin. The slides were dehydrated with successive grades of ethanol and counter stained with 0.5% (*w*/*v*) fast green (fast green dissolved in 1:1 (*v*/v) clove oil and absolute ethanol; O'Brien and Mccully, 1981). The slides were further differentiated in absolute ethanol and xylol series, cleared with pure xylol and finally mounted with Canada balsam. Histological observations of the sectioned materials were made using an Olympus CH20i light microscope and photographed.

2.6.2. Genomic DNA extraction and PCR amplification

DNA was extracted using cetyl trimethyl ammonium bromide (CTAB) with slight modifications by the method (Murray and Thompson, 1980). Approx. 1 g of fresh and young leaves was taken from nature grown mother plant as well as *in vitro* regenerated plantlets (60-day-old) and subsequently ground in liquid nitrogen using mortar and pestle along with 2% (*w*/*v*) PVP (polyvinylpyrrolidone) and 0.2% (*v*/v) β-mercaptoethanol. To the ground leaf powder, 5–6 ml of extraction buffer (2% CTAB, 100 mM Tris–HCl, 25 mM EDTA and 1.5 M NaCl, pH 8.0) were added and incubated at 65 °C for 90 min. The above samples were extracted with equal volumes of Tris saturated phenol (pH 8.0), chloroform and iso-amyl alcohol (25:24:1) and centrifuged at 10,000 rpm for 10 min. The supernatant was transferred to a fresh tube and subsequently chilled isopropanol was added and incubated overnight at −20 °C. Following centrifugation the pellet was washed with 70% alcohol and re-suspended in TE buffer and treated with 5 μl RNase (10 μg/ml) for 1 h at 37 °C. These were extracted with equal volumes of chloroform and iso-amyl alcohol (24:1) and precipitated with chilled ethanol and 3 M sodium acetate (pH 5.2) at −20 °C for 30 min. The pellet was washed twice with 80% ethanol and dissolved in TE buffer. The concentration of DNA was quantified by a UV–vis spectrophotometer and quality of genomic DNA was checked through electrophoresis on 0.8% agarose gel.

PCR amplifications for RAPD were performed in a thermal cycler. Amplification of RAPD fragments was performed using 8 random decanucleotide primers (Operon Technologies Inc., USA). The reaction was carried out in a 25 μL of reaction mixture containing 0.5 μL (50 ng) template DNA, 2.5 μL 1× PCR Assay buffer (GeNei™), 0.5 μL 10 mM dNTP mix (GeNei™), 1.5 μL 25 mM $MgCl_2$ (GeNei™), 7.5 μL (1.5 U) Taq DNA polymerase (GeNei™) and 0.75 μL 10 μM/L primer stock (final conc. 0.5 μM/L) (Fermentas, USA) and 11.75 μL PCR grade water. The PCR reaction program consisted of initial denaturation at 94 °C for 3 min followed by 45 cycles of denaturation at 94 °C for 45 s, primer annealing at 37 °C for 1 min, extension at 72 °C for 1 min and final extension at 72 °C for 10 min.

Amplified DNA was loaded on 1.8% agarose gel in 1× TAE buffer, stained with 0.5 μg/mL ethidium bromide and photographed on a Gel documentation system. The RAPD experiments were repeated at least twice and only the distinct, well-resolved and reproducible bands were scored. The RAPD data generated with the 4 primers were used to calculate pairwise similarity coefficients (Jaccard, 1908) using the binary data *via* XLSTSAT software package ver. 2009.06.01. Based on the matrix of genetic similarity, cluster analysis was performed and the generated similarity coefficients were applied for constructing dendrogram using the unweighted pair group method with arithmetic average (UPGMA).

2.6.3. Experimental design, data collection and analysis

The experiments were set according to completely randomized design and all experiments were performed in triplicates with 12 treatments. The results are expressed as mean ± SEM. Observations were recorded after 25–30 days of interval. The data were analyzed using analysis of variance (ANOVA) and means were separated at $p = 0.05$ level of significance using Duncan's multiple range test (DMRT) using the statistical software IBM SPSS 20.

3. Results

3.1. Germination of seeds

The seeds germinated within one week of culture and the highest germination percentage of 91% were obtained on cotton soaked with liquid MS medium as compared to that on semi solid MS medium and filter paper (Fig. 1).

3.2. Micropropagation

3.2.1. Effect of type of explants on shoot regeneration

Preliminary experiments were conducted for selection of optimum explant for higher shoot production. Direct and indirect organogenesis from different explants showed variable results when BAP was tested in combination with KN, IAA, IBA and NAA. On PGR-free basal MS medium (control) both apical and axillary bud explants produced single shoots, while a rhizogenic response was initiated from leaf and internode explants. These findings suggested that endogenous levels of hormones present in these explants are not sufficient to sustain their growth in the basal medium. Both apical and axillary bud explants of *G. abyssinica* responded within a week of culturing with basal swelling (Fig. 2a, b), however, leaf and internode explants swelled in 2 and 3 weeks respectively.

The swollen leaf explants developed green shoots two weeks later without callus formation (Fig. 2c). The formation of shoot buds occurred on the abaxial surface, when in contact with the medium. Swollen internode explants produced green shoots after 3 weeks with little callus formation (Fig. 2d).

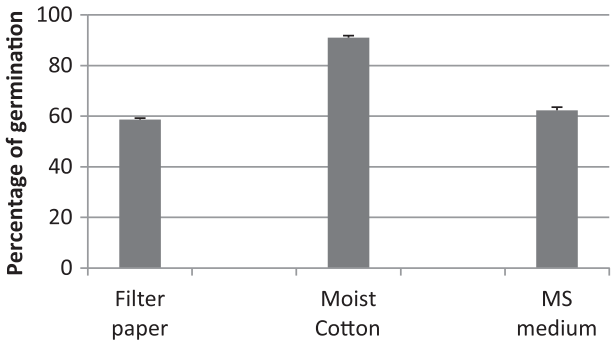

Fig. 1. Effect of different medium is right on percentage of germination in *Guizotia abyssinica* Cass. The maximum percentage of germination was obtained from moist cotton.

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Fig. 2. Direct regeneration of *Guizotia abyssinica* Cass. from apical and axillary bud explants and indirect regeneration from leaf and internode explants. a Shoot bud initiation from apical bud explant after 1 week. b Bud breakage from axillary bud explants after 1 week. c shoot regeneration from leaf explants after 2 week. d Shoot bud induction with little callusing from internode explants after 3 week. e Multiple shoot formation from apical bud on BAP (2.22 μM) + Kn (2.32 μM) 3 week. f Shoot multiplication from axillary bud on BAP (2.22 μM) + Kn (2.32 μM) 3 week. g Multiple shoot formation from leaf on BAP (2.22 μM) + Kn (2.32 μM) 2–3 weeks. h Shoot initiation with BAP (0.44 μM) + Kn (23.2 μM) from internode explants in 2–3. i Multiple shoot initiation on BAP (0.44 μM) + Kn (23.2 μM) 4 week. j–k Complete multiple shoot formation with BAP (0.44 μM) + Kn (23.2 μM) 5 weeks from internode explants through brown callusing.

3.2.2. Effect of cytokinin combinations on shoot regeneration

The highest percentage of shoot initiation and multiplication was obtained on BAP (2.22 μM) + Kn (2.32 μM) from apical, axillary buds and leaf explants (Fig. 2e–g). As the concentration of BAP and Kn increased or decreased, the number of shoot bud decreased (Table 1). Thus, the optimal medium for shoot bud proliferation from apical, axillary and leaf explants was MS medium containing BAP (2.22 μM) and Kn (2.32 μM). However, shoot elongation on this medium was minimal. While from internode explants the highest shoot initiation (69.44 ± 2.27) (Fig. 2h, i) and shoot multiplication (2.41 ± 0.1) (Fig. 2j, k) was obtained on medium supplemented with BAP (0.44 μM) + Kn (23.2 μM) with little brown callusing.

