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# *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*

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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).

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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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