3.2.3. Effect of cytokinin–auxin combinations on shoot regeneration

To evaluate the synergistic effect of cytokinin–auxin combination on multiple shoot induction, different concentrations of auxins (NAA, IAA and IBA) were added with the different concentrations of BAP. Among the auxins tested, IAA was found to be the most effective (Table 2) followed by NAA and IBA (data not provided). High frequency of shoot initiation and shoot multiplication were induced on MS medium supplemented with 2.22 μM BAP + 2.85 μM IAA. On this combination apical bud (80.55 ± 2.26, 4.16 ± 0.4) (Fig. 3a, b), axillary bud (83.33 ± 2.26, 4.02 ± 0.6) (Fig. 3c, d) and leaf (80.55 ± 4.53, 3.16 ± 0.3) (Fig. 3e, f) explants yielded maximum frequency of shoot initiation and multiplication. However, internode explants responded only on 2.22 μM BAP + 28.55 μM IAA (Fig. 3g, h) with comparatively lower shoot initiation (69.44 ± 4.53) and multiplication (2 ± 0.3). BAP was found to be necessary for multiplication as medium devoid of it (auxins alone) did not show any multiplication (data not provided) except for the growth of single shoot with roots. Increasing concentrations of BAP increased the number of shoots up to a conc. of 4.44 μM but a further increase in BAP concentration (8.88 μM), however, reduced the number of shoots produced and instead induced callusing.

3.2.4. Effect of subculturing on shoot regeneration and multiplication

When apical bud from the mother explants were subcultured on the fresh shoot multiplication medium (MS) containing BAP (2.22 μM) + IAA (2.85 μM), the shoot number increased significantly in the next three subcultures. The maximum number of shoots (6.52 ± 0.09) was produced during the third subculture (Fig. 4, Table 3).

3.2.5. Effect of cytokinin on shoot elongation

Maximum elongation of shoot buds into shoots was achieved on MS medium fortified with lower concentrations of BAP (0.44 μM) and Kn (0.46 μM) (Fig. 5a–f). This medium was, therefore, designated as the "Shoot elongation medium". This led to the conclusion that media

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Table 1
Effect of BAP + KN combination on shoot proliferation and elongation from apical, axillary bud, leaf and internode explants of *Guizotia abyssinica* Cass.

PGR Conc. (μM)		Frequency of shoot initiation (%)				Shoot number (mean ± SE)				Shoot length (cm) (mean ± SE)			
BAP	Kn	ApB	AxB	Leaf	Internode	ApB	AxB	Leaf	Internode	ApB	AxB	Leaf	Internode
0.44	0.46	47.22 ± 2.27^{d}	61.10 ± 4.54^{cde}	44.44 ± 2.27^{d}	–	1.22 ± 0.1^{f}	1.36 ± 0.1^{g}	1.33 ± 0.1^{c}	–	**4.57 ± 0.4^{a}**	**3.99 ± 0.4^{a}**	**2.10 ± 0.2^{a}**	–
	2.32	69.44 ± 2.27^{bc}	55.55 ± 4.54^{de}	30.55 ± 2.27^{e}	–	2.77 ± 0.2^{bc}	1.83 ± 0.2^{f}	0.61 ± 0.2^{f}	–	2.42 ± 0.2^{cd}	2.54 ± 0.3^{c}	0.28 ± 0.01^{def}	–
	4.64	77.77 ± 2.27^{ab}	72.22 ± 2.27^{bc}	33.33 ± 2.27^{e}	–	3.30 ± 0.3^{a}	3.58 ± 0.4^{a}	1.05 ± 0.1^{de}	–	3.86 ± 0.3^{b}	3.72 ± 0.4^{a}	0.13 ± 0.01^{ef}	–
	23.2	44.44 ± 2.27^{de}	61.10 ± 4.54^{cde}	69.44 ± 2.27^{a}	**69.44 ± 2.27^{a}**	2.08 ± 0.2^{de}	2.27 ± 0.1^{d}	2.16 ± 0.2^{b}	**2.41 ± 0.1^{a}**	1.08 ± 0.1^{fg}	1.28 ± 0.1^{e}	0.67 ± 0.1^{bc}	**1.41 ± 0.1^{a}**
2.22	0.46	69.44 ± 2.27^{bc}	77.77 ± 2.27^{ab}	55.55 ± 4.54^{b}	–	2.58 ± 0.1^{c}	2.88 ± 0.3^{b}	1.19 ± 0.1^{cde}	–	2.16 ± 0.3^{de}	2.96 ± 0.3^{b}	0.48 ± 0.1^{cd}	–
	2.32	**86.10 ± 2.78^{a}**	**86.10 ± 2.78^{a}**	**72.22 ± 2.78^{a}**	–	**3.77 ± 0.3^{a}**	**3.41 ± 0.2^{a}**	**2.61 ± 0.3^{a}**	–	1.91 ± 0.2^{e}	1.96 ± 0.3^{d}	0.77 ± 0.1^{b}	–
	4.64	61.10 ± 5.56^{c}	49.88 ± 2.78^{ef}	19.44 ± 2.78^{f}	–	2.13 ± 0.1^{de}	1.80 ± 0.2^{f}	0.97 ± 0.01^{e}	–	0.61 ± 0.1^{hi}	2.02 ± 0.2^{d}	0.26 ± 0.01^{def}	–
	23.2	80.55 ± 2.78^{a}	61.10 ± 4.54^{cde}	44.44 ± 2.27^{d}	–	3.22 ± 0.3^{a}	2.11 ± 0.2^{de}	1.22 ± 0.2^{cd}	–	1.45 ± 0.1^{f}	0.67 ± 0.01^{fg}	0.32 ± 0.01^{de}	–
4.44	0.46	69.44 ± 2.27^{bc}	69.44 ± 0^{bcd}	55.55 ± 4.54^{b}	–	2.24 ± 0.2^{d}	1.99 ± 0.1^{ef}	1.19 ± 0.1^{cde}	–	2.51 ± 0.1^{cd}	2.42 ± 0.1^{c}	0.19 ± 0.01^{def}	–
	2.32	72.22 ± 2.27^{bc}	69.44 ± 4.54^{bcd}	47.22 ± 2.27^{bc}	–	2.90 ± 0.3^{b}	2.58 ± 0.2^{c}	1.21 ± 0.1^{cd}	–	2.70 ± 0.1^{c}	2.81 ± 0.1^{bc}	0.70 ± 0.01^{bc}	–
	4.64	61.10 ± 4.54^{c}	66.60 ± 4.54^{bcd}	27.11 ± 1.72^{ef}	–	1.99 ± 0.2^{e}	1.80 ± 0.1^{f}	1.10 ± 0.1^{cde}	–	1.01 ± 0.01^{g}	1.05 ± 0.1^{ef}	0.36 ± 0.01^{de}	–
	23.2	61.10 ± 4.54^{c}	63.88 ± 2.27^{cd}	72.22 ± 2.27^{a}	–	2.08 ± 0.1^{de}	2.24 ± 0.1^{de}	2.35 ± 0.2^{b}	–	1.26 ± 0.1^{fg}	0.56 ± 0.01^{gh}	0.21 ± 0.01^{def}	–
8.88	0.46	36.10 ± 2.27^{ef}	30.55 ± 2.27^{g}	–	–	1.14 ± 0.01^{fg}	1.16 ± 0.01^{gh}	–	–	0.23 ± 0.1^{ij}	0.20 ± 0.01^{hi}	–	–
	2.32	38.88 ± 2.27^{def}	38.88 ± 2.27^{fg}	–	–	1.02 ± 0.01^{fg}	1.02 ± 0.01^{h}	–	–	0.63 ± 0.1^{h}	0.12 ± 0.01^{i}	–	–
	4.64	30.55 ± 2.27^{fg}	30.55 ± 2.27^{g}	–	–	0.94 ± 0.01^{g}	0.94 ± 0.01^{h}	–	–	0.20 ± 0.01^{j}	0.12 ± 0.01^{i}	–	–
	23.2	22.22 ± 2.27^{g}	16.66 ± 2.27^{h}	–	–	0.91 ± 0.01^{g}	0.94 ± 0.01^{h}	–	–	0.07 ± 0.01^{j}	0.07 ± 0.01^{i}	–	–

Means followed by same letters in each column are not significantly different according to Duncan's multiple range test ($P \leq 0.05$). Data was recorded after 4 weeks of culture on shoot multiplication medium. The bold data showed the best results. Abbreviations used: ApB- Apical Bud, AxB- Axillary Bud.

Table 2
Effect of BAP + IAA combination on shoot proliferation and elongation from apical, axillary bud, leaf and internode explants of *Guizotia abyssinica* Cass.

PGR Conc. (μM)		Frequency of shoot initiation (%)				Shoot number (Mean ± SE)				Shoot length (cm) (Mean ± SE)			
BAP	IAA	ApB	AxB	Leaf	Internode	ApB	AxB	Leaf	Internode	ApB	AxB	Leaf	Internode
0.44	0.57	75 ± 0.01^{ab}	61.10 ± 4.53^{de}	47.22 ± 2.27^{e}	–	2.69 ± 0.1^{e}	2.41 ± 0.1^{d}	1.02 ± 0.01^{f}	–	1.88 ± 0.2^{d}	2.23 ± 0.2^{bc}	0.25 ± 0.1^{e}	–
	2.85	69.44 ± 2.27^{bc}	69.44 ± 2.27^{bcd}	–	–	2.16 ± 0.2^{f}	1.85 ± 0.1^{e}	–	–	1.16 ± 0.1^{f}	1.27 ± 0.01^{de}	–	–
	5.71	74.99 ± 4.09^{ab}	72.22 ± 2.26^{abcd}	72.21 ± 2.26^{ab}	–	2.94 ± 0.2^{e}	2.63 ± 0.2^{cd}	1.60 ± 0.2^{d}	–	**3.17 ± 0.3^{a}**	**3.62 ± 0.4^{a}**	0.37 ± 0.01^{de}	–
	28.55	69.44 ± 2.27^{bc}	61.10 ± 4.53^{de}	–	–	2.13 ± 0.2^{f}	1.49 ± 0.1^{ef}	–	–	2.40 ± 0.2^{c}	0.61 ± 0.1^{fg}	–	–
2.22	0.57	69.44 ± 2.27^{bc}	75 ± 0.01^{abc}	61.10 ± 4.53^{cd}	–	3.52 ± 0.4^{cd}	3.96 ± 0.8^{a}	3.05 ± 0.3^{a}	–	1.89 ± 0.1^{d}	1.77 ± 0.1^{cd}	1.47 ± 0.2^{a}	–
	2.85	**80.55 ± 2.26^{a}**	**83.33 ± 2.26^{a}**	**80.55 ± 4.53^{a}**	–	**4.16 ± 0.4^{a}**	**4.02 ± 0.6^{a}**	**3.16 ± 0.3^{a}**	–	1.59 ± 0.1^{e}	1.92 ± 0.2^{c}	0.71 ± 0.1^{c}	–
	5.71	77.77 ± 2.26^{ab}	69.44 ± 2.27^{bcd}	72.22 ± 2.27^{ab}	–	4.05 ± 0.4^{ab}	3.19 ± 0.3^{b}	2.24 ± 0.1^{c}	–	1.71 ± 0.1^{de}	1.83 ± 0.2^{c}	0.66 ± 0.1^{c}	–
	28.55	72.22 ± 2.27^{ab}	72.22 ± 2.27^{abcd}	55.55 ± 4.53^{de}	**69.44 ± 4.53^{a}**	3.33 ± 0.3^{d}	3.08 ± 0.2^{b}	1.30 ± 0.5^{e}	**2 ± 0.3^{a}**	2.84 ± 0.2^{b}	2.36 ± 0.2^{bc}	0.47 ± 0.2^{d}	0.25 ± 0.1^{a}
4.44	0.57	74.99 ± 2.27^{bc}	72.22 ± 2.27^{abcd}	69.44 ± 2.27^{bc}	–	3.60 ± 0.5^{cd}	2.96 ± 0.2^{bc}	2.77 ± 0.2^{b}	–	1.29 ± 0.01^{f}	2.60 ± 0.3^{b}	0.33 ± 0.01^{e}	–
	2.85	69.44 ± 2.27^{bc}	80.55 ± 2.27^{ab}	47.11 ± 2.36^{e}	–	4.13 ± 0.5^{a}	3.61 ± 0.3^{a}	1.02 ± 0.01^{f}	–	0.46 ± 0.01^{g}	0.85 ± 0.1^{ef}	0.25 ± 0.1^{e}	–
	5.71	72.21 ± 4.53^{ab}	72.22 ± 2.27^{abcd}	–	–	3.83 ± 0.6^{abc}	3.22 ± 0.3^{b}	–	–	0.31 ± 0.01^{gh}	0.38 ± 0.01^{fgh}	–	–
	28.55	75 ± 2.27^{ab}	80.55 ± 2.26^{ab}	77.77 ± 2.26^{ab}	–	3.74 ± 0.3^{bc}	3.63 ± 0.5^{a}	2.74 ± 0.3^{b}	–	0.51 ± 0.01^{g}	0.53 ± 0.01^{fgh}	1.01 ± 0.1^{b}	–
8.88	0.57	47.11 ± 2.36^{d}	55.55 ± 4.53^{e}	–	–	1.10 ± 0.1^{g}	1.13 ± 0.1^{fg}	–	–	0.13 ± 0.01^{hi}	0.60 ± 0.01^{fg}	–	–
	2.85	61.10 ± 4.53^{c}	63.88 ± 2.27^{cde}	–	–	2.02 ± 0.2^{f}	1.02 ± 0.01^{g}	–	–	0.16 ± 0.01^{hi}	0.19 ± 0.01^{gh}	–	–
	5.71	–	–	–	–	–		–	–	–	–	–	–
	28.55	–	–	–		–		–	–	–	–	–	–

Means followed by same letters in each column are not significantly different according to Duncan"'s multiple range test ($P \leq 0.05$). Data was recorded after 4 weeks of culture on shoot multiplication medium. The bold data showed the best results. Abbreviations used: ApB — Apical Bud, AxB — Axillary Bud.

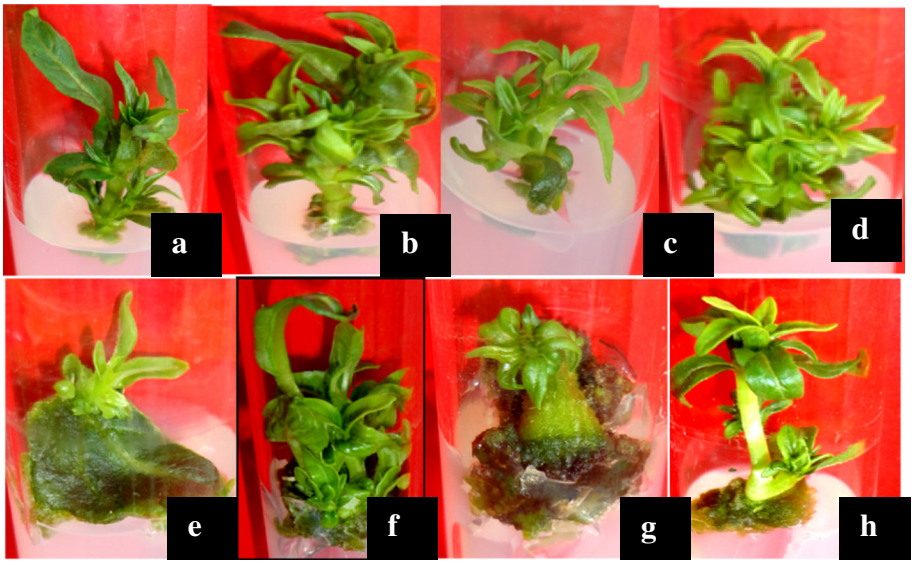

Fig. 3. a–b Shoot initiation and Multiple shoot formation with BAP (2.22 μM) + IAA (2.85 μM) from apical bud explants 2 week. c–d Shoot initiation and multiple shoot formation with BAP (2.22 μM) + IAA (2.85 μM) from axillary bud explants 2 week. e Shoot initiation with BAP (2.22 μM) + IAA (2.85 μM) 2 week from leaf explants. f Multiple shoot formation with BAP (2.22 μM) + IAA (2.85 μM) 3–4 weeks. g–h Shoot initiation and multiple shoot formation with BAP (2.22 μM) + IAA (28.5 μM) from internode explants.

with lower concentration of BAP (0.44 μM) and Kn (0.46 μM) favored the elongation of shoots (Table 1).

3.2.6. In vitro rooting

Elongated *in vitro* regenerated shoots were subcultured on to media with half-strength MS (alone) and with different auxins (IBA, NAA and IAA) for root induction. *In vitro* rooting was also observed on medium (half-strength MS) devoid of auxin but frequency of root initiation was low. *In vitro* shoots (3–4 cm long) not only produced roots but an increase in its shoot length was also observed when grown on half strength MS medium supplemented with different concentrations of IBA, NAA and IAA. In our study NAA (2.68 μM) was responsible for producing the highest frequency as well root length in *in vitro* regenerated shoots (Fig. 6 (a), Table 4).

3.2.7. Hardening and acclimatization

Planting substrate (vermiculite, soil, sand and farm yard manure mixture) plays an important role in acclimatization of plantlets as a

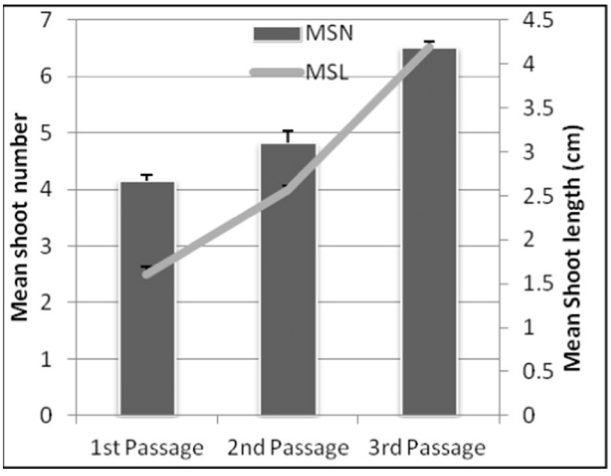

Fig. 4. Effect of subculture passages on mean shoot number and mean shoot length in *Guizotia abyssinica* Cass. on BAP (2.22 μM) + IAA (2.85 μM) from ApB. Experiment was repeated thrice with 12 replicates. Values represent mean ± standard error. Mean values followed by different superscript letters are significantly different at P ≤ 0.05, as determined by Duncan's multiple range test (DMRT).

higher survival rate (97.33%) (Fig. 6b–d) was obtained on it as compared to sand, soil, farm yard manure and vermiculite alone (Table 5). The regenerated plants did not show detectable variation in morphological or growth characteristics compared to the parent plant (Fig. 6e–g).

3.3. Histological analysis

Histology of basal tissue of all explants like, apical, axillary bud, leaf and internode was studied to ascertain the mode of regeneration and origin of *de novo* shoot formation. The histological sections revealed a very clear and distinctive feature of morphogenesis. The histological studies revealed a multicellular dermal origin of the neoformations. The actively dividing cells, which were densely cytoplasmic and deeply stained, divided mitotically, forming meristematic zones (Fig. 7a). Meristematic zone enlarged in size because of fast meristematic activity leading to the formation of nodular structures. Later this meristematic zone developed into prominent epidermal bulge which subsequently formed primary shoot primordia and later grew into a shoot bud (Fig. 7b–d). Shoot apex was clearly observed. Meristematic tissues were composed of dense cytoplasm and were deeply stained meristematic cells beneath the epidermis, which later produce multiple shoots (Fig. 7e–g).

During the study an unorganized nature of parenchymatous cells of internode explants revealed callogenic nature of the tissue (Fig.7h(a)). During later stages of development the callus cells showed distinct meristematic regions with cluster of actively dividing cells of the nodule characterized by densely stained regions consisting of small cells (Fig. 7h(b)). These regions were referred to as meristemoids. Meristemoids in the calli

Table 3
Effects of subculture passages on shoot regeneration, number of shoots and shoot length of *Guizotia abyssinica* Cass. obtained from apical bud on MS medium fortified with selected concentrations and combination of BAP and IAA.

	BAP + IAA (2.22 + 2.85 μM)		
Passages	Frequency of shoots	No. of shoots	Shoot length (cm)
I	80.55.1 ± 2.26^{a}	4.16 ± 0.10^{a}	1.59 ± 0.09^{a}
II	83.33 ± 0.01^{a}	4.83 ± 0.20^{b}	2.57 ± 0.04^{b}
III	**94.44 ± 2.27^{b}**	**6.52 ± 0.09^{c}**	**4.20 ± 0.05^{c}**

Means followed by same letters in each column are not significantly different according to Duncan's multiple range test (P ≤ 0.05). Data were recorded after 4 weeks of culture. The bold data showed the best results.

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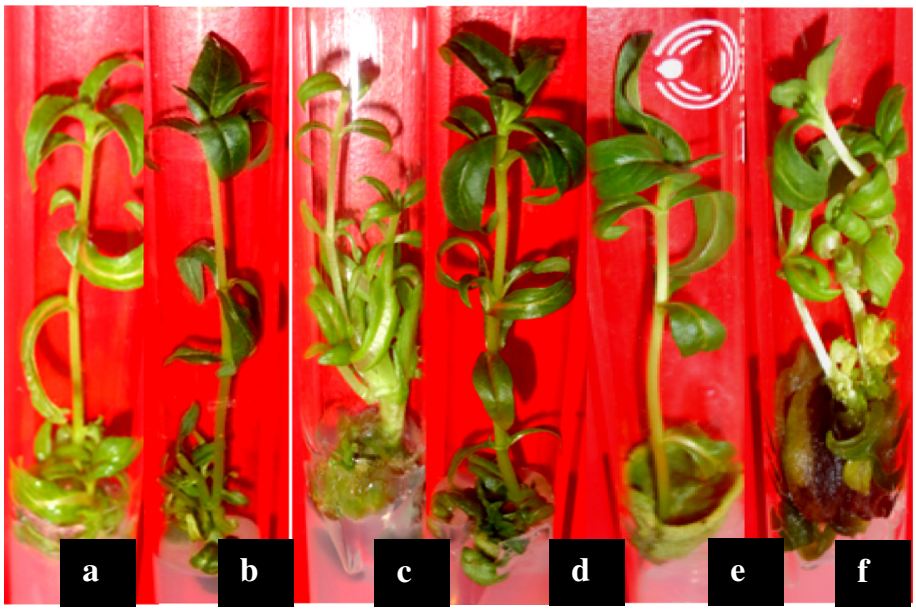

Fig. 5. a–b Shoot elongation on BAP (0.44 μM) + Kn (0.46 μM) from apical bud explants 4 week. c–d Elongation of shoots from axillary bud on BAP (0.44 μM) + Kn (0.46 μM) 4 week . e Shoot elongation on BAP (0.44 μM) + Kn (0.46 μM) 4 week from leaf explants. f Shoot elongation on BAP (0.44 μM) + Kn (23.2 μM) from internode explants in 6 weeks.

later started initiating shoot buds in the form of bulging. These bulging later developed into well differentiated shoot buds consisting of meristematic apical dome with two leaf primordia flanking both the sides of the dome (Fig. 7h(c)). Further, differentiation of the adjoining cell layers resulted in the formation of multiple shoot bud with well developed apical meristem and leaf primordia (Fig. 7h(d)).The shoot meristem gave rise to leaf primordia which protruded above the surface of each responding explants.

3.4. RAPD analysis

The present study was carried out to assess the genetic nature of *G. abyssinica* parent (nature grown plant) as well as the *in vitro* regenerated plantlets through RAPD analysis. DNA fingerprint analysis of the parent plant (S1), direct *in vitro* regenerated plant from apical and axillary bud (S3) as well as indirect regenerated plant from leaf (S2) and internode (S4) were carried out initially employing 8 primers of which 4 primers (RBA13, RAN2, RAN10, RBA10) generated distinct and reproducible amplified fragments. Each primer produced a unique set of amplified products and these fragments were characterized based on their sizes, ranging from approx. 120–1500 kb. A total of 197 RAPD bands were produced in PCR amplification. The number of bands scored in each primer ranged from 47 to 52 with an average of 49.25 bands per primer. All the primers tested produced polymorphic bands. The differences between the 4 plants *viz.* S1, S2, S3 and S4 is in the presence of new, or absence of, specific amplification products as shown in the profiles of primers RBA13, RBA10, RAN2 and RAN10 (Fig. 8). Maximum polymorphism (*i.e.*, 44%) was observed in the amplification pattern of the primer RAN10 followed by RBA10 which produced 33.33% polymorphic bands, followed by RAN2 which depicted 23% polymorphism and the least polymorphism was observed with primer RBA13 which showed 14.89% polymorphism (Fig. 8; Table 6).

From this data, similarity indices and distance matrix was developed. Based on this data, a dendrogram was constructed. The Jaccard's similarity co-efficient between the genomes of parent plant (S1) and those of direct regenerated plant from apical and axillary bud (S3) was 0.577, whereas between the parent plant (S1) and leaf regenerated plant (S2) was 0.556, while between the parent plant (S1) and indirect regenerated plant from internode (S4) was 0.27. This analysis indicated that genetic variation was higher in indirect regenerants as compared to the direct regenerated plants (Fig. 9 Table 7).

Analysis of the RAPD profiles revealed the genetic distance levels ranging from 0.00 to 0.57 between the parent *G. abyssinica* and its analyzed plants, and among the micropropagated, by different plants the matrix value ranged from 0.27 to 0.57, with a mean value of 0.42 indicating the genetic similarity at low to moderate level. The similarity coefficients were used as input data to generate a phylogenetic dendrogram by UPGMA cluster analysis using XLSTSAT program, in order to determine the genetic relationships. Maximum genetic similarity of 0.57 was observed between the parent plant (S1) and those of direct regenerated plant from apical and axillary bud (S3) while the least similarity of 0.27 was observed between the parent plant (S1) and indirect regenerated plant from internode (S4).

4. Discussion

Sterilization of a material (explant/seeds) before subjecting them to *in vitro* procedure is essential for the production of 'clean' *in vitro* plantlets that ensures the reduction of the contaminants as well as high survival rate of explants (Srivastava et al., 2010; Sharma et al., 2014).

In the present study, labolene (1%) and bavistin (1%) (fungicide) were used as surfactants. While ethanol (70%) and $HgCl_2$ (0.1%) were used as the sterilizing agents. Labolene is a surfactant normally used to increase the tissue penetration capability of sterilizing agents. Bavistin is a common systemic fungicide belonging to the benzamidazole group which is very commonly used to control different latent fungal contaminations under field conditions (Moghaddam et al., 2011; Sen et al., 2013a). To improve the effectiveness in sterilization procedure, ethanol is generally used prior to treatment with other compounds. It has been reported that alcohols are rapidly bactericidal rather than bacteriostatic against vegetative forms of bacteria; they are tuberculocidal, fungicidal and virucidal as well but do not destroy bacterial spores (Eziashi et al., 2014). The motility rate of microbes depends upon the concentration and time exposure of $HgCl_2$ and also varies according to explant type *i.e.* softness and hardness of the tissue and plant parts. There are many reports of surface sterilization using the most frequently used sterilizer mercuric chloride in various herbaceous species (Sairkar et al., 2009; Sharma et al., 2010; Anburaj et al., 2011; Preethi et al., 2011; Sen et al., 2013b; Mohanta and Sahoo, 2014 and Zamir and Rab, 2014). Higher

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Fig. 6. Rooting, hardening and acclimatization of *Guizotia abyssinica* Cass. a Root formation on NAA (2.68 μM). b–d 10, 20 and 30 day old hardened plantlets. e–f 3 month old hardened plant with flowering. g A group of *in vitro* regenerated plants grown in potted soil producing flowers.

Table 4
Effect of auxins on rooting of *in vitro* raised shoots of *Guizotia abyssinica* Cass.

PGR	Conc. (μM)	Frequency of rooting (mean ± SE)	No. of roots per shoot (mean ± SE)	Root length (cm) (mean ± SE)
½MS	Control	75.0 ± 0.1^{a}	30 ± 0.1^{c}	3.8 ± 0.22
IAA	0.57	80.55 ± 2.27^{b}	40.66 ± 2^{b}	1.65 ± 0.2^{c}
	2.85	83.33 ± 2.27^{b}	36.77 ± 2.1^{a}	1.16 ± 0.1^{b}
	5.71	72.22 ± 2.27^{a}	50.83 ± 3.8^{c}	1.10 ± 0.01^{b}
	28.55	69.44 ± 2.27^{a}	55.99 ± 2.5^{d}	0.61 ± 0.01^{a}
IBA	0.49	89.44 ± 2.27^{b}	24.86 ± 2^{a}	2.59 ± 0.2^{c}
	2.46	72.22 ± 2.27^{a}	28.83 ± 1.8^{b}	2.14 ± 0.^{b}
	4.92	69.44 ± 2.27^{a}	30.41 ± 1.6^{c}	2.81 ± 0.2^{c}
	24.60	66.66 ± 4.54^{a}	41.49 ± 2.5^{d}	0.96 ± 0.1^{a}
NAA	0.53	80.55 ± 2.27^{ab}	50.80 ± 3^{c}	2.52 ± 0.2^{b}
	2.68	**97.22 ± 2.27^{c}**	**66.86 ± 2.6^{d}**	**3.02 ± 0.1^{b}**
	5.37	86.10 ± 2.27^{b}	40.49 ± 1.5^{b}	2.48 ± 0.1^{b}
	26.84	72.22 ± 2.27^{a}	19.05 ± 0.8^{a}	1.26 ± 0.1^{a}

Values represent mean ± standard error. Mean values followed by different superscript letters is significantly different at P ≤ 0.05, as determined by Duncan's multiple range test. Data were recorded after 4 weeks of culture. The bold data showed the best results.

exposure of mercuric chloride has an adverse effect on plant tissue while lesser time ends with contamination. Therefore, adequate time duration of mercuric chloride was required to sterilize the seeds. In the present investigation the treatment of explants with 0.1% (*w*/*v*) of $HgCl_2$ (5–6 min.) has been found suitable for sterilization of seeds.

In the present study, apical and axillary bud explants failed to induce multiple shoot buds on PGR-free basal MS medium (control) while leaf

Table 5
Evaluation of different planting substrates for hardening of *in vitro* raised plantlets of *G. abyssinica* Cass. after 4 weeks of transfer.

Planting Substrate	No. of plants transferred	% Survival
Vermiculite (alone)	50	61.33 ± 1.09
Soil:sand:manure (1:1:1)	50	86 ± 1.63
Vermiculite:soil:sand:manure (1:1:1:1)	50	**97.33 ± 1.09**

Data represent mean ± SE. Means sharing the same letter within columns are not significantly different (P = 0.05) using Duncan's multiple range test (DMRT). The bold data showed the best results.

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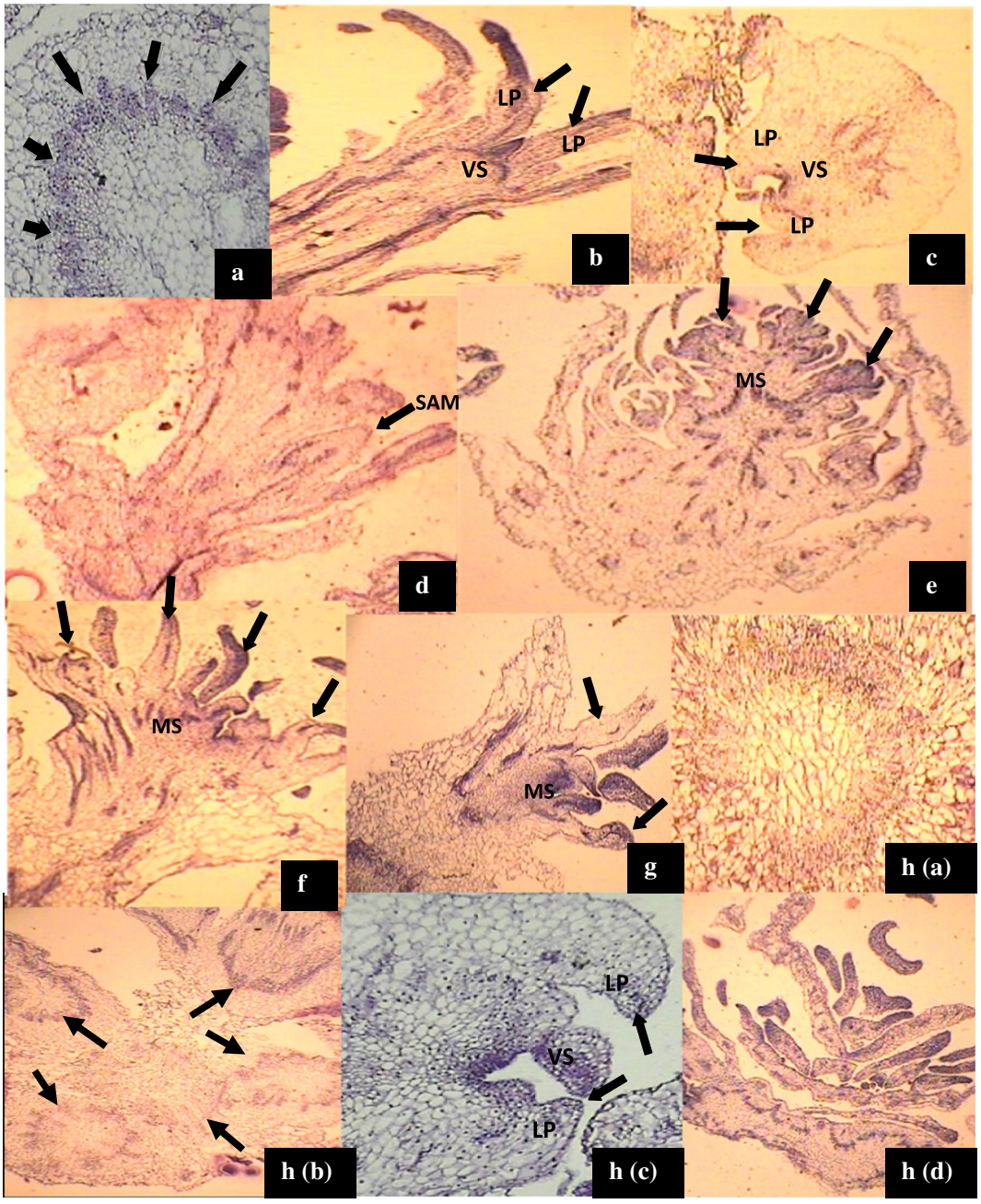

Fig. 7. Histological study of regenerating tissues. a Cells showing distinct meristematic regions (arrows) referred as meristemoids (MS) with cluster of actively dividing cells. b–c Development of well differentiated shoot bud (SB) from the meristemoid with a pair of leaf primordial (LP) with developed meristem dome (m) on the surface of apical and axillary bud explants. d Histological section showing shoot initiation and development from leaf explants e–f Histological section showing multiple shoot formation from both apical and axillary bud explants. g Histological section showing multiple shoot formation from leaf explant. h Histological study of regenerating tissues. h(a) Section of shoot tip derived basal internode tissue of plant showing undifferentiated parenchymatous cells revealing the callogenic nature of tissue. h(b) Callus cells showing distinct meristematic regions (arrows) referred to as meristemoids (MS) with cluster of actively dividing cells. h(c) Initiation of shoot buds and development of well differentiated shoot bud (SB) from the meristemoid with a pair of leaf primordial (LP). h(d) Multiple shoot formation from the differentiated shoot buds. *LP* leaf primordium, *VS* provascular stand, *SAM* shoot apical meristem, and MS multiple shoots.

and internode explants showed only rhizogenesis (Baghel and Bansal, 2013, 2014). Therefore, it was mandatory to augment the culture medium with BAP combined with Kn or with auxins to induce multiple shoot buds. The nature of the explant has been proved to be an important factor for *in vitro* growth and development of plant species, affecting callus induction and adventitious bud induction as well as shoot regeneration. The apical and axillary bud explants were found to be more effective for shoot initiation but in terms of shoot multiplication the former was more responsive than the latter. This differential morphogenetic response could be due to differences between the physiological states of the buds on different regions of a stem (Vieitez et al., 1985; Akter et al., 2013). The apical buds are better than the axillary bud for multiple shoot production because of the higher cytokinin to auxin ratio present in the shoot tip (Kavitha et al., 2012). Similar results were also reported in *Stevia rebaudiana* (Hossain et al., 2008) and *Solanum nigrum* (Kavitha et al., 2012).

The apical and axillary bud proliferation through *in vitro* technique is reported to be the safest and faithful strategy which maintains genetic integrity of developing progenies (Salvi et al., 2002; Anis et al., 2003). Leaf explants produced multiple shoots without callus formation while internode explants produce shoots with little callusing. The differential responses of the explants are probably due to endogenous

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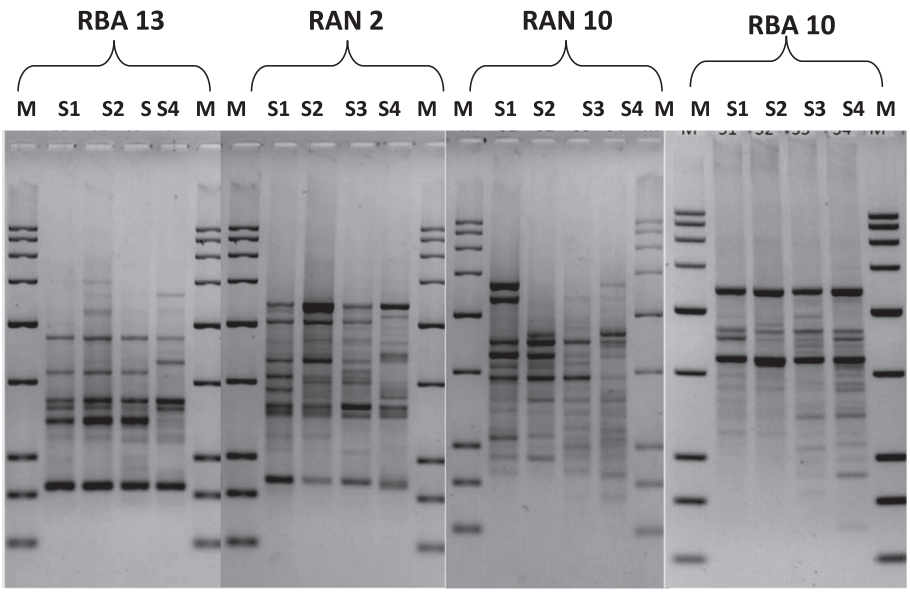

Fig. 8. RAPD profiles of mother plant (S1), direct *in vitro* regenerated plant from apical and axillary bud (S3), indirect regenerated plantlets from leaf (S2) and callus regenerated plantlets from internode (S4) of *Guizotia abyssinica* Cass. with primer RBA13, RAN2, RAN10 and RBA10. Lane M molecular weight ladder.

hormonal balance in plant tissues (Gattapaglia and Machado, 1998). These differences in the four explants can be explained by changes in the levels of endogenous hormones and the expression of genes encoding hormone receptors, as proposed by Close and Gallagher-Ludeman (1989).

It is well known that cytokinins stimulate plant cell division and participate in the release of lateral bud dormancy, in the induction of adventious bud formation in the growth of lateral buds and in the cell cycle control (Gaspar et al., 2003). To encourage the growth of axillary buds and reduce apical dominance in shoot cultures, one or more cytokinins are usually incorporated into the medium at initial establishment stage (George, 1993). Therefore, in the present study, response of seedling explants to various concentrations of BAP and Kinetin combinations was observed. The benefits of using a combination of cytokines, rather than a single compound, may be an indication of differences in uptake, recognition by the cells, or mechanism of action of the compounds (Huetteman and Preece, 1993). Similar effect of cytokinin combination on multiple shoot proliferation was observed in *Eclipta alba* (Baskaran and Jayabalan, 2005); *Paederia foetida* (Alam et al., 2010); *Abelmoschus moschatus* (Lithy et al., 2011); *Scoparia dulcis* (Premkumar et al., 2011); *S. rebaudiana* (Verma et al., 2011; Mehta et al., 2012; Razak et al., 2014) and in *Pluchea lanceolata* (Arya and Patni, 2013).

In the internode explants most of the cytokinin treatment failed to give response and observed basal callus browning might provide a potential sink for cytokinin conjugates that are inhibitory to further proliferation of adventitious shoots. This finding indicates the specificity of explants and PGR interaction (Rahman and Bhadra, 2011).

Table 6
Description of 4 primers used for RAPD analysis of mother plant (S1), direct regenerated (S3) and indirect regenerated from leaf (S4) and internode (S2) callus regenerated plantlets of *Guizotia abyssinica* Cass.

Primer	Primer sequence (5′–3′)	Scorable bands	Monomorphic bands	Polymorphic bands	Fragment size scored (bp)
RBA13	-CCGGCCATA-	47	40	7	210–1500
RAN2	-GGCACCATT-	52	40	12	210–1200
RAN10	-GTGCCCGAT-	50	28	22	180–1400
RBA10	- CCCGCCTTC-	48	32	16	160–1300
	Total	197	140	57	

It is well established that proper ratio of auxin and cytokinins is necessary for morphogenesis leading to the formation of complete plantlets (George and Sherrington, 1984). The requirement for exogenous auxin and cytokinin in the process varies with the tissue system, apparently depending on the endogenous levels of the hormones present in the tissue (Norstrog, 1970). In the present study, BAP in combination with IAA had a significant effect on the number of shoots produced from all explants. These results confirmed the positive effect of hormones on adventitious bud induction. Cytokinins have been defined as substances that stimulate cell divisions in plants and interact with auxin in determining the direction of cell differentiation (Wareing and Phillips, 1981). It is known that a balance between auxin and cytokinin normally induces effective organogenesis. Though the nature of interaction between the two plant growth regulators is not completely understood, cell division seems to be regulated by their interactions affecting

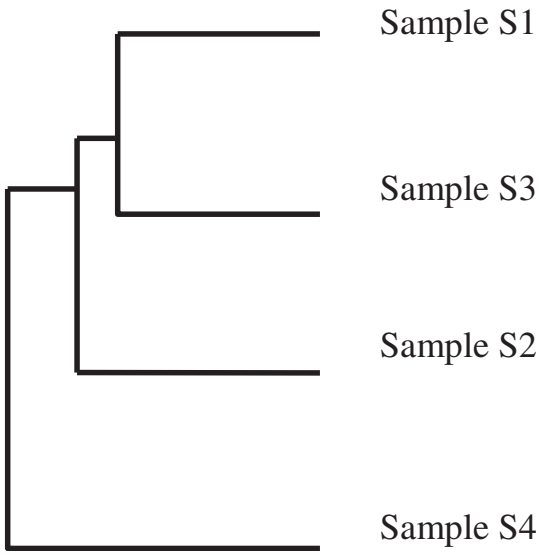

Fig. 9. Dendrogram depicting micropropagated *Guizotia abyssinica* plantlets (S2, S3, S4), and the mother plant (S1) based on genetic distance generated by 4 random RAPD primers using UPGMA module of XLSTSAT.

Please cite this article as: Baghel, S., Bansal, Y.K., *In vitro* regeneration of *Guizotia abyssinica* Cass. and evaluation of genetic fidelity through RAPD markers, South African Journal of Botany (2017), http://dx.doi.org/10.1016/j.sajb.2017.01.002

Table 7
Similarity matrix of the direct regenerated (S3) and from leaf (S2)and internode (S4) *via* callus regenerated plant (S1) of *Guizotia abyssinica* Cass. and their corresponding mother plant (S1) based on Jaccard's similarity coefficient.

Sample	S1	S2	S3	S4
S1	1	0.556	0.577	0.278
S2		1	0.433	0.333
S3			1	0.343
S4				1

different phases of cell cycle. While auxins are known to exert an effect on DNA replication, cytokinin exerts some control over the events leading to mitosis (Pasternak et al., 2000). Moreover the positive effect of cytokinin and auxin combination is explained by the enhanced RNA synthesis. The applied combination provokes the peak of RNA synthesis. This peak is associated with the appearance of first bud primordial thus conditioning bud formation (Bisht et al., 2012). Synergistic effect of BAP and IAA for shoot multiplication is well documented in previous studies in *Ocimum gratissimum* (Gopi et al., 2006); *Withania somnifera* (Supe et al., 2006); *Scoparia dulcis* (Hassan et al., 2008; Majumder et al., 2011); *Scrophularia takesimensis* (Sivanesan et al., 2008); *Ocimum basilicum* (Asghari et al., 2012); *Enicostema axillare* (Randive, 2013) and others. Narayanaswamy (1977) and Asghari et al. (2012) reported that the toxicity caused by an excess of growth regulators in the culture medium, or the extended period of time in which the culture was exposed to them, might lead to genetic, physiological and morphological changes, resulting in reduction of the proliferation rate *in vitro*. The reduction in shoot formation at higher concentrations of BAP may be related to the toxicity of BAP at higher concentrations. The reduced number of shoots could also be due to inhibition of adventitious meristem elongation due to high BAP concentration as stated by Borchetia et al. (2009).

Repeated subculturing of explants through the first three culture passages enabled continuous production of a healthy callus free shoots without any decline in multiplication rate. The enhanced multiplication of shoots during subsequent subculturing substantiates the earlier report on *Bacopa monniera* (Tiwari et al., 2001); *Eclipta alba* (Husain and Anis, 2006); *Clitoria ternate* (Mukhtar et al., 2012); *Stevia rebaudiana* (Thiyagarajan and Venkatachalam, 2012) and *Withania somnifera* (Udayakumar et al., 2014). The increase in shoot number due to successive transfer of mother cultures may be owing to suppression of apical dominance during subcultures that induced the basal dormant meristematic cells to form new shoots (Phulwaria et al., 2012). Higher cytokinin concentrations promoted shoot multiplication and at lower concentrations shoot elongation was observed. Similar effects of lower concentrations of cytokinins on shoot elongation have also been reported by Arya et al. (2008), Gayathri et al. (2009), Uranbey et al. (2010), Attia et al. (2012) and Meena et al. (2012).

The success of *in vitro* regeneration protocol relies on an efficient rooting in regenerated shoot and their subsequent acclimatization. In the present study, half-strength MS medium in combination with different concentrations of three auxins *viz.*, IAA, IBA and NAA was selected for root induction. The requirement of half-strength culture medium for root induction has also been reported in many plant species including *Cichorium intybus* (Nandagopal and Ranjitha Kumari, 2007); *Plectranthus barbatus* (Thangavel et al., 2011); *Acorus calamus* (Verma and Singh, 2012); *Caralluma adscendens* (Aruna et al., 2012) and *Chrysanthemum morifoium* (Verma, 2012). The frequency of rooting response was high in all the treatments and also in medium without a growth regulator. The ease of root formation on auxin-free medium may be due to the availability of endogenous auxin in the *in vitro* shoots (Minocha, 1987). Root formation (30 ± 0.1c) was also observed in shoots transferred on half-strength MS medium without growth regulators (Baghel and Bansal, 2013, 2014). However, in the present study, the treatment of NAA was most effective for callus free *in vitro* root induction. It has been established that auxin stimulates lateral root initiation by activating quiescent pericycle cells to initiate division and expansion which facilitates lateral root emergence (Fukaki and Tasaka, 2009). Therefore, appropriate synthesis, signaling and transport of auxins are all required for root formation (Peret et al., 2009). Auxin increases the number of lateral roots (Lewis et al., 2011). NAA is considered to be a potential auxin that induces rooting in *in vitro* regenerated shoots in this plant earlier by Ganapathi and Nataraja (1993), Nikam and Shitole (1993), and Bhandari et al. (2009). Within five to six days of culture on rooting media, all the shoots produced branched roots.

Histological investigation proved useful in ascertaining the direct and callogenic nature of the regenerating nodular tissue formed at the basal cut end of apical, axillary bud, leaf and internode explant. Sequential regeneration process was revealed through the histological sections of tissues at various stages of development. Formation of meristemoids in the developing callus ensured high frequency of adventitious shoot regeneration. The histological observations suggest that shoots developed following a normal pattern of organogenesis, similar to that previously described by Thorpe (1980), Mroginski et al. (2004), and Parveen and Shahzad (2011).

The survival of the plantlets under field condition depends not only on the proper environmental conditions but also on the proper growth and development conditions of the regenerated plants (Baksha et al., 2003). Planting substrate (vermiculite, soil, sand and farm yard manure) played an important role in acclimatization of plantlets and produced a better survival rate (97.33%) as compared to sand, soil and farm yard manure already reported by us (Baghel and Bansal, 2014).

Among the PCR based molecular techniques, RAPD is considered as a simple and cost effective method for certification of genetic fidelity of *in vitro* propagated plants (Gupta and Roy, 2002). Presently, RAPD primers were employed to assess the genetic similarity among the *in vitro* regenerated clones with respect to the mother plants of all four accessions. In our study 4 primers were analyzed for assessing genetic stability in micropropagated plants. As a result 197 amplified products were produced, ranging in size from 120 to 1500 bp. Among them, total percentage of the polymorphism obtained was 57%. The common amplicons in the agarose profile differ only in the intensity of their bands in the RAPD spectra (Kozyrenko et al., 2001). Smith (1998) reviewed the factors contributing to this variation and divided these into two, an intrinsic factor which largely depends on the genetic stability of the explant and an extrinsic factor depending on culture media and particularly growth regulators.

Micropropagation through organized meristems is generally considered to be a low risk method for genetic stability (Pierik, 1991), because the organized meristems are generally more resistant to genetic changes as compared to unorganized callus under *in vitro* conditions (Shenoy and Vasil, 1992; Bairu et al., 2011). However, there are numerous reports on the incidence of somaclonal variations among microproagated plants of *Curcuma longa* (Salvi et al., 2001), *Ocimum americanum* (Rady and Nazif, 2005), *Musa paradisca* (El-Dougdoug et al., 2007) and *Bacopa monnieri* (Karthikeyan et al., 2011; Ramesh et al., 2011). In contrast RAPD markers have also indicated the maintenance of genetic integrity among micropropagated plants in *Elaeis guineensis* (Rival et al., 1998); *Uraria picta* (Rai et al., 2010); *Alpinia galangal* (Parida et al., 2011); *Gerbera jamesonii* (Minerva et al., 2012); *Ocimum gratissimum* (Saha et al., 2012) and *Solanum melongena* (Mallaya and Ravishankar, 2013). The polymorphism in amplification products which represents one allele per locus may be the result of changes in either the sequences of the primer binding site (*e.g.*, point mutation) or changes which alter the size or prevent successful binding of the primer on the target DNA. The polymorphism was due to the occurrence of variation in only four plants which are not morphologically indistinguishable. The present study provides the first information on genetic variation among micropropagated *G. abyssinica* using RAPD analysis.

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5. Conclusion

The present study provides an efficient protocol for the mass multiplication of *G. abyssinica via* direct and indirect organogenesis. The highest frequency of shoot bud induction was observed from apical bud explants followed by axillary bud, leaf and hypocotyls respectively. BAP in combination with IAA enhanced shoot induction and multiplication in *in vitro* culture of niger. Well developed plantlets were successfully propagated in the normal environment. The present study is the first report on genetic fidelity analysis of micropropagated plants of *G. abyssinica.* Our result reveals the application of testing *in vitro* culture procedures for variation before applying them for commercial purposes. Moreover, the detection of morphologically indistinguishable offtypes by RAPD markers in the present study warrants additional testing of tissue culture propagated plants at the molecular level. This work demonstrates the scope of selecting improved clones of Niger with high oil yield and quality through somaclonal variation and suitability of RAPDs for detecting gross genetic changes in somaclonal variants at DNA level.

Conflict of interest

The authors hereby declare no conflict of interest.

Acknowledgments

One of us (S. Baghel) acknowledges funding received under the scheme Rajiv Gandhi National Fellowship (RGNF) of the University Grants Commission (UGC), New Delhi, India for carrying out this research.

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Please cite this article as: Baghel, S., Bansal, Y.K., *In vitro* regeneration of *Guizotia abyssinica* Cass. and evaluation of genetic fidelity through RAPD markers, South African Journal of Botany (2017), http://dx.doi.org/10.1016/j.sajb.2017.01.002

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Please cite this article as: Baghel, S., Bansal, Y.K., *In vitro* regeneration of *Guizotia abyssinica* Cass. and evaluation of genetic fidelity through RAPD markers, South African Journal of Botany (2017), http://dx.doi.org/10.1016/j.sajb.2017.01.002

